BMP signaling regulation by GREMLIN 2 promotes proliferation and differentiation of human iPS cell-derived cardiac progenitors to cardiomyocytes

By

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Chapter 1

Introduction

Cellular Reprogramming to Pluripotency and Regenerative Medicine

With the awarding of the Noble Prize in Physiology or Medicine to Shinya Yamanaka and Sir John Gurdon in 2012, an international spotlight shone on their pioneering work in the field of cellular reprogramming. A worldwide audience recognized the significance of their contribution to our understanding of the elegant and intricate mechanisms that underlie cellular plasticity and our ability to manipulate such mechanisms to achieve consistent and predictable effects (Gurdon, 1962; Gurdon and Melton, 2008; Gurdon et al., 1958; Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Wernig et al., 2007; Yu et al., 2007). Such work has implications in basic biology, biotechnology, and the field of regenerative medicine, which seeks to find ways to improve treatments for disease and re-growing organs to replace those that are damaged and/or failing.

The major thrust of the field of regenerative medicine has been towards generating tissues that in a normal physiological context intrinsically lack a robust regenerative potential (Mao and Mooney, 2015). Such tissues remain in a somewhat senescent state, performing homeostatic maintenance functions but lacking the ability to recover, in part or wholly, from major cellular and tissue losses caused by disease or acute injury. Among these slowly regenerating tissues are the brain, nervous and spinal

tissue, and the heart, Fig 1 (Kajstura et al., 1998; Rando, 2006; Bergmann et al., 2009; Leri et al., 2011; Sánchez Alvarado and Yamanaka, 2014; Bergmann et al., 2015; Mao and Mooney, 2015).

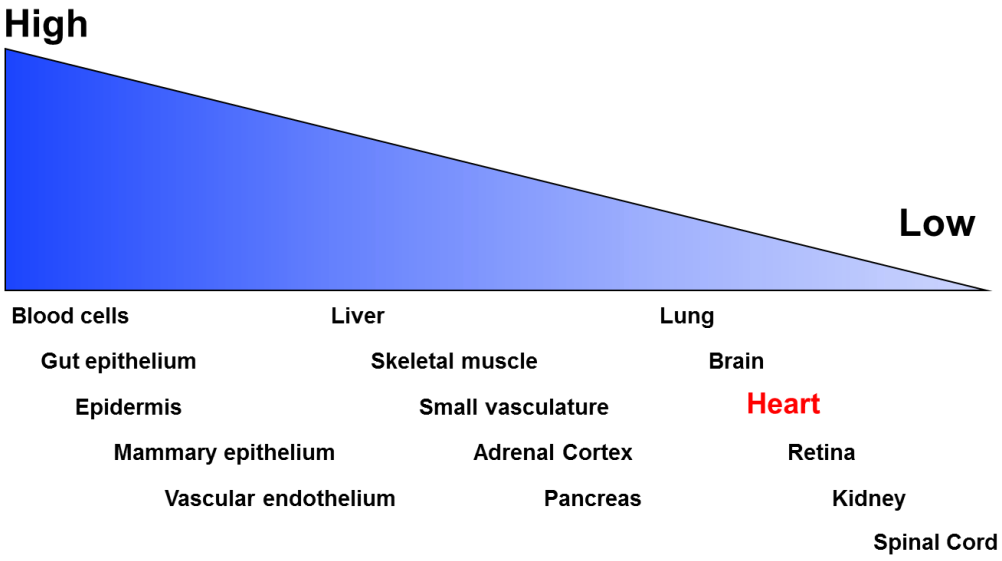


Figure 1: **Regenerative capacity of human organs and tissues.** A schematic representation of the capacity for selected organs and tissue types to regenerate. At the left, labeled “High”, are organs and tissues with resident stem cell niches that enable a very robust regenerative potential. In the case of blood cells, for example, regeneration can happen in as few as 24-48 hours (red blood cells). On the right, labeled “Low”, are organs and tissue types that have little to no regenerative capacity. The heart for instance has been reported to turn over (completely replace its cells) at as low a rate as 50% per lifetime. Adapted from Rando, TA et. al. (2008) *Stem cells, ageing and the quest for immortality* Nature.

Due to the limited regenerative capacity of adult human hearts, access to human cardiac tissue is severely limited. Yet human heart tissue is in demand. In the pharmaceutical industry, high fidelity models of functional human cardiac tissue are needed for cardioactive drug screening, safety pharmacology, and cell therapies used to replace damaged tissues (Amanfu and Saucerman, 2011; Mason et al., 2011; Matsa et al., 2014). In academia, continuing research into the mechanisms of cardiac

development, tissue engineering techniques, regenerative medicine, and cardiac disease requires access to abundant sources of human cardiac cells and tissues (Shim et al., 2012; Feaster et al., 2015; Dees and Baldwin, 2016; Passier et al., 2016; Ruan et al., 2016).

Efforts to regenerate heart tissue have focused on first understanding the processes by which heart tissue forms during development, and then on mimicking this process *in vitro* where pluripotent stem cell cultures are exposed to a regimen of growth factors, cellular matrix substrates, and small molecules that induce differentiation of the cells through types representative of the primary germ layer or mesoderm, then on to cardiac mesoderm and finally to functional cardiomyocytes (van Berlo and Molkentin, 2014; Mercola et al., 2011; Ruan et al., 2016; Ye et al., 2013). The remainder of this chapter will briefly cover cardiac development and the signaling pathways that govern it. Extended discussion of the TGFβ and BMP signaling pathways are included to provide context for the work described in Chapters 3 - 9.

Cardiac Development

While our understanding of the anatomical and morphological features of heart development spans more than a century, it is only recently that we have begun to unlock the cellular and molecular mechanisms of this amazingly complex process (Dees and Baldwin, 2016).

In the post-gastrulation embryo the heart and circulatory system are the first fully functional organ system to form, pumping nutrient rich blood to cells within developing

tissues. The adult heart is made up of several cell types. The endocardium is a cellular layer lining the chambers, valves, and vessels of the heart that is primarily comprised of endothelial cells. The myocardium consists of cardiomyocytes that are responsible for generating contractile force in the heart. Cardiomyocytes within the myocardium can be categorized into subtypes. Atrial myocytes are primarily responsible for regulating blood flow into and out of the ventricles. Ventricular myocytes provide the contractile force generated during a heartbeat. Connecting the atria and ventricles are bundles of mostly "non-working" cardiomyocytes that initiate and propagate the electrical signals which cause the heart to beat. These non-working cells are generally referred to as conduction system cells.

A fully formed heart passes through what may be described as 4 major stages of development: specification of early cardiac progenitors and their coalescence into cardiogenic regions called heart fields, fusion of these fields at the midline to form the heart tube, cardiac looping and primitive chamber formation, and finally cardiac remodeling, including septation and valve formation.

The first of these 4 stages of heart development is the specification and migration of cardiac progenitor cells from the mesoderm of the gastrula. These early cardiogenic regions appear around embryonic day 6.5 in mice and about 22 days post-fertilization in humans (Hamburger and Hamilton, 1992; Sissman, 1970). At the earliest stages, cardiac progenitors are not easily distinguished from other cells in the mesoderm. However, by day 7.5 in the mouse these progenitors have migrated into the midline of the embryo to form the first distinguishable cardiac structure, the cardiac crescent (Harvey, 2002; Vincent and Buckingham, 2010). At the cardiac crescent stage cardiac

progenitor cells reside in two distinct regions, called the primary and secondary heart fields (PHF and SHF, respectively) (Vincent and Buckingham, 2010). The cells in these fields will become most of the heart. Cells in the PHF become the left ventricle and a portion of the atria and the right ventricle. The SHF is located lateral to the PHF and contributes to the remainder of the atria and right ventricle as well as the outflow tract. In addition to spatial regulation, the cells of the primary and secondary heart fields are also temporally regulated. The cells derived from the primary heart field are the first to differentiate into cardiomyocytes and are known as first lineage cells. The SHF cells remain in an undifferentiated state until after the PHF cells begin to form the heart tube. During tube formation and throughout looping, SHF cells continue to differentiate and make contributions to the atria, ventricles and outflow tract (Kelly, 2012).

The second stage of heart development is cardiac tube formation. This process starts around embryonic day 8 in mice and is indicated morphologically as a budlike structure emerges at the cranial portion in the primary heart field of the cardiac crescent (Harvey, 2002). This budlike structure then enlarges as the crescent begins to fuse at the midline, gaining volume while expanding cranially and caudally (Buckingham et al., 2005). At the caudal end of the crescent PHF cells migrate downward in a bisymmetric fashion on the right and left sides of the emerging heart, forming an inverted Y. At the top of this inverted Y the budding PHF cells, with contributions from the SHF, expand downward to form the heart tube. This tube is a cylindrical structure composed of developing myocardium on the outside and a layer of endothelium called endocardium on the interior. While the PHF is expanding to form the anterior portions of the emerging heart, the SHF is also expanding cranially and posterior to the PHF. The cells of the

expanding SHF make some contribution to the mostly PHF-derived heart tube but also form a distinct structure at the top of the inverted Y that will eventually become the outflow tract. The cranial and caudal portions of this tube are known respectively as the arterial and venous poles. Once the heart tube is completely fused between the correctly oriented arterial and venous poles, it begins to act as a rudimentary pump.

At this point the heart detaches itself from the mediastinum and orients itself properly for chamber and valve formation. This reorientation process occurs during the third major stage in heart development, cardiac looping. In mice this looping process begins around embryonic day 9.0 and in humans around 5 weeks post-fertilization. In mice this process takes about 1-2 days to complete. As already described, the newly formed heart tube is at this point an asymmetric structure consisting of a bifurcated region at the venous pole and a straight tube with rudimentary SHF-derived outflow tract at the arterial pole. During looping the venous pole of the heart moves dorsally and cranially while twisting to the right, bringing it to a cranial position just above the developing ventricles. While the venous pole is reorienting as described, the arterial pole containing the outflow tract is also moving, albeit caudally and ventrally in an opposite and complementary fashion. These movements have now oriented the two poles in an asymmetric dorsal-ventral configuration with the outflow tract on the right and a common atrium and inflow tract on the left. These two regions are connected by a loop of bulbous, asymmetric cardiac tissue that includes the atrioventricular (AV) canal. This bulbous loop will eventually become the left and right ventricles. The former poles will become the atria and major vessels located at the base of the heart (Buckingham et al., 2005; Harvey, 2002).

The final step of development is cardiac remodeling and completion of the cardiac chambers. At this point the common atrium expands to form two appendages. While these atrial appendages are forming, the outflow tract is imposed upon their junction by continued spiraling of the heart tube. The outflow tract and atria fuse, producing two distinct atrial chambers. At the same time the inflow region insinuates itself between the bulging ventricular regions on the dorsal side of the heart. The inflow tract then fuses with the cardiac loop creating separate left and right ventricles. During this stage the valves and septa that separate the chambers from one another are also forming. The endocardial cushions in the AV canal give rise to the tricuspid and mitral valves while those in the outflow tract form the septum dividing the outflow tract into the aorta and pulmonary artery (Harvey, 2002).

These four steps highlight the complexity of the heart forming process. Although the procession through these four stages is complete by around E14 in the mouse, the heart continues to grow and mature through the remaining week prior to birth and post-natally. For the purpose of this work, these four basic steps provide the proper context for our discussion of the cell types that populate the heart during development and the molecular mechanisms that drive their expansion and differentiation. It is this understanding of molecular mechanisms that regulate heart development that has allowed us to generate cardiomyocytes *in vitro* from pluripotent stem cells.

Molecular Events in Heart Development

In order to appreciate how our understanding of the major events in cardiogenesis has been applied to pluripotent stem cell differentiation *in vitro*, it is

important to describe the major cell types involved in each of the developmental stages of the heart in terms of their origin, movements and eventual fates. Coordinating the differentiation and movement of cells during the four stages outlined above are major developmental signaling pathways. From mesoderm derivation to remodeling and chamber formation, these signaling pathways act to sequentially regulate cardiac transcription factor networks. These transcription factor networks regulate specific structural and functional genes that eventually characterize the mature cardiac cells (Bruneau, 2013). Thus the molecular and cellular events that drive cardiogenesis are best thought of in terms of these signaling pathways and the transcription factors under their control. In the context of cardiomyocyte differentiation from pluripotent stem cells, signals and factors regulating the origin and differentiation of early cardiac progenitor cells are most relevant.

Molecular Regulation of Mesoderm Specification

Prior to gastrulation, stem cells from the inner cell mass of the blastula differentiate to one of the earliest embryonic tissue types, the epiblast. Cells of the epiblast then give rise to the three primary germ layers. Epiblast cells first form the primitive streak and the earliest of the three germ layers, the ectoderm. The cells of the primitive streak then go on to form the endoderm and mesoderm. This transition of embryonic stem cells towards mesodermal tissue may be classified as the first important transition *in vivo* that guides our understanding of how to generate cardiomyocytes *in vitro* from pluripotent stem cells.

As gastrulation progresses, epiblast cells migrate through the primitive streak and expand outward between the ectoderm and endoderm (Arnold and Robertson, 2009). Mesodermal cells continue to expand laterally into the developing space between the ectoderm and endoderm, eventually segmenting into four different sections (Gilbert, Scott F., 2010). Various signaling pathways direct the specification, migration, and expansion of mesoderm from epiblast through the primitive streak and into its final position between ectoderm and endoderm, including Activin/Nodal, Wnt, BMP and FGF (Arnold and Robertson, 2009; Thiery et al., 2009).

Early induction of mesoderm is associated with the activity of Activin/Nodal signaling. Nodal is expressed in the epiblast where it activates BMP signaling that in turn activates Wnt. Later, Nodal and BMP act together to control the expression of Wnt antagonists such as Dkk1 to restrict Wnt signaling to the primitive streak, where it directs the formation of mesoderm. As mesodermal tissue continues to form, Wnt signaling is detected towards the posterior end of the primitive streak around E6.5 and then expands into the entire primitive streak and much of the mesoderm by E7.5 (ten Berge et al., 2008). Loss of Wnt signaling components, including Wnt ligands, co-receptors and β-Catenin (the nuclear protein mediating Wnt transcriptional control) results in a failure to form mesodermal tissue (Haegel et al., 1995; Kelly et al., 2004; Liu et al., 1999; Takada et al., 1994; Yoshikawa et al., 1997).

Wnt signaling affects the formation of mesoderm mainly via the Wnt ligand Wnt3A. Wnt3A regulates the activation of the essential mesodermal transcription factors T-brachyury (T-bry) and Eomes (Yamaguchi et al., 1999). In fact Wnt signaling acts directly on T-bry via translocation of β -catenin to the nucleus and activation of

gene transcription (Arnold et al., 2000). These transcription factors play a critical role in cell migration during mesoderm specification and are used as definitive markers of mesodermal tissue (Tam and Loebel, 2007).

FGF signaling also plays an essential role in the early formation of mesoderm. Loss of FGF ligands such as FGF8 and receptors such as FGFR1 leads to a failure of mesoderm formation because of defective cell migration during the primitive streak stage (Ciruna and Rossant, 2001; Sun et al., 1999; Yamaguchi et al., 1994). The effect of FGF signaling on mesoderm formation is likely mediated via its regulation of the transcription factors Snail, T-bry, and Tbx6, three genes required for mesoderm migration, specification, and patterning.

Transcription Factors in Cardiac Development

Cardiac precursors can be detected in the anterior region of the primitive streak during the early stages of mesoderm formation. These early cardiac cells are present on either side of the embryonic midline and express the transcription factors Mesp-1 and Mesp-2. Loss of Mesp expression in these cells aborts migration of emerging cardiac progenitor cells (Bondue and Blanpain, 2010; Buckingham et al., 2005; Saga et al., 1996).

Expression of Mesp-1 in the cardiogenic mesoderm is a master signal that induces the cardiac transcription factor signaling network (Bondue and Blanpain, 2010). These transcription factors include Nkx2-5, Hand2, Myocardin (Myocd), Gata4, Mef2c, Tbx20, FoxH1, Foxc1, SRF and Foxc2 among others (Bondue and Blanpain, 2010). The mechanism behind this activation lies in the ability of Mesp-1 to bind directly within

the regulatory regions of its target genes including Nkx2-5, Hand2 and Myocd. Myocd in turn activates Mef2c and SRF, which go on to activate the essential cardiac structural genes Myh6, Myl1, Myl2, Myl7, and cardiac troponin 2 (Tnnt2) (Bondue et al., 2008; Lemonnier and Buckingham, 2004).

Each of the transcriptional events in the formation of cardiac progenitors is dependent on Mesp-1 expression beginning in the early mesoderm and persisting through the expression of the cardiac-specific structural and functional components that define mature cardiomyocytes. Thus, cardiogenesis on a cellular level occurs via the carefully orchestrated, stepwise differentiation of progenitor cells towards mature cardiac subtypes, directed by cardiac transcription factors. As will discuss later, it is upon this principle that most protocols for production of *de novo* cardiomyocytes from pluripotent stem cells depend.

Each of these transcription factors is expressed in specific regions of the developing heart during particular periods of heart development and plays a distinct role during cardiogenesis.

For example, Nkx2-5, Hand1 and 2, Gata4, Tbx1, 5 and 20 are expressed in the PHF. These transcription factors direct the differentiation of cells within the PHF as they populate the heart tube and differentiate during looping morphogenesis (Buckingham et al., 2005; Harvey, 2002; Vincent and Buckingham, 2010). Nkx2-5 is critical for atrial-ventricular chamber compartmentalization and formation of ventricular tissue. Hand1 and 2 play a role in the formation of the left and right ventricles, respectively. Gata4 expression is essential for looping morphogenesis, septation of the valves and formation of ventricular myocardium. At the stage of heart tube formation cardiac cells

must migrate and fuse at the midline. Here, Gata4 and a companion transcription factor, Foxp4, are essential for proper tube formation as loss of function mutations in these genes lead to a failure of the cardiac fields to fuse and form a proper heart tube (Harvey, 2002).

The Tbx transcription factors comprise a whole class that plays diverse roles in cardiogenesis. These roles include patterning and specification of conduction system cells, sinoatrial nodal cells and distinguishing between atrial and ventricular myocardium. In fact patterning via the Tbx genes is the major driver for distinguishing between working and non-working myocardium during the later stages of cardiac development (Evans et al., 2010). Tbx transcription factors are also critical to the formation of the outflow and inflow tracts and neuralization of the atrium at later stages of heart development (Plageman and Yutzey, 2005; Stennard and Harvey, 2005). Tbx20 regulates proliferation of myocardial progenitor cells during early myocardial expansion. Mutant embryos lacking Tbx20 expression present a large decrease in the number of terminally differentiated cardiomyocytes (Cai et al., 2005). Tbx18 is expressed in the extreme posterior regions of the SHF(Vincent and Buckingham, 2010). Cells that express Tbx18 give rise to smooth muscle, myocardium, and interventricular septum (Vincent and Buckingham, 2010).

The Isl-1 transcription factor is expressed in the SHF and plays an essential role in many cardiac developmental processes. Mutations that inactivate Isl-1 result in major cardiac defects. These include lack of septation in the atrial and ventricular compartments, abnormal atria, and no outflow tract (Cai et al., 2003). Isl-1, along with Gata4, can directly control the expression of Mef2c by binding a genomic enhancer

element upstream of its coding sequence (Dodou et al., 2004). Incidentally, Isl-1 expression has been detected in the PHF as well as the SHF (where it was originally identified), though it does not seem to be required for formation of PHF lineages (Prall et al., 2007). When Isl-1 expression is knocked down in mice the PHF and its associated derivatives form normally (Cai et al., 2003).

The Forkhead transcription factors (known as Fox) are also important for cardiac development. Foxh1 is expressed in the anterior region of the secondary heart field and plays a role in formation of the outflow tract and right ventricle (Seo and Kume, 2006). Foxh transcription factors also play a role in regulating other transcription factors such as Mef2c (von Both et al., 2004). Mef2c is involved in the formation of the outflow tract, inflow tract and proper development of the right ventricle (Lin et al., 1997).

Pitx transcription factors also regulate myocardial formation. Pitx2c expression is detectable throughout the left side of the SHF during early cardiogenesis and is later detected extensively in the ventricular and atrial myocardium. Pitx2c is essential in the formation of left-right asymmetry in the heart, and loss of Pitx2c expression leads to right atrial isomerism and defects in ventricular maturation (Tessari et al., 2008). Pitx2c also regulates the formation of left *versus* right atrial chambers by repression of right atrial identity.

Atrial and ventricular cells are being patterned at very early stages of cardiac development. Cells in the secondary heart field that will eventually become part of the atria require the expression of the transcription factor Nfatc1 in order to fully develop (Schubert et al., 2003). At later stages of heart development the transcription factors Irx4 and Hey2 are expressed in emerging ventricles and play a role in ventricular

myocyte formation. In the atria the transcription factors Hey1 and Coup-TFII are expressed and regulate atrial myocyte formation (Evans et al., 2010; Kokubo et al., 2007; Wu et al., 2013).

Transcription factors not only regulate the proliferation, patterning, differentiation and final maturation of cardiomyocytes and their progenitors during development, they are also indispensable as markers of distinct differentiation stages from progenitor populations and mature cardiac subtypes. Understanding the role of transcription factors in tissue specific compartments allows us to track the development of cardiomyocytes *in vitro*, thereby allowing the successful translation of developmental paradigms into the culture dish for efficient cardiomyocyte differentiation.

Major Developmental Signaling Pathways in Cardiac Development

The timing and expression of the transcription factor networks described above is coordinated by major developmental signaling pathways. Many of the same pathways already discussed as important for mesoderm formation also contribute to the differentiation and maturation of cardiac progenitors. These pathways control diverse processes including the expansion of progenitor populations and differentiation of progenitors to myocardial cells. TGFβ, WNT, Notch, Retinoic Acid, and Hippo signaling pathways all play important roles in cardiac development (Bartram et al., 2001; Cohen et al., 2008; Cunningham and Duester, 2015; Dobaczewski et al., 2011; Gessert and Kuhl, 2010; Luxán et al., 2016; Niessen and Karsan, 2008; Rhinn and Dollé, 2012; Singh et al., 2016; Zhou et al., 2015). For the scope of this work, attention will be

focused on the prominent role played by the TGFβ super family and specifically the subfamily of the Bone Morphogenic Proteins (BMP).

Transforming Growth Factor Beta (TGFβ) Signaling

TGFβ is a superfamily of secreted factors that includes TGFβ, Activin/Nodal, Growth and Differentiation Factors (GDF), and Bone Morphogenetic Protein (BMP) ligands. Due to their phylogenetic similarities, BMP and GDF signaling molecules are commonly grouped together, with some members having both a BMP and GDF designation (Miyazono et al., 2005).

TGFβ ligands bind to two types of transmembrane receptors, classified as Type I and Type II. Upon ligand binding, oligomerization of the type I and type II receptors causes activation of the receptor complex which then activates downstream effector molecules within the cytoplasm (Horbelt et al., 2012).

TGFβ receptors are serine/threonine kinase receptors that span the cellular membrane, consisting of a single transcellular domain, an extracellular domain, and an intracellular cytoplasmic domain (Kitisin et al., 2007). Cystine-rich binding motifs on the extra-cellular domain serve as active sites for binding of TGFβ ligands to the type II receptor (Hinck, 2012). Upon ligand binding, conformational changes lead to oligomerization of type II receptors with their type I counterparts. Receptor oligomerization leads to transphosphorylation of the Type I receptor by the Type II receptor. Phosphorylated type I receptors then phosphorylate cytoplasmic signal transduction proteins known as Smads (Kitisin et al., 2007). Activated Smads form nuclear translocation complexes that regulate the transcriptional activity of downstream

gene targets including ID1, ID2, and ID3. TGFβ signaling is regulated by TGFβ type III receptors (TGFβRIII or Betaglycan), and endoglin, a transmembrane glycoprotein that binds with high affinity to the TGFβRI and TGFβRIII (Guerrero-Esteo et al., 2002; Massagué, 2012; Morén et al., 1992).

Members of the TGFβ superfamily play important roles in heart development. Activin signaling directs formation of primitive streak and mesodermal cells from the epiblast (Kattman et al., 2011; Ladd et al., 1998). Concentration gradients of Nodal direct specification of nascent mesoderm towards cardiogenic mesoderm (Brennan et al., 2001). TGFβ receptors and ligands themselves are required for proper coronary artery and heart valve formation and atrioventricular cushion transformation (Brown et al., 1999; Clark et al., 2016; Lai et al., 2000).

Bone Morphogenetic Protein (BMP) Signaling

Among TGFβ signaling pathway family members, the BMP sub-family is the largest. BMP ligands consist of a series of receptor binding molecules with a conserved cystine-knot binding motif including BMP2, BMP4, BMP5-7, BMP10, and BMP12-14 (Miyazono et al., 2005; Wang et al., 2014).

BMP signaling is highly regulated and plays a role in a host of biological processes (Wang et al., 2014). Traditional BMP signaling regulation can occur by cell-specific expression of selected BMP ligands, varied binding affinities for BMPRI and BMPRII receptors, interaction with a host of antagonists including members of the CAN family (GREM2 and DAN), chordin, and Noggin, (Fig 2; Balemans and Van Hul, 2002; Hung et al., 2012; Kattamuri et al., 2012a; Miyazono et al., 2005; Sieber et al., 2009).

In canonical BMP signaling, BMP ligands bind to the extracellular domain of transmembrane receptors. Following the common paradigm of TGFβ super family members, these transmembrane receptors are known as type I or type II receptors. While BMP ligands can bind with some affinity to a variety of TGFβ family receptors, the two Type I receptors BMPR-1A (also known as ALK2), and BMPR1B (also known as ALK6), and the type II receptor BMPR-2 are specific to BMP signaling (Miyazono et al., 2010). Both BMPR-1 and BMPR-2 receptors exist on the cell membrane as dimers. As is characteristic of TGFβ signaling family members, ligand dependent oligomerization of receptor dimers activates the BMP signaling cascade. However an extra level of regulation is achieved in BMP signaling by receptor subtype selectivity among BMP ligands. For example, BMP6 and 7 follow the traditional TGFβ signaling paradigm wherein binding affinity is higher for the type II receptor. But in the case of BMP2 and 4, binding affinity is higher for the type I receptor (Miyazono et al., 2010). In both cases binding leads to receptor oligomerization and receptor transphosphorylation, however downstream targets can be different depending on which receptor was bound first. Adding to the complexity of receptor ligand interactions is the existence in some cases of pre-formed tetrameric receptor complexes that, when bound, are able to cause phosphorylation of p38 MAPK (Gilboa et al., 2000; Nohe et al., 2002).

An additional level of signaling regulation occurs via cross-over binding of BMP ligands with traditional TGFβ receptors. For example, the BMP ligands BMP-2, BMP-4, BMP-7, and GDF-5, can all bind to the TGFB type III receptor (TβRIII or Betaglycan) with ligand binding domains and kinetics that are highly conserved when compared to that of TGFβ ligands (Kirkbride et al., 2008).

BMP can also signal through non-canonical pathways and affect a wide range of additional signaling pathways, including p38, JNK, and other MAP kinases (Sieber et al., 2009; Wang et al., 2014; Yuan et al., 2015).

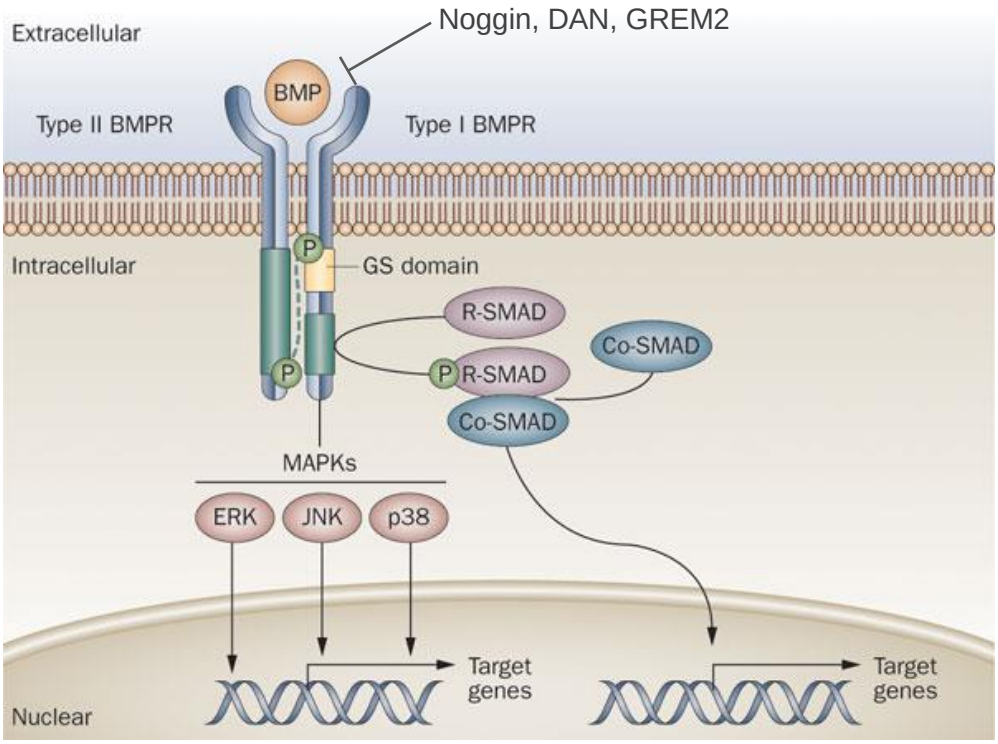


Figure 2: **BMP signaling.** BMP signaling is activated when ligand binding facilitates the formation of a heterotetrameric receptor complex. Upon complex formation, the type II receptor transphosphorylates the type I receptor which in turn phosphorylates receptor SMAD proteins. Activated SMAD proteins regulate transcription of target genes. BMP inhibitors such as Noggin, DAN, and GREM2 directly bind BMP ligands, thereby preventing receptor binding and tetrameric complex formation. Adapted from Shore, E. M. & Kaplan, F. S. (2010) Inherited human diseases of heterotopic bone formation
Nat. Rev. Rheumatol.

In the heart, BMP ligands and receptors are expressed in cardiac progenitor cells and the mature heart. BMP factors are secreted by endoderm during gastrulation as mesodermal cells move towards the cardiac crescent and SHF (Lough and Sugi, 2000).

BMP2 induces the expression of Nkx2-5 in the cardiac crescent, which then directly interacts with the early BMP effector Smad to auto-regulate BMP2 expression in the SHF (Liberatore et al., 2002). BMP expression is also important for regulating elongation of the linear heart tube, heart chamber formation and cushion formation as well as interacting with Nodal signaling to establish left-right asymmetry (van Wijk et al., 2007). These interactions between BMP and Nkx2-5 regulate early cardiac expansion by first stimulating proliferation and then limiting the number of cardiomyocytes in the heart tube (Prall et al., 2007).

Specific BMPs exert pleiotropic effects on cardiac morphogenesis and cardiomyocyte maturation (Pater et al., 2012). BMPs 4, 5 and 10 regulate cardiac looping, proper outflow-tract septation, branchial-arch artery remodeling, valve formation, cushion remodeling, and ventricular development (Breckenridge et al., 2001; Chen et al., 2004; Chocron et al., 2007; Kim et al., 2001; Liu et al., 2004; McCulley et al., 2008). BMP5 and 7 play a redundant role in regulating heart development as single knockout of either gene results in normal development while double knockout causes cardiac defects (Solloway and Robertson, 1999). BMP antagonists such as Noggin are also necessary for cardiac development. Mice lacking Noggin have thicker myocardium than wild types (Choi et al., 2007). This phenotype could be rescued by halving the gene dosage of Bmp4, indicating that fine-tuning the strength, or confining the zone of BMP activity is required for proper cardiac development (Choi et al., 2007).

Chapter Summary

The complexity of heart formation is reflected in the varied morphogenetic and cellular movements during heart formation as well as the transcription factors and developmental signaling pathways that drive them. While further research into the mechanisms of myocardial subtype specification as well as cardiac maturation and maintenance remains to be done, our current understanding of these processes allows their application towards the successful generation of cardiomyocytes *in vitro* from pluripotent stem cells.

Chapter 2

Pluripotent Stem Cells

Unique Features of Pluripotent Stem Cells

Pluripotent stem cells (PSCs) possess two unique and defining properties that distinguish them from other cell types. The first is that they are self-renewing, able to proliferate practically indefinitely in their undifferentiated state to generate new pluripotent cells. The second defining property of pluripotent stem cells is their ability to differentiate and form mature, specialized cell types (Mummery et al., 2012). In contrast to multi-potent stem cells, whose capacity to differentiate is confined to specific subtypes (Wagers and Weissman, 2004; Avgustinova and Benitah, 2016), pluripotent stem cells have a broader differentiation potential and can give rise to most tissue-specific cell types (Ludwig et al., 2006; Sánchez Alvarado and Yamanaka, 2014). These features make pluripotent stem cells a viable source for production of cardiomyocytes *in vitro*.

Pluripotent Stem Cell Sources

Pluripotent stem cells generally come from two major sources. The first are the embryonic stem cells (ESCs) that are derived from the inner-cell mass of blastocysts prior to implantation (Yu and Thomson, 2008) . These cells are both self-renewing and pluripotent, able to produce all the cell types of the body. Due to ethical issues and technical constraints, most early research conducted on embryonic stem cells in culture

was focused on those isolated from mouse embryos (Martin, 1981; Yu and Thomson, 2008). As technology has advanced and our ability to deal with the political and ethical concerns surrounding research using human embryos has evolved, research involving human embryonic stem cells has become less restricted, but still poses many practical and ethical challenges. Research in mouse and human ES cells has identified a large network of transcription factors that are required for pluripotency, self-renewal and suppression of differentiation (Boyer et al., 2005; Loh et al., 2006). In a seminal discovery by Yamanaka, it was shown that forced expression of four of these factors: Oct4, Klf4, Sox2 and c-Myc is sufficient to reprogram somatic, terminally differentiated cells to pluripotent, embryonic-like stem cells. These reprogrammed cells, called induced pluripotent stem cells (iPSCs) were first derived from mouse embryonic and adult fibroblasts using a subset of defined factors exogenously expressed in cultured cells (Takahashi and Yamanaka, 2006; Wernig et al., 2007). Shortly thereafter the same technique was shown to be effective at generating pluripotent stem cells from human adult fibroblasts (Takahashi et al., 2007; Yu et al., 2007; Park et al., 2008).

iPSCs have proven to be highly similar to ESCs in their ability both to self-renew and to differentiate to a variety of specific cell types (Boulting et al., 2011; Passier et al., 2016). iPSC technology offers the advantages of being both less ethically challenging and more patient-specific. Patient-specific stem cells open the way for generating cardiac tissue from patients suffering from genetic cardiac diseases in order to study these diseases *in vitro* and eventually the creation of patient-matched tissue for transplantation that is resistant to immune rejection (Murry and Keller, 2008; Passier et al., 2016).

Since the initial reprogramming protocol, various modifications have been made to simplify and increase the efficiency of pluripotent stem cell induction. These methods include using small molecules and proteins to aid the reprogramming process, generating new gene expression vectors that do not require viral integration and modulating regulation of pluripotency (Chen et al., 2011; Esteban et al., 2010; Jia et al., 2010; Liang et al., 2010; Okita et al., 2011; Rais et al., 2013; Yamanaka and Blau, 2010). These advances have made generating induced pluripotent stem cells practical enough to be done in a routine fashion.

Maintaining Pluripotency

Prior to differentiation, stem cells are maintained in an undifferentiated, pluripotent state. Maintenance of this pluripotent state is critical for taking advantage of the self-renewing capacity of stem cells and for the efficient directed differentiation of cardiomyocytes *in vitro* (Ojala et al., 2012). If pluripotent stem cells begin to differentiate in culture prematurely, they rapidly lose their capacity for self-renewal and spontaneously acquire features of differentiated cells in a more or less random fashion. In such a state they are not effective for use in directed differentiation. The tendency of pluripotent stem cells to spontaneously differentiate in culture must be controlled until they are ready to be used for directed differentiation (Burridge et al., 2012).

Both mouse and human cells have a tendency to spontaneously differentiate. Growth of pluripotent stem cells on feeder cells (an inactivated layer of cells that is unable to divide but provide extracellular matrix and secreted growth factors) such as fibroblasts, also used for derivation of embryonic and induced pluripotent stem cells,

helps to suppress premature differentiation to some degree; however, use of feeders introduces an undefined component to the culture system without completely eliminating differentiation (Xu et al., 2001).

The discovery that the protein LIF (Leukemia Inhibitory Factor) was sufficient to prevent differentiation of mouse embryonic stem cells when added to the culture medium presented a major advancement to the field of embryonic stem cell culture (Niwa et al., 1998). LIF is effective even in the absence of feeder cells and is therefore an essential component for the undifferentiated culture of mESCs in feeder-free conditions. Culture of stem cells in the absence of feeders eliminates the variability associated with the undefined role they play in maintenance of pluripotency and their effect on the eventual fate of stem cells during differentiation (Xu et al., 2001; Nakagawa et al., 2014).

It is critical to note that LIF is not sufficient to prevent spontaneous differentiation of cultured human pluripotent stem cells (hPSC). In order to support the feeder-independent culture of hPSCs more complex mixtures of defined factors have been developed. These formulations are based on our understanding of the signaling pathways that maintain pluripotency *in vivo* and include a combination of ligands such as bFGF and TGFβ-1 (Ludwig et al., 2006; Vallier et al., 2005). These strategies also employ artificial extracellular matrices such as matrigel or vitronectin in order to preserve pluripotency and enhance cell survival and attachment (Mummery et al., 2012). Feeder-free culture of human pluripotent cells in defined media allows for more precise control of pluripotency and differentiation (Burridge et al., 2012).

In summary, the unlimited proliferative capacity and broad differentiation potential of pluripotent stem cells are defining characteristics distinguishing them from other stem cell types. As will be discussed in the following section, these characteristics make them ideal for use as a source for cardiomyocytes *in vitro*.

Inducing Cardiomyocyte Differentiation

Upon induction of differentiation, cultured pluripotent stem cells proceed through a well ordered progression highly similar to that observed in the early stages of embryonic development *in vivo* (Schulz et al., 2009). This includes the sequential formation of cells with characteristics of mesodermal progenitors, mesoderm, cardiac mesoderm, cardiac progenitor cells, immature cardiomyocytes and finally more mature contracting myocytes (Burridge et al., 2012). While differentiating cultures are not purely made up of cardiac cells, among cardiomyocytes the differentiation of PSCs recapitulates the *in vivo* program with such fidelity it has been used to yield valuable insight into the basic mechanisms driving cardiac development (ten Berge et al., 2008; Kattman et al., 2006, 2007; Vliet et al., 2012).

Tracking the expression of transcription factors characteristic of pluripotent cells, mesoderm, cardiac progenitors and mature myocytes is an important example of how *in vivo* developmental paradigms aid in the derivation of cardiomyocytes from pluripotent stem cells. Over the first few days of cardiac differentiation, gene expression patterns indicate that pluripotency genes are down regulated while gastrulation gene expression is induced. Starting around day 2 and peaking at day 3 in mESCs and a little later in human stem cells the mesodermal marker T. bry is expressed, indicating the formation

of mesoderm (Ojala et al., 2012; Rai et al., 2011). After day 4 in both human and mouse stem cells, early cardiac markers such as Mesp-1 and Nkx2-5 can be detected. By day 10 cardiac-specific structural genes, including the sarcomeric light and heavy myosin chains as well as cardiac conduction channels, are detected. Thus pluripotent stem cells pass through the necessary developmental stages discussed in the first section of this chapter that lead to cardiomyocyte formation (Martin-Puig et al., 2008).

In order to coax stem cells from their pluripotent state through each of these stages to yield cardiomyocytes, differentiation strategies typically focus on mimicking the *in vivo* differentiation process. The earliest of these techniques relies on the formation of embryoid bodies (EBs): small spheres of pluripotent cells induced to form in droplets of cell suspension media hung on the lids of petri dishes, or forced to aggregate in microwells (Burridge et al., 2011; Fuegemann et al.). The strategy behind using such a system is to reconstruct the spherical nature of the early post-implantation embryo from which pluripotent stem cells are originally derived, in order to induce the process of gastrulation. In fact, differentiating EBs give rise to layers of tissue representative of mesoderm, endoderm and ectoderm and will eventually produce cells representative of all tissues of the body (Evans and Kaufman, 1981). The EB method is used for the differentiation of both human and mouse pluripotent stem cells. Its effectiveness in both systems and its relative scalability have made the EB method a popular format for induction of differentiation.

Monolayer differentiation techniques have also been used to induce the differentiation of pluripotent stem cells. These techniques have the advantage of being less complex, more accessible to proteins and other molecules used for cardiac

induction and more amenable for use in tissue engineering and other applications where thin films are preferable to aggregates (Burridge et al., 2014; Laflamme et al., 2007a; Palpant et al., 2017; Shim et al., 2012; Zhang et al., 2012). Monolayer differentiation does not inherently produce embryonic germ-layer tissues as do EBs. Thus, these protocols rely heavily on directed differentiation techniques.

Directed Cardiomyogenesis

EB formation alone is enough to induce some cardiogenesis. After induction of differentiation small clusters of contracting cells are usually observed between days 8-10 of differentiation in mESCs and a little later in hPSCs (Laflamme et al., 2007a; Maltsev et al., 1994; Mummery et al., 2002). However, only a small portion of cells in differentiating EBs will spontaneously become cardiomyocytes, usually around 5-10% (Zwi et al., 2009). Non-cardiomyocyte populations include other mesodermal lineages such as endothelial cells and smooth muscle cells, and those of the other germ layers including neurons. Thus, merely forming EBs and allowing them to differentiate is not sufficient to generate large numbers of relatively pure cardiomyocytes from pluripotent stem cells *in vitro*. As already mentioned, monolayers of PSCs also form few if any cardiomyocytes when simply allowed to spontaneously differentiate in culture.

In order to generate higher percentages of cardiomyocytes, further cues from embryonic development are used to drive or enhance the progression of cells to form mesoderm, cardiac progenitors and functional cardiomyocytes. The most effective current methods employ a refined approach that relies on the addition of exogenous factors and small molecules in defined media at precise times during the course of

differentiation (Burridge et al., 2012). These factors include protein and small molecule agonists and antagonists of specific developmental signaling pathways including Activin/Nodal, Wnt, BMP, FGF, and TGFβ (Burridge et al., 2012; Mummery et al., 2012).

The first step in directing pluripotent stem cells towards becoming cardiomyocytes involves the induction of mesoderm. As discussed in chapter 1, one of the earliest events in the differentiation of cells to cardiomyocytes *in vivo* is the coordinated action of Activin/Nodal, BMP and FGF signaling pathways to induce expression of the mesodermal transcription factors T bry, Eomes and SnaiI. Indeed successful protocols for the efficient induction of mesoderm include addition of the Nodal ligand ActivinA, the BMP ligand BMP4 and the FGF ligand FGF2 (Burridge et al., 2012). Once mesoderm has been formed, specification of cardiac progenitors can be induced using a combination of Wnt, TGFβ and BMP inhibitors with VEGF. These molecules block the negative regulatory effect of their respective pathways at this stage of differentiation, allowing for the more robust transition of cardiac mesoderm to cardiac progenitor cells (Kattman et al., 2011; Yang et al., 2008a). To drive cardiomyocyte differentiation from progenitor cells to functional, contracting myocytes, VEGFA, FGF2 and the Wnt inhibitor Dkk1 are added. As in the case of enhancing progenitor cell specification, these factors maintain the positive regulatory activity of VEGF and FGF signaling while preventing the negative regulatory effects of Wnt.

Other molecules outside of the traditional pathway modulators have also been empirically determined to enhance cardiomyocyte differentiation. These include Vitamin C to enhance proliferation of cardiac progenitors, cardiogenic small molecules identified using high-throughput screening methods, and polymers such as polyvinyl alcohol

(PVA) that modify surface tension (Cao et al., 2012; Elliott et al., 2011; Wu et al., 2004). While these molecules do not necessarily have developmental cognates, their use illustrates the flexibility and utility of *in vitro* differentiation methods for the development of novel approaches to increasing cardiomyocyte yields in culture.

The most effective methods for generating monolayers of cardiomyocytes are those that are robust and transferrable from laboratory to laboratory. The Matrix Sandwich protocol, developed in the Kamp Laboratory at UW Madison, relies on both the stepwise differentiation of pluripotent stem cells by the timely addition of exogenous Activin, BMP4 and bFGF in combination with a layer of Matrigel below and above the cellular monolayer consistently produces high yields of cardiomyocytes (Zhang et al., 2012). Another highly reproducible protocol is the GiWi method, which relies on the timely activation of wnt signaling via GSK3β inhibition (Gi) followed by wnt inhibition (Wi) via the addition of small molecules to the differentiation medium (Lian et al., 2013). These methods have been built upon to generate more refined versions that produce high yields of cardiomyocytes or cardiomyocytes for specialized applications (Burridge et al., 2014; Feaster et al., 2015).

Despite the relatively high yield of cardiomyocytes produced using these protocols, there remains a significant amount of heterogeneity among the resultant cardiomyocytes. Cultures often consist of other cell types, typically of the vascular lineage, including endothelial and fibroblast cells. Among the cardiac cells functional phenotypes representing ventricular, atrial, and nodal cells can be detected. This heterogeneity presents a challenge for practical use of pluripotent stem cell-derived cardiomyocytes (Lian et al., 2013).

The first source of heterogeneity arises from the fact that even though over 80% cardiomyocyte populations can be achieved by careful optimization of growth factor concentration and timing of treatment, the other 20% of cells are mainly mesoderm-derived cells such as endothelial cells (Burridge et al., 2012). Thus cardiomyocytes do not necessarily make up the entire population. To address this lack of homogeneity, purification strategies are employed to enrich the number of cardiomyocytes present in the culture by either selectively eliminating unwanted cell types by inserting antibiotic resistant cassettes behind cardiac-specific genes, or by physically removing persistent cardiac progenitor cell populations using pan-vascular pre-cursor cell markers like kinase insert domain receptor (KDR) (Elliott et al., 2011; Kattman et al., 2011; Yang et al., 2008a). Even more recently, protocols have been developed which allow enrichment of mature, functional cardiac myocytes using metabolic selection. Cardiac myocytes are unique among other cell types for their ability to metabolize lactate to generate ATP instead of glucose (Burridge et al., 2014; Tohyama et al., 2013). By completely removing glucose from the cell culture media non-cardiac cells starve and die out. These selection methods yield the highest purity (up to 99%) of cultured cardiomyocytes. However, some of these methods carry some risk of enhanced tumorigenicity and are therefore most useful for applications that do not include clinical therapies (Burridge et al., 2012).

Another source of heterogeneity among PSC-derived cardiomyocytes arises from the heterogeneous nature of cardiomyocytes themselves. Cells of the atria, ventricles, nodes and conduction system have distinct functional and structural properties (Maltsev et al., 1994). PSC-derived cardiomyocyte populations contain both types of working

myocardium as well as conduction system and nodal cells (Zhang et al., 2009). In order to address this heterogeneity by directing differentiation towards specific cardiac subtypes, cues from development have guided development of protocols for efficient subtype specification. These protocols are in the very earliest stages of research and remain to be fully understood, but some attempts have shown that this specification may be possible soon. Modulation of signaling pathways such as retinoic acid and BMP can bias the differentiation of maturing cardiac cells towards specific subpopulations of cardiomyocytes (Kolossov et al., 2005; Wiese et al., 2011; Zhang et al., 2011). These protocols remain somewhat inefficient, however, and current research into the mechanisms that regulate cardiomyocyte subtype differentiation is required.

Our understanding of the signaling mechanisms that drive the development of cardiomyocytes *in vitro* continues to evolve. Still unknown are the specific roles played by all of the varied signaling pathways and their respective signaling molecules in this complex and well-coordinated process. The potential to uncover further insights into the mechanisms that drive cardiac subtype specification, cardiac progenitor cell expansion, and cardiac progenitor cell maturation is great.

Chapter Summary

Both short and long-term applications for the use of human pluripotent stem cells are emerging from the state of the art. In the short term, use of induced pluripotent stem cells derived from patients with traditionally intractable cardiac illnesses will allow large numbers of disease- and patient-specific cardiomyocytes to be generated (Itzhaki et al., 2011). These myocytes can be used to study the pathology and development of

various cardiomyopathies *in vitro*, as well as to test drugs and other therapeutics for their efficacy as treatments. Research into the mechanisms of long QT syndrome, for example, and the effect of drugs on this phenotype has already yielded insights into the molecular mechanisms underlying this disease (Itzhaki et al., 2011).

Another near-term application for human pluripotent stem cell-derived cardiomyocytes is in toxicology and drug screening. Most drugs that fail in late stages of development and clinical trials or are recalled after release cause unacceptable cardiac toxicity as an off-target effect (Wilke et al., 2007). By screening drugs in a human cardiomyocyte model system early in the drug development process, these off-target cardio-toxic effects can be discovered and development efforts shifted accordingly (Liang et al., 2013).

In the more long-term future, hPSC-derived cardiomyocytes hold promise as a source of cells for regenerative therapies. One can envision new hearts grown using cardiomyocytes derived from individual patient iPSCs; cardiac patches made from engineered myocytes on biopolymer scaffolds; and injection of engineered ventricular myocytes into post-ischemic tissues to replenish working myocardium (Karakikes et al., 2015; Palpant et al., 2017).

Heart development is a complex process on the morphological, cellular and molecular levels. Our understanding of these cellular and molecular mechanisms has improved greatly as molecular biology and biochemical techniques have been applied to elucidate the developmental signaling pathways and transcription factor networks that drive cardiogenesis. This understanding has been applied to the culture and directed cardiac differentiation of pluripotent stem cells *in vitro*. Continued research into the

mechanisms behind cardiomyocyte maturation and subtype specification combined with improved culture techniques will bring us closer to achieving the promising applications for pluripotent stem cell-derived cardiac myocytes.

Chapter 3

Gremlin2/PRDC Function in Development and Cardiac Differentiation

GREM2 Structure and Signaling

Gremlin 2 (GREM2), also called Protein Related to Dan and Cerberus (PRDC), is a secreted BMP antagonist that belongs to a family of functionally and structurally-related antagonists known as the CAN (Cerberus And Dan) or DAN (Differential screening-selected gene in neuroblastoma) family. Comprising this family are Dan (also known as Nbl1 and DAND1), Cerberus or Cerberus-like 1 (Cer1), SOST (Sclerostin), Dante (DAND5, Coco), USAG-1 (Uterine sensitization-associated gene-1 or Wise), Gremlin 1 (Grem1, Drm) and Gremlin2 (Avsian-Kretchmer and Hsueh, 2004; Nolan and Thompson, 2014a; Pearce et al., 1999).

A defining structural feature of the DAN protein family is a cystine knot motif that is similar to that found in BMP ligands, that consists of 8-cystines (Nolan and Thompson, 2014a). Structural analysis of GREM2 shows that it is highly similar to BMP ligands such as BMP2 and 4 (Sudo et al., 2004). Unlike BMP ligands however, which form as di-sulfide linked dimers, functional GREM2 protein is a highly stable, noncovalent homodimer that doesn't require disulfide bonding between cystines within the knot motif to stabilize its dimeric structure (Kattamuri et al., 2012a). Instead, GREM2 dimerization is stabilized by a combination of zipper-like β-sheets at the protein core

and unique N-terminal α-helices that interact with one another as monomers align in an anti-parallel fashion (Nolan and Thompson, 2014a). These unique properties of the GREM2 functional dimer highlight the complexity of its structure and suggests that GREM2 has a distinct function among its fellow DAN family members.

Functionally, DAN family proteins are related by their traditional identification as antagonists of BMP signaling through direct binding interaction with BMP ligands (Avsian-Kretchmer and Hsueh, 2004; Hung et al., 2012; Nolan and Thompson, 2014a). GREM2 selectively inhibits activation of Smad-mediated BMP signaling by BMP2, BMP4, BMP6, and BMP7 but has no effect on that of TGFβ, GDF9 or Activin (Sudo et al., 2004). GREM2 has been shown to interact directly with BMP2, BMP4, and GDF5 (also known as BMP14) (Nolan et al., 2016; Sudo et al., 2004). Crystal structures of the GREM2, GDF5 complex show that GREM2 is able to directly interact with the BMPRII and BMPRI binding domains of GDF5, though with a higher affinity for the BMPRII binding domain. These structural studies also revealed that when GREM2 dimers bind GDF5 ligands, one GREM2 monomer remains exposed to bind another GDF5 ligand. This can lead to multiple, alternate binding events between GREM2 and GDF5 to form long protein chains. Similarly, because GREM2 binding of the BMPRII site leaves the BMPRI site exposed, GREM2-GDF5-BMPRI complexes are also able to form (Nolan et al., 2016). While other members of the DAN family have also shown to form these oligomeric chains, none were able to do so to such a high degree as GREM2. This cooperative binding strategy, wherein GREM2 combines with BMP ligands to generate a new oligomeric protein complex, contrasts with that of Noggin, which sequesters BMP ligands, completely covering their receptor binding sites (Groppe et al., 2002).

GREM2 Function *In Vivo*

The primary sequence of GREM2 is highly conserved across many species, suggesting that its function has been preserved throughout evolution (Müller et al., 2006). In mice, GREM2 expression has been detected in commissural neurons of the developing spinal cord and in lung mesenchyme (Lu et al., 2001; Minabe-Saegusa et al., 1998). In zebrafish embryos, GREM2 expression is detected beginning around 17 hours post fertilization (hpf) and continues through 72 hpf. It is highly expressed in the eye, swim bladder and in the pharyngeal arch mesoderm adjacent to the developing heart suggesting a role in heart development (Müller et al., 2006).

In vivo studies using mice and zebrafish have implicated GREM2 in follicle development, placode neurogenesis, osteogenic differentiation and craniofacial patterning (Ideno et al., 2009; Kriebitz et al., 2009; Sudo et al., 2004; Zuniga et al., 2011). Further studies in zebrafish showed that GREM2 plays a pivotal role in heart development. Examination of GREM2 expression by *in situ* hybridization shows that GREM2 expression domains occur in ventral pharyngeal arch mesoderm, adjacent to the cardiac field as cardiac progenitor cells are migrating from the midline to the left side of the embryo. The spatial and temporal expression of GREM2 puts it into the proper position to influence cardiac development from the cardiac progenitor stage through cardiac specification and maturation. Indeed loss of function approaches showed that GREM2 depletion caused randomization of the cardiac axis, varied the shape of the heart tube, diminished cardiac chamber volume, and resulted in incomplete looping and

randomized jogging (Müller et al., 2013). These defects were rescued when GREM2 mRNA was introduced back into GREM2-deficient embryos.

In addition to anatomical defects, loss of GREM2 resulted in reduced expression of the chamber-specific cardiac genes *amhc* and *vmhc*, upregulation of the laterality factor *pitx2*, and loss of *lefty2*, which is normally expressed on the left side of the embryo as the cardiac tube jogs leftward. Loss of GREM2 also resulted in an increase in the amount of phosphorylated Smad1/5/8 protein present in the heart. Treatment with the chemical BMP inhibitor dorsomorphin led to reductions in phosphorylated Smad1/5/8 levels and rescued loss of function mutants from cardiac chamber defects. These data showed that GREM2 is able to mediate cardiac development through modulation of BMP signaling (Müller et al., 2013).

Over expression of GREM2 in zebrafish embryos caused an increase in the expression of cardiac genes and ectopic formation of cardiac tissues in the posterior portion of the trunk. Interestingly, this ectopic tissue was found to be entirely composed of atrial myocytes. The atrium was also expanded while the ventricle was significantly diminished. These results showed that GREM2 expression served as an atrializing factor and that it was necessary and sufficient for proper atrial tissue development and differentiation (Müller et al., 2013).

In humans, a hypermorphic GREM2 variant, GREM2 Q76E, wherein the glutamine residue of normal GREM2 has been substituted for a glutamate, has been associated with familial atrial fibrillation (Darbar et al., 2007; Müller et al., 2013). This suggests that abnormal GREM2 activity can cause cardiac arrhythmias. mRNA injection

of the human Q76E variant resulted in slower cardiac contraction rates, abnormal atrial contraction velocity, and distorted wavefront propagation in zebrafish.

Taken together these data show that GREM2 is an essential molecular component of proper cardiac formation. It is both necessary and sufficient *in vivo* to produce cardiac tissue, and dysfunctional GREM2 can cause human cardiac defects.

GREM2 Promotes Cardiogenic Differentiation of Mouse Embryonic Stem Cells (mESCs)

The work described in the rest of this chapter investigating the role of GREM2 in mESCs has been published in the journal Stem Cells (Tanwar et al., 2014). As co-author with Dr. Tanwar, I took part in experimental design, data collection, data analysis, figure composition, and writing the manuscript. In the following section I will summarize our findings. For a complete listing of author contributions please refer to Appendix B.

The data discussed so far show that GREM2 is a BMP antagonist with distinct structural and functional properties and that it is essential for proper heart development *in vivo*. To explore the possible effect of GREM2 on promoting cardiogenic differentiation *in vitro*, mouse embryonic stem cells (mESC's) were differentiated using The Embryoid Body Method and the expression pattern and effects of GREM2 treatment were investigated (Tanwar et al., 2014).

As already described, GREM2 expression can be observed *in vivo* early in the cardiac differentiation process. In mESCs, genes that are typically expressed during the gastrulation phase of development, such as Cerberus-like 1 (Cer1) and T-brachyury (T-bra), are transiently induced around day 3-4 of differentiation, followed by early mesendoderm markers, including Bmp2, Foxf1 and Foxa2, at day 4. These are followed by expression of the first hematopoietic and vascular-specific genes Gata1 and Vegfr2, followed by expression of the early cardiac-specific genes Nkx2.5 and Myocardin (Myocd). Markers of mature cells, i.e., Hemoglobin Hbb-Y, Vascular Endothelial cadherin (VE-cadherin, or Cdh5), α - Myosin heavy chain (Myh6) and alpha Fetoprotein (Afp) are initially detected between days 5-7 (Schulz et al., 2009; Tanwar et al., 2014). GREM2 expression starts around day 4 and becomes prominent from day 5 onwards, thus appearing during the early differentiation stages of mesodermal and endodermal progenitor cells, marked by expression of Foxf1 and Foxa2 respectively, and early cardiac progenitor cells marked by Nkx2.5 and Myocd (Tanwar et al., 2014).

Treatment of differentiating mESCs with GREM2 starting at differentiation day 4, when GREM2 transcripts are first detected, increased the number of cardiomyocytes in culture. Appearance of spontaneously contracting cells was accelerated in treated cultures (Fig. 3A). αMHC-DsRed mESCs, a reporter stem cell line expressing DsRed fluorescent protein under control of the cardiac-specific alpha-myosin heavy chain promoter, showed a 20-fold increase in the number of positive cells in cultures treated with GREM2 compared to untreated controls (Fig. 3B). And GREM2 treated cultures stained with antibodies recognizing the cardiac-specific α-Actinin (Fig. 3C) and Troponin

T2 (Tnnt2) proteins had a 20 and 120-fold increase in positive cells respectively when compared to control, untreated cells (Tanwar et al., 2014).

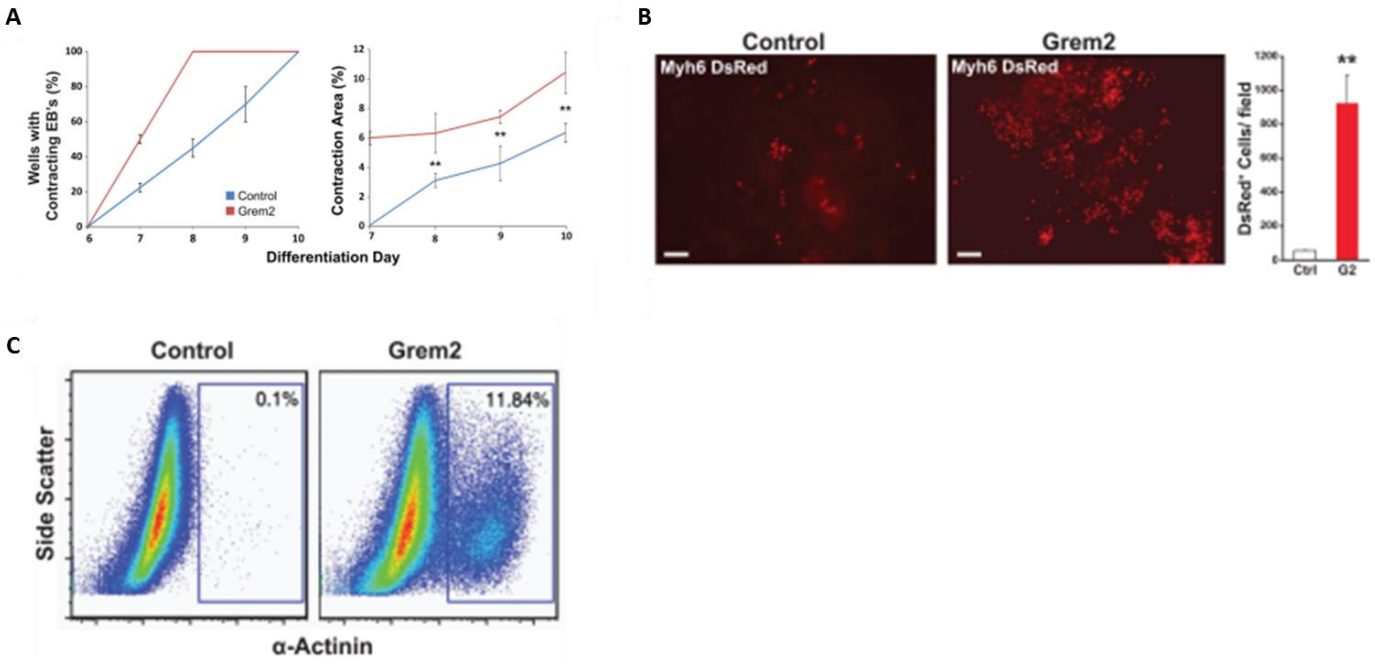


Figure 3: **Grem2 stimulates cardiac differentiation.** CGR8 embryonic stem cells (ESCs) were allowed to form EBs with or without addition of Grem2 starting at differentiation day 4. **(A):** Grem2 accelerates the appearance (left graph) and expands the size of contracting areas (as percent of the total culture area) in differentiating EBs (right). **, $p < .01$. **(B):** CGR8 ESCs were stably engineered to express the *DsRed2 fluorescent protein* gene fused to a nuclear localization signal under the *Myh6 promoter* (*Myh6-DsRed*) to specifically mark cardiomyocytes. Grem2 (G2) increases 20-fold the yield of cells with nuclear fluorescence compared to control. Scale bar = 50 μm. **, $p < .01$. **(C):** Grem2 increases 120-fold the number of α-Actinin$^+$ cardiomyocytes, quantified by flow cytometry analysis. Abbreviations: EB, embryoid body; Grem2, Gremlin 2; *Myh6*, myosin heavy chain 6. Used with permission from Tanwar et al., 2014.

RNA analysis of control (untreated) and GREM2-treated cells by quantitative PCR using gene-specific primers at early and late differentiation stages (day 6 and day 10 of differentiation respectively) showed that GREM2 treatment induced both early and late cardiomyocyte-specific markers such as Nkx2.5 (7-fold), Gata4 (5-fold), Tnnt2 (27-fold) and Myh6 (96-fold). GREM2 treatment also had a statistically significant inhibitory effect (2-5 fold) on the expression levels of both early and late hematopoietic- (Gata1,

Hbb-y), endothelial- (Vegfr2, CD31) and endodermal- (Foxa2, Afp) genes. Thus GREM2 increased the number of cardiac myocyte cells at the expense of other cardiovascular and even non-mesodermal lineages (Fig. 4) (Tanwar et al., 2014).

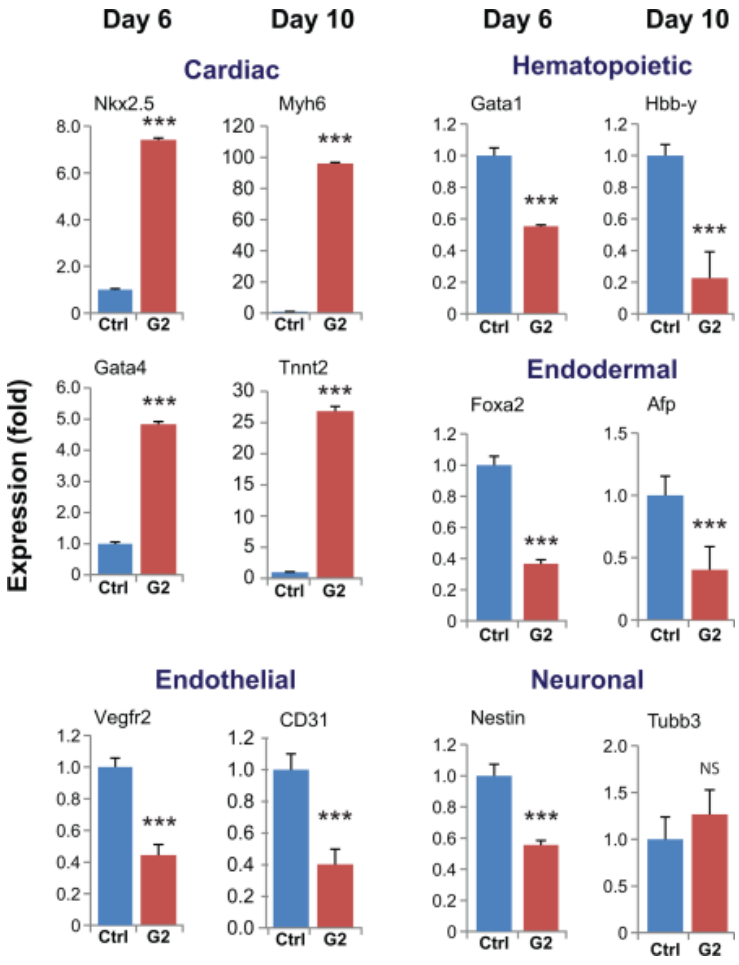


Figure 4: **Grem2 specifically expands cardiac differentiation.** CGR8 embryonic stem cells (ESCs) were allowed to differentiate untreated (Control, Ctrl) or treated with Grem2 (G2) between differentiation days 4 and 10. Quantitative polymerase chain reaction (PCR) analysis of RNA samples at day 6 or 10 of differentiation shows induction of cardiac genes (*Nkx2.5, Gata4, Myh6, Tnnt2*) and decrease in the expression levels of hematopoietic (*Gata1, Hbb-y*), endothelial (*Vegfr2, CD31*) and endodermal (*Foxa2*, *Afp*), as well as a subset of neuronal (*Nestin*, *Tubb3*) genes. Bars represent SE of at least three independent biological repeats in triplicates. ***, $p < .001$; NS, not significant. Abbreviations: *Afp,* alpha Fetoprotein*; CD31*, cluster of differentiation 31, or platelet endothelial cell adhesion molecule-1; *Foxa2*, Forkhead box A2; G2, Gremlin 2; *Gata4*: GATA binding protein 4; *Hbb-y*, hemoglobin Y, beta-like embryonic chain; *Myh6*, Myosin heavy chain 6; *Tnnt2*, Troponin T type 2; *Tubb3*, tubulin, beta 3 class III; *Vegfr2*, vascular endothelial growth factor receptor 2. Used with permission from Tanwar et al., 2014.

GREM2 treatment also had a qualitative effect on the type cardiomyocytes generated in treated mESC cultures. This was initially determined by the visual observation that GREM2 treated cultures contracted at higher rates than untreated cells. Because the rate of contraction is a function of the physiological properties of the

cells themselves, it was surmised that perhaps the cells had distinct electrophysiological properties (Maltsev et al., 1994; Mummery et al., 2002, 2012). Isolated cardiomyocytes from differentiated Myh6-DsRed CGR8 cultures treated with GREM2 were subjected to patch-clamp electrophysiology to measure action potentials. Untreated, control cells displayed a wide range of action potentials, either short, typical of atrial cells, or with a long plateau phase, characteristic of ventricular cells. In contrast, GREM2-treated differentiating ES cells gave rise to a relatively uniform cardiomyocyte population with short, atrial-like, action potentials (He et al., 2003; Hescheler et al., 1997). Further analysis of genes typically associated with atrial cardiomyocytes showed that GREM2 induced genes that encode atrial contractile proteins (Myl7 or Mlc2a), gap junction proteins (Gja5) and ion channels (Kcnj5, Cacna1d), as well as Nppa and Sarcolipin (Fleischmann et al., 2004; Houweling et al., 2002; Kubalak et al., 1994; Leaf et al., 2008; Minamisawa et al., 2003; Tanwar et al., 2014; Zhang et al., 2005).

GREM2 was able to induce cardiac differentiation and produce cardiac myocytes that exhibited characteristics of the atrial lineage. This induction was further found to be a specific property of the Gremlin subfamily of BMP antagonists. Differentiating mESCs treated with other BMP agonists or antagonists including BMP2, BMP4, Noggin, DAN, and Chordin failed to produce the same results as those observed with GREM2. Interestingly, Grem1, a protein closely related to GREM2 was also observed to have a cardiogenic effect on differentiating stem cells (Tanwar et al., 2014).

Among BMP inhibitors tested, only GREM2 was able to promote cardiogenesis in treated cultures. Thus BMP antagonism likely isn't the primary mechanism behind the cardiogenic effect observed in GREM2 treated cultures. As discussed in chapter 1,

BMP signaling is induced when BMP ligands bind to Type II receptors, forming a complex that engages the Type I receptors, which subsequently undergo transphosphorylation by the constitutively active kinase domain of Type II receptors. This leads to recruitment and phosphorylation of cytoplasmic Smads 1/5/8 (P-Smads), which then heterodimerize with Smad4 and translocate to the nucleus, acting as transcriptional regulators of BMP target genes such as Id2. Besides canonical, Smad-mediated signaling, activated BMP receptors can also stimulate several other signaling cascades involving p38, JNK and other MAP kinases (Sieber et al., 2009).

While GREM2 was able to equally antagonize canonical BMP signaling in mESCs when compared with other BMP antagonists, Western blot analysis of differentiating stem cells starting at day 4 against phospho-JNK (an indicator of non-canonical BMP signaling activity) showed that while Noggin and GREM2 treated samples could inhibit Smad-mediated canonical BMP signaling (Fig. 5 A), GREM2 treated samples had a robust upregulation of phospho JNK over the course of 96 hours from treatment (Fig. 5 B). This induction of JNK signaling was not observed in Noggin-treated cells. When JNK activation was blocked in GREM2-treated differentiating mESCs using two structurally unrelated small molecule JNK inhibitors, the cardiogenic effects of GREM2 treated were abolished (Fig. 5 C, D, and E). These results showed that GREM2 treated cells have an increase in JNK signaling activation after treatment with GREM2 and that this increase in JNK signaling is required for the cardiac induction observed with GREM2 (Fig. 5 E) (Tanwar et al., 2014).

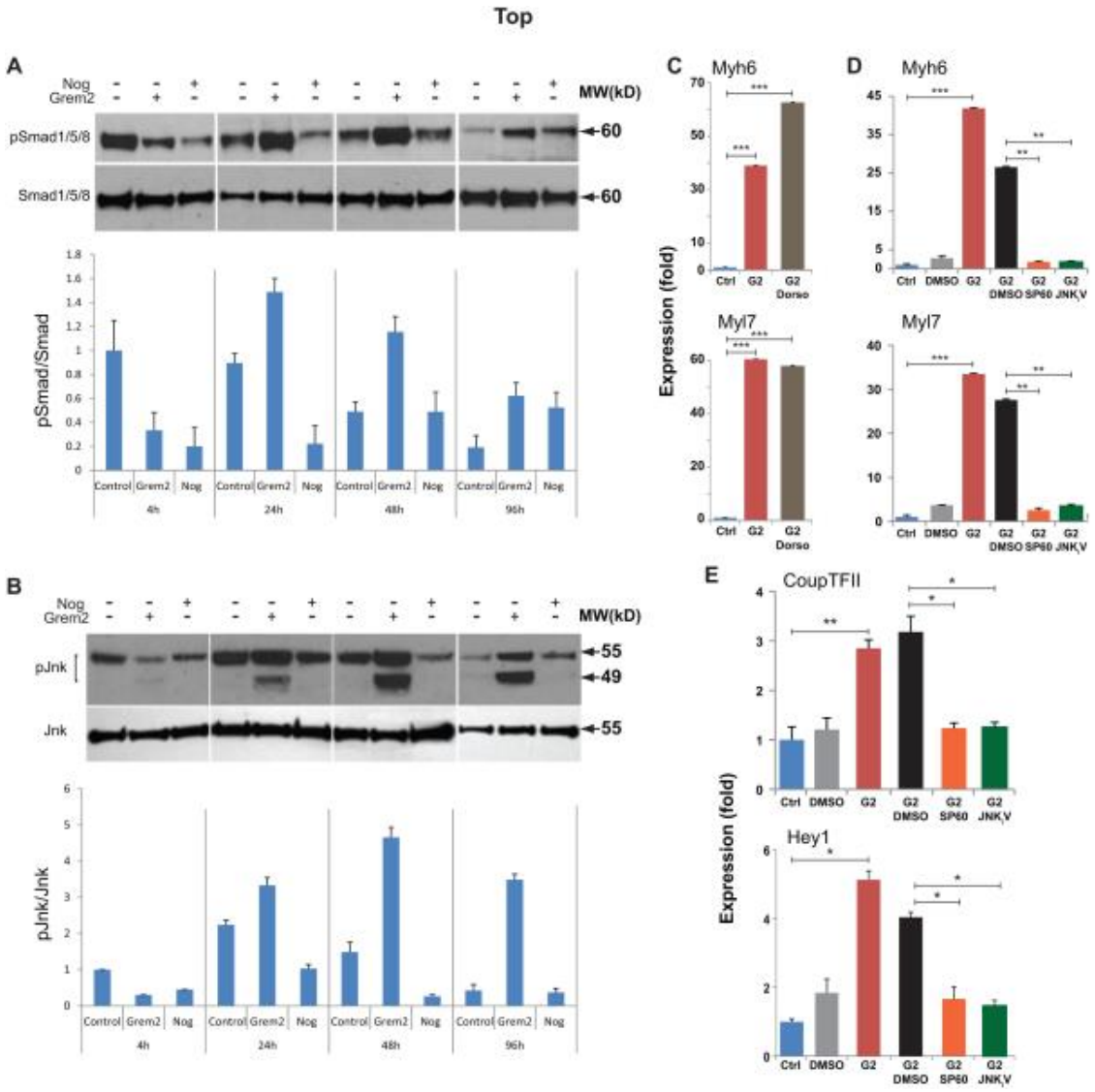


Figure 5: **JNK signaling activation by Grem2 is required for atrial cardiogenesis. (A):** CGR8 embryonic stem cells (ESCs) were allowed to differentiate untreated (Control) or treated between day 4 and 10 with Noggin (Nog) or Grem2. Western blot analysis of embryoid body (EB) protein lysates collected at sequential differentiation time points and blotted with antibodies recognizing total and phosphorylated forms of Smad1/5/8 [expected molecular weight (MW) 60 kD] and pJNK isoforms (49 and 55 kD, respectively). Both Noggin and Grem2 effectively inhibit Smad phosphorylation. However, in both Noggin- and Grem2-treated samples Smad phosphorylation recovers at later time points. In contrast, only Grem2 treatment leads to strong upregulation of JNK phosphorylation. Graphs below the blots represent quantification of phosphorylated Smad and JNK forms relative to total protein levels. **(C, D):** CGR8 ESCs were allowed to differentiate untreated (Control, Ctrl) or treated between days 4 and 10 with Grem2 (G2). Grem2 samples were also treated with the Smad1/5/8 inhibitor dorsomorphin (Dorso) and the JNK inhibitors SP600125 (SP60) and JNK_i V. The JNK inhibitors' vehicle (DMSO) was also used as a control. Quantitative polymerase chain reaction (PCR) analysis of *Myh6* and *Myl7* expression at differentiation day 10 shows that JNK inhibition suppresses Grem2-induced cardiac and atrial genes, whereas dorsomorphin has no effect. Control values were arbitrarily set at 1. **(E):** Quantitative polymerase chain reaction analysis at D10 shows that JNK inhibition suppresses Grem2-induced activation of atrial regulatory genes *CoupTFII* and *Hey1*. *, $p < .05$; **, $p < .01$; ***, $p < .001$, comparing samples as indicated by horizontal bars. Abbreviations: *CoupTFII*: COUP transcription factor 2; DMSO, dimethyl sulfoxide; Grem2, Gremlin 2; *Hey1*: hairy/enhancer-of-split related with YRPW motif 1; JNK, c-Jun N-terminal Kinase; *Myh6*, Myosin heavy chain 6; *Myl7*, myosin light chain 7; Nog, Noggin. Used with permission from Tanwar et al., 2014.

Conclusion and Hypothesis

The data discussed so far demonstrate that the GREM2 morphogen regulates proper cardiac camber formation, establishment of cardiac rhythm during embryonic development, and can be employed to promote differentiation of mouse ESCs to cardiomyocytes. The cardiogenic effect of GREM2 is unique among BMP antagonists and relies on an upregulation of non-canonical JNK-mediated BMP signaling activation. (Tanwar et al., 2014). To date all of these data represent studies conducted in non-human model systems. While these models can yield general mechanistic insights into the differentiation of cardiac myocytes, in order to translate these discoveries into a more clinical relevant context, a human model is ideal. I hypothesized that GREM2 plays a critical role in human cardiac differentiation and can be used as a cardiogenic agent in human pluripotent stem cells.

Chapter 4

GREM2 is Expressed in Human Cardiac Progenitor Cells and Cardiac Myocytes

Introduction

Chapters 4-8 describe my thesis work, completed in the laboratory of Dr. Antonis Hatzopoulos and reported in the journal Stem Cells and Development (Bylund et al., 2017). The text and figures from the published report have been used in chapters 4-8 with permission from the publisher. The work reported in these chapters represents the contributions of a number of collaborators and colleagues. A detailed description of contributions is found in Appendix B.

Expression of GREM2 in a variety of developmental contexts, and most importantly that of cardiac development, has already been discussed in the previous chapter. To date no studies have been carried out looking at GREM2 expression in the context of human cardiac development.

Studies of human development in general are limited by a lack of access to tissues, reproductive characteristics untenable for common laboratory research, and important ethical considerations (Greely et al., 1989). Human pluripotent stem cells have been used as a proxy for studying human development *in vitro* (Dvash et al., 2006). Human embryonic (ES) and induced pluripotent stem (iPS) cells differentiate to a variety of distinct tissue-specific cell types, providing a unique resource to study human

embryonic development and disease mechanisms (Bellin et al., 2012; Golos et al., 2010; Sayed et al., 2016; Sterneckert et al., 2014).

Thus pluripotent stem cells may be used as a model of human cardiac development. In order to establish a context for GREM2 expression and function in human cardiac development, I analyzed human pluripotent stem cells that were differentiated to cardiac myocytes using established cardiac differentiation protocols and using gene expression and immunostaining assays to investigate the pattern and cell specificity of GREM2 expression.

MATERIALS & METHODS

In situ hybridization

Mouse E8.0 embryos were prepared and analyzed for in situ hybridization analysis using GREM2-specific riboprobes using standard protocols (Müller et al., 2006, 2013).

Human pluripotent stem cell culture

Human induced pluripotent stem cell lines iMR90 and DF 19-9-11 from WiCell were cultured under feeder free conditions in mTeSR1 or Essential 8 (E8) media (Stem Cell Technologies) as previously described (Beers et al., 2012). Briefly, cells were thawed and seeded onto 6-well plates coated with 8.7 µg/cm^2 growth factor reduced Matrigel (Corning) in mTeSR1 or E8 media supplemented with 10 µM Y-27632 dihydrochloride (Tocris Bioscience) to promote cell survival and attachment. Media was exchanged daily until cells reached approximately 60-70% confluence. Cells were

passaged using Versene EDTA cell dissociation reagent (Thermo Fisher Scientific/Gibco) and seeded onto Matrigel-coated plates in mTeSR1 or E8 media supplemented with 10 µM Y-27632 dihydrochloride at a split ratio of 1:15 - 1:20. Cells were kept in a copper-lined humidified incubator (Thermo Fisher Scientific) at 37˚C with a 5% CO_2 atmosphere.

Human embryonic stem (hES) cells WA07 were cultured in conditioned media as previously described (Xu et al., 2001). Briefly, cells were cultured in mouse embryonic fibroblast- conditioned medium (MEF-CM) supplemented with basic fibroblast growth factor (bFGF) (8 ng/ml). Cells were cultured for 4-6 days or until colonies occupied 75-80 % of well surface area. Cells were then passaged by rinsing with DPBS and incubating with collagenase (200 units/ml) for 5-10 min at 37°C to dissociate them into small clumps. Clumps were then plated onto 6-well cell culture plates pre-coated with 1ml/well of 50 µg/ml Matrigel (Corning). Cells were kept in a copper-lined humidified incubator (Thermo Fisher Scientific) at 37˚C with a 5 % CO_2 atmosphere.

hPS cell differentiation

Human iPS cells were differentiated using either the “Matrix Sandwich” method, the GiWi method, or the BMP/ACTIVIN A method as already described (Laflamme et al., 2007b; Lian et al., 2013; Zhang et al., 2012). In all differentiation methods, cells were allowed to become 80-90 % confluent then dissociated by incubating in Versene EDTA cell dissociation reagent (Thermo Fisher Scientific/Gibco) for 10 min at room temperature. After incubation, cells were triturated to dissociate into a single cell

suspension. Single cell preparations were centrifuged for 5 min at 200 x g and cell pellets were re-suspended in cell culture media for plating.

For the “Matrix Sandwich” method, cells were re-suspended in E8 or mTeSR1 media (Stem Cell Technologies) supplemented with 10 µM ROCKi (Y-27632 dihydrochloride, Tocris) and plated onto Matrigel-coated (8.7 µg/cm^2) 12-well culture plates at a density of 500,000 cells per well. Fresh E8 or mTeSR1 media was given daily until cells became 90 % confluent. When 90 % confluent, cells were overlayed with 8.7 µg/cm^2 growth factor reduced Matrigel (Corning) in E8 or mTeSR1 media. After 24 hrs the matrix coating solution was removed and fresh E8 or mTeSR1 media were added until cells were 100 % confluent. Cells were then treated with 1 ml/well of Day 0 media (RPMI 1640 media supplemented with B27 minus insulin, coated with 8.7 µg/cm^2 Matrigel, and 100 ng/ml ACTIVIN A, R&D Systems). Exactly 24 hrs later Day 0 media was aspirated and cells were treated with 1.5 ml/well of Day 1 media (RPMI 1640 media supplemented with B27 minus insulin (Life Technologies), 5 ng/ml of hBMP4 (R&D Systems), and 10 ng/ml hbFGF (Life Technologies). Four days after addition of Day 1 media, cells were treated with 1 ml/well basal differentiation media (RPMI 1640 supplemented with B27 plus insulin). Cells treated with GREM2 received 1 ml/well of RPMI 1640 media with B27 minus insulin supplemented with 150 ng/ml GREM2 exactly 48 hrs after adding Day 1 media (day 3). At day 5, GREM2-treated wells received basal differentiation medium with 150 ng/ml of GREM2. Media in all wells was replaced daily. Cells were treated with similar fashion with 50 ng/ml NOGGIN or 1.5 µg/ml DAN, based on their specific activities (R&D Systems). GREM2 wild-type protein and mutated

versions of GREM2 were synthesized, purified and measured for activity by the laboratory of our collaborator Dr. Thomas Thompson as previously described (Kattamuri et al., 2012a, 2012b; Nolan et al., 2013).

The BMP/ACTIVIN A method followed the same protocol as described for the "Matrix Sandwich" method, but without the Matrigel overlay steps.

For the GiWi method, cells were re-suspended in E8 media (Stem Cell Technologies) supplemented with 10 µM ROCKi (Y-27632 dihydrochloride, Tocris) and plated onto Matrigel-coated (8.7 µg/cm^2) 12-well culture plates at a density of 500,000 cells per well. Once cells were 100 % confluent (typically 3-4 days after seeding) differentiation was started by adding 2 ml/well of Day 0 media (RPMI 1640 with B27 minus insulin and 12 µM CHIR 99021). Exactly 24 hrs after adding Day 0 media, cells were treated with 2 ml/well early differentiation media (RPMI 1640 with B27 minus insulin). After 48 hrs 1 ml/well of conditioned media was removed from differentiating cells and combined with 1 ml early differentiation media and supplemented with 2 µM IWR-1 endo (Tocris). After 48 hrs, cells were treated with 2 ml/well of late differentiation media (RPMI 1640 with B27 plus insulin). Late differentiation media was then replaced daily.

Human ES cells were differentiated as previously described (Xu et al., 2011). Briefly, WA7 hESCs were rinsed with 2 ml DPBS and incubated with 2 ml Versene (EDTA, Life Technologies) for 10 min at 37°C. Versene was aspirated and replaced

with 1 ml/well of mouse embryonic fibroblast conditioned media supplemented with 8 ng/ml of bFGF. hESCs were triturated to produce a single cell suspension and seeded onto 24-well Matrigel-coated (8.7 µg/cm^2) plates at a density of 400,000 cells per well. Cells were given fresh media daily until 100 % confluent. Once 100% confluent, cells were given 1 ml/well Day 0 medium (RPMI 1640 with 2% B27 minus insulin and 100 ng/ml ACTIVIN A). Cells were incubated at 37°C for 24 hrs and then treated with 1 ml/well of Day 1 medium (RPMI 1640 with 2% B27 minus insulin and 10 ng/ml BMP-4). After 4 days, the medium was replaced with late differentiation medium (RPMI 1640 with 2% B27). 1 ml/well fresh late differentiation media was added to each well every other day.

Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR)

Cells were collected from each well using Tryp-LE Select (Thermo Fisher Scientific) and centrifuged at 200 x g for 5 min to pellet. Cell pellets were lysed using RLT buffer and RNA was isolated using the RNeasy Mini Kit following the manufacturer's instructions (Qiagen). cDNA was generated by reverse transcription of 1-3 µg of RNA as I have previously reported (Tanwar et al., 2014). cDNA samples were amplified using GoTaq qPCR master mix (Promega) in a Bio-Rad CFX thermocycler. Relative gene expression levels were calculated using the delta-delta Ct method (Beck et al., 2008; Livak and Schmittgen, 2001). Relative primer efficiencies were determined using the Real-time PCR Miner algorithm and confirmed experimentally using the slope of the standard curve from plotting log(DNA copy number) vs. Ct value (Zhao and Fernald, 2005). Amplification primer sequences are reported in Table 1.

Immunofluorescence

Cells were seeded onto Matrigel-coated (8.7 µg/cm^2) 12-well plastic culture plates (Thermo Fisher Scientific) at a density of 500,000 cells per well and differentiated as described above for the “Matrix Sandwich” method. Cells were fixed at the desired time points (differentiation days 4, 5, 6 and 10) by rinsing with 500 µl DPBS and incubating in 4 % PFA in PBS at 4^{o}C for 5 min. The PFA solution was then aspirated and cells were rinsed with 1X PBS 5 times. Fixed cells were permeabilized by incubating with 400 µl permeabilization buffer (0.2 % Triton X-100 in 1X PBS) in each well at room temperature for 1 hr. Nonspecific binding was then blocked using 400 µl/well of blocking solution (5 % non-fat dry milk in permeabilization buffer) for 2 hrs at room temperature with gentle rocking. For all subsequent steps, a minimum of 350 µl of solution was added to each well. Cells were then washed 3 x 5 min with 400 µl/well of 1X PBS at room temperature. After washing, cells were incubated with primary antibodies in incubation buffer (0.1 % Triton X-100, 1 % BSA in 1X PBS) overnight at 4°C with gentle rocking. Next, cells were washed 3 x 3 min each, with PBST (0.2 % Tween-20 in 1X PBS) followed by 3 x 3 min each with 1X PBS. Cells were then incubated with secondary antibodies diluted in incubation buffer for 1 hr at room temperature in the dark. Finally, cells were then washed 2 times, 3 min each, with 1X PBS and stored in 400 µL 1X PBS for imaging.

Imaging was done using a Leica DM IRB Inverted Microscope or a Zeiss Laser Scanning Microscope (LSM 880). Image analysis and volume rendering were done using NIS Elements, Zen, ImageJ/FIJI, and Imaris software suites. Primary antibodies

recognizing NKX2.5 (Santa Cruz Biotechnology, sc-8697, 1:50), GREM2 (ProteinTech, 13892-1-AP, 1:100), and α-ACTININ (Sigma, A7811, 1:500) were applied. I have also tested anti-GREM2 rabbit polyclonal antibodies from GeneTex (GTX108414), Abcam (ab102563) and R&D Systems (AF2069) at 1:100 dilution. The fluorescent dye 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) was added during the final 15 mins of secondary incubation at a 1:10,000 dilution to stain nuclei.

RESULTS

GREM2 is expressed in differentiating human pluripotent stem cells during cardiac progenitor cell expansion and differentiation

We have previously shown that *GREM2* expression in zebrafish embryos first appears within pharyngeal arch mesoderm, adjacent to the cardiac field, during the early stages of heart tube formation (Müller et al., 2006, 2013). To test whether this expression is conserved in mouse embryos, I performed *in situ* hybridization analysis during the early stages of cardiac development. I found that *GREM2* expression appears around embryonic day 8 and overlaps with the nascent cardiac field area [Fig. 6, (Tanwar et al., 2014)]. These data are consistent with those observed in zebrafish, with GREM2 appearing at a similar developmental stage and embryonic area.

Figure 6: **In situ hybridization analysis of E8.0 mouse embryos** using anti-sense (left) and sense (right) Grem2 riboprobes shows Grem2 expression in the cardiac field area (arrow).

To determine whether GREM2 is associated with early human cardiac development, I cultured and differentiated the human induced pluripotent stem cell line DF 19-9-11 using the “Matrix Sandwich” method that was designed for efficient cardiomyocyte differentiation (Zhang et al., 2012). I then isolated RNA samples at consecutive days of differentiation and analyzed the temporal program of cardiogenic development by RT-qPCR. As shown in Fig. 7, expression of the *T BRY* gene that marks mesoderm formation is transiently induced at days 2-3 after initiation of differentiation, followed by sequential expression of markers specific to early cardiogenic mesoderm (*MESP-1*), cardiac progenitor cells (*NKX2.5*, *ISL1*, and *KDR* or *VEGFR2*), and cardiomyocytes (*TNNT2*, *MYH6*). Expression of endothelial and hematopoietic genes (*CD31*, *VE-CADHERIN*, *HBBY*) were at low levels (data not

shown), likely because the “Matrix Sandwich” method is optimized specifically for cardiac differentiation.

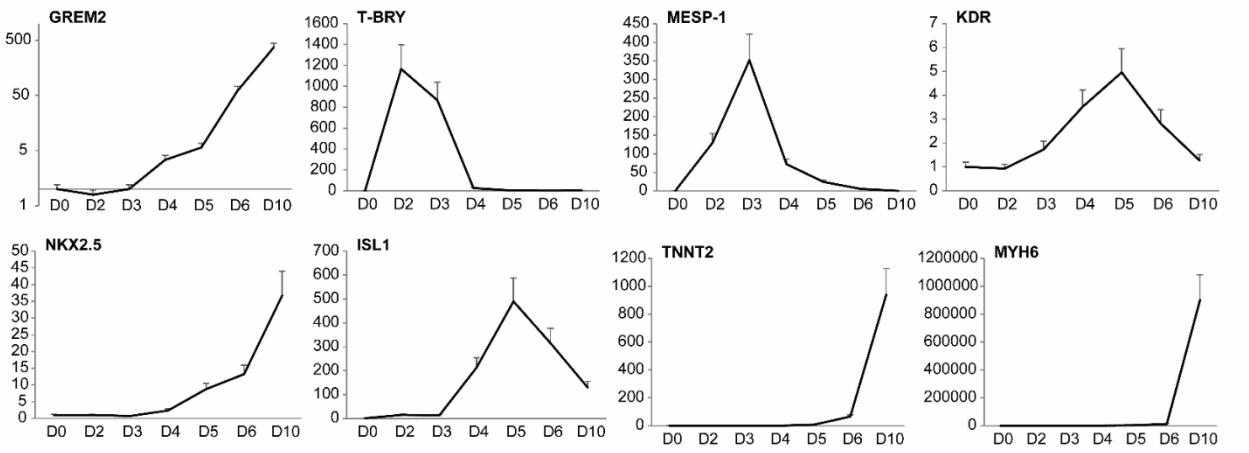


Figure 7: ***GREM2* expression during human iPS cell cardiac differentiation follows the expression pattern of cardiac-specific genes.** RT-qPCR gene expression analysis in DF 19-9-11 human induced pluripotent stem (iPS) cells during cardiac differentiation [day 0 (D0) to day 10 (D10)] using the “Matrix Sandwich” method indicates cardiogenic mesoderm specification (*MESP-1*) and the appearance of cardiovascular progenitor cell gene markers (*KDR*, *NKX2.5*, *ISL1*). *GREM2* expression appears concurrently with cardiovascular progenitor cells and increases during differentiation to cardiomyocytes marked by *TNNT2*, and *MYH6* genes. N= 3 replicates per condition. Abbreviations: GREM2: GREMLIN 2; ISL-1: INSULIN GENE ENHANCER PROTEIN 1; KDR: KINASE INSERT DOMAIN RECEPTOR; MESP-1: MESODERM POSTERIOR bHLH TRANSCRIPTION FACTOR 1; MYH6: MYOSIN HEAVY CHAIN 6; NKX2.5: NK2 HOMEOBOX 5; T-BRY: T-BRACHYURY; TNNT2: CARDIAC TROPONIN T 2.

GREM2 expression in the context of BMP signaling components

To place *GREM2* expression in the context of other BMP signaling components during hiPS cell differentiation, I analyzed expression of select BMP ligands, BMP receptors and BMP antagonists. Our results show that *CERBERUS LIKE 1* and *CHORDIN* are transiently induced during mesoderm formation, in accordance with their

expression patterns in other species (Biben et al., 1998; Hsu et al., 1998; Sasai et al., 1994; Tanwar et al., 2014). I also found that *NOGGIN* is expressed at later time points of differentiation. *GREM2* expression appears after mesoderm formation, coincidently with early cardiac markers such as *NKX2.5*, and continues to rise during cardiac differentiation (Fig. 8). The expression of *BMP2* and *BMP4* are consistent with their respective roles in cardiac differentiation in mice, with *BMP2* being expressed first during cardiogenic specification, and followed by *BMP4* (Ma et al., 2005; McCulley et al., 2008). In contrast to the dynamic expression of BMP ligands and BMP antagonists during cardiac differentiation, BMP receptors (*BMPR2*, *ALK3*, *ALK6*) appear to be expressed at constant levels throughout the differentiation process.

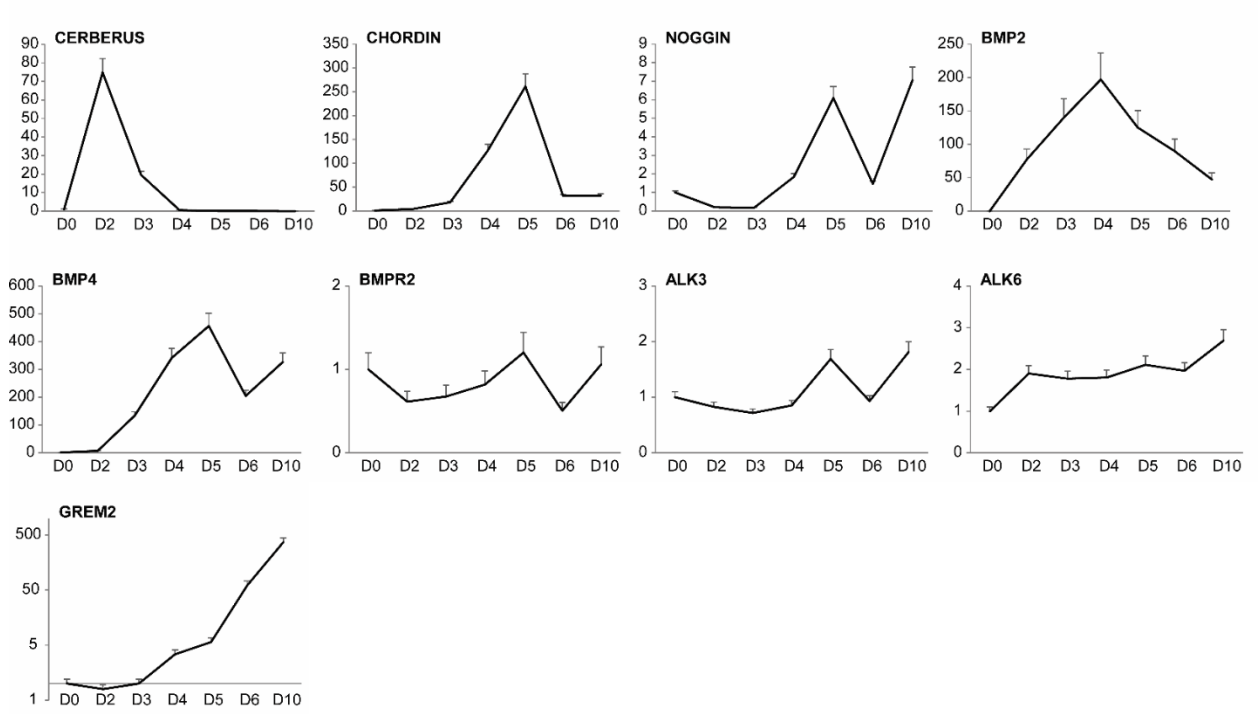


Figure 8: ***GREM2* expression during human iPS cell cardiac differentiation in the context of BMP signaling.** The expression pattern of BMP ligands BMP2 & BMP4, BMP antagonists CERBERUS-LIKE 1 and NOGGIN, as well as BMP receptors type 2 (BMPR2) and type 1 (ALK3 and ALK6) are indicated. N= 3 replicates per condition. Abbreviations: ALK3 or 6: ACTIVIN A RECEPTOR TYPE II-LIKE KINASE 3 or 6, also BMPR1A or BMPR1B; BMP2 or 4: BONE MORPHOGENETIC PROTEIN 2 or 4; BMPR2: BONE MORPHOGENETIC PROTEIN RECEPTOR 2; GREM2: GREMLIN 2.

GREM2 expression pattern is consistent across stem cell lines and differentiation protocols

A similar expression pattern for *GREM2* was also observed in 1) the WA07 human embryonic stem cells and 2) the independently reprogrammed human induced pluripotent stem cell line iMR90 C4 (Fig. 9). Furthermore, because the "Matrix Sandwich" method of cardiac cell differentiation depends on timely activation and withdrawal of BMP signaling, which may influence expression of BMP ligands and

antagonists, I used the distinct GiWi differentiation protocol that relies on sequential activation and inhibition of canonical Wnt signaling (Lian et al., 2013). The results show

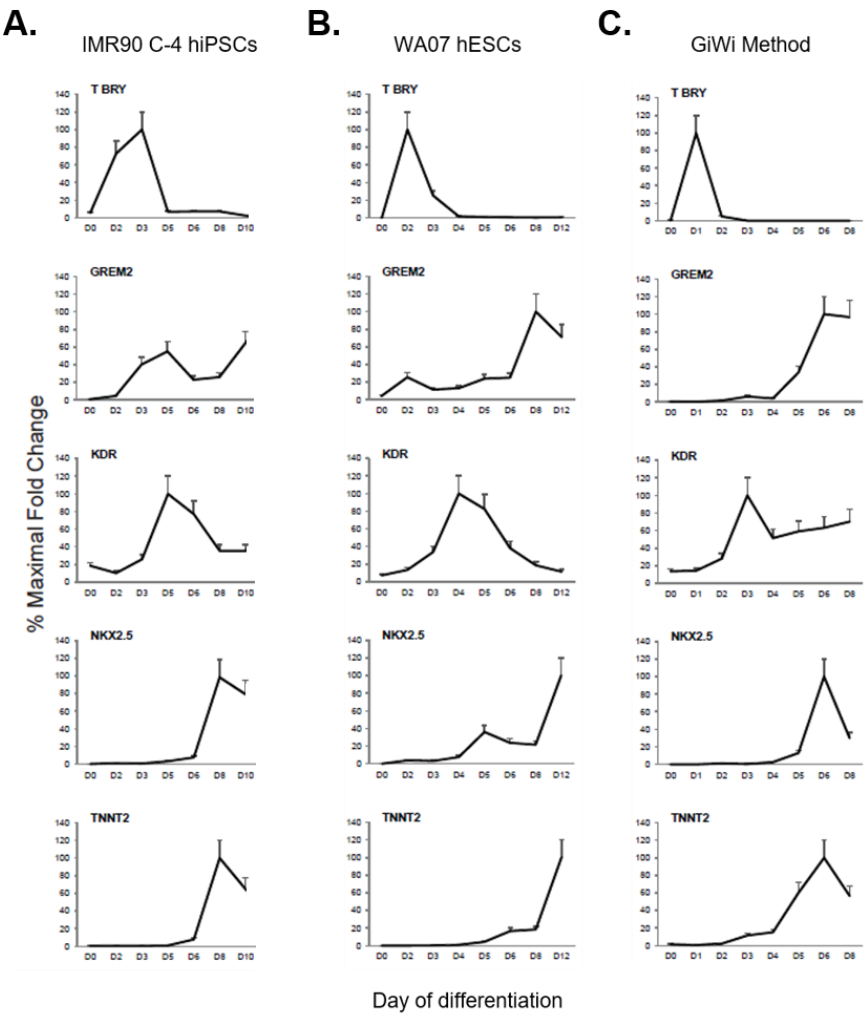


Figure 9: ***GREM2* expression pattern during cardiac differentiation is conserved among pluripotent stem cell lines and independent of differentiation method.** RT-qPCR analysis of *GREM2*, *T-BRY*, *KDR*, *NKX2.5 and TNNT2* genes at distinct differentiation time points from day 0 (D0) as indicated. (A) Gene expression during differentiation of human iMR90-C4 iPS cells. (B) Gene expression during differentiation of human embryonic stem (hES) cells WA07. (C) Gene expression during differentiation of DF 19-9-11 human iPS cells using the small-molecule Wnt modulation or GiWi method. N=3 replicates per condition.

a similar induction profile of *GREM2* during cardiac differentiation following the GiWi protocol, indicating that the *GREM2* expression pattern is independent of the differentiation method used or the pluripotent cell line used for differentiation (Fig. 9).

Taken together, my data demonstrate that *GREM2* expression starts after mesoderm formation, during the initial phase of cardiac progenitor cell (CPC) specification, and is retained throughout CPC expansion and differentiation to cardiomyocytes. The GREM2 expression pattern is unique among BMP antagonists, although *NOGGIN* is also expressed within specific time windows of cardiac differentiation. The *GREM2* expression pattern is consistent with our previous studies in zebrafish and mouse development, as well as mouse ES cells differentiation (Müller et al., 2006, 2013; Tanwar et al., 2014), indicating that *GREM2* expression, and possibly function, have been conserved during evolution from lower vertebrates to humans. Furthermore, the *GREM2* expression pattern is consistent among different human ES and iPS cell lines and independent of the applied differentiation protocol, suggesting GREM2 expression during early cardiac differentiation in hiPSCs is conserved and not cell-line or protocol dependent.

GREM2 is expressed in human cardiac progenitor cells and differentiated cardiomyocytes

To determine the cellular source of GREM2, I performed immunofluorescence (IF) staining on fixed cells during early (day 5 and 7) and late (day 10) differentiation stages using primary antibodies against GREM2, and antibodies recognizing typical

cardiac-specific proteins, such as the transcription factor NKX2.5 and the sarcomeric component α-ACTININ.

To identify antibodies specific for detection of human GREM2 protein in cultured cells, I screened four distinct, commercially available, anti-GREM2 primary antibodies. To this end, I used HEK 293 cells transiently transfected with a plasmid expressing the human *GREM2* cDNA under the regulation of the CMV promoter, or empty vector as control. Transfected cells were fixed, stained, and imaged using a fluorescence microscope (Fig. 10). Three antibodies were observed to have a GREM2-specific signal. Among these antibodies, signal strength was observed using fluorescent microscopy and the antibody that generated the highest contrast between true signal and background was subsequently selected for immunolabeling of differentiating iPS cells.

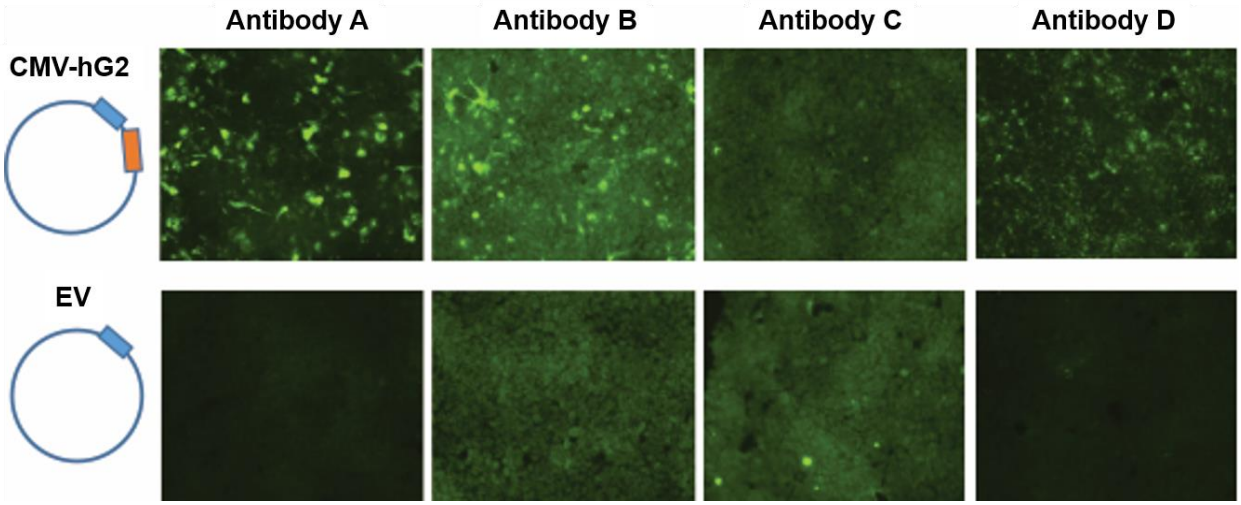


Figure 10: **Specificity and robustness screen of available antibodies recognizing human GREM2 protein.** Plasmid constructs containing the coding region of the human *GREM2* gene under the CMV promoter (CMV-hG2) and empty vector (EV) as control were used for transfection of human embryonic kidney (HEK293) cells. Four distinct antibodies against GREM2 from different commercial sources were used to immunostain transfected HEK293 cells. Antibody A, rabbit polyclonal Grem2 antibody (ProteinTech, 13892-1-AP) gave the best noise to signal ratio and was used in subsequent staining experiments. The remaining anti-GREM2 antibodies tested were rabbit polyclonal antibodies from GeneTex (Cat# GTX108414), Abcam (ab102563) and R&D Systems (AF2069).

I found that GREM2 is expressed in NKX2.5^{+} cells at differentiation day 5 (Fig 7A). This expression continued through differentiation day 7 (Fig. 12 B). GREM2 protein expression persists in α-ACTININ^{+} cardiomyocytes at day 10 of differentiation (Fig. 11 B and Fig. 12 C). GREM2 was not detected in NKX2.5^{-} cells, suggesting GREM2 expression is confined to the cardiac lineage during iPS cell differentiation and acts in autocrine fashion.

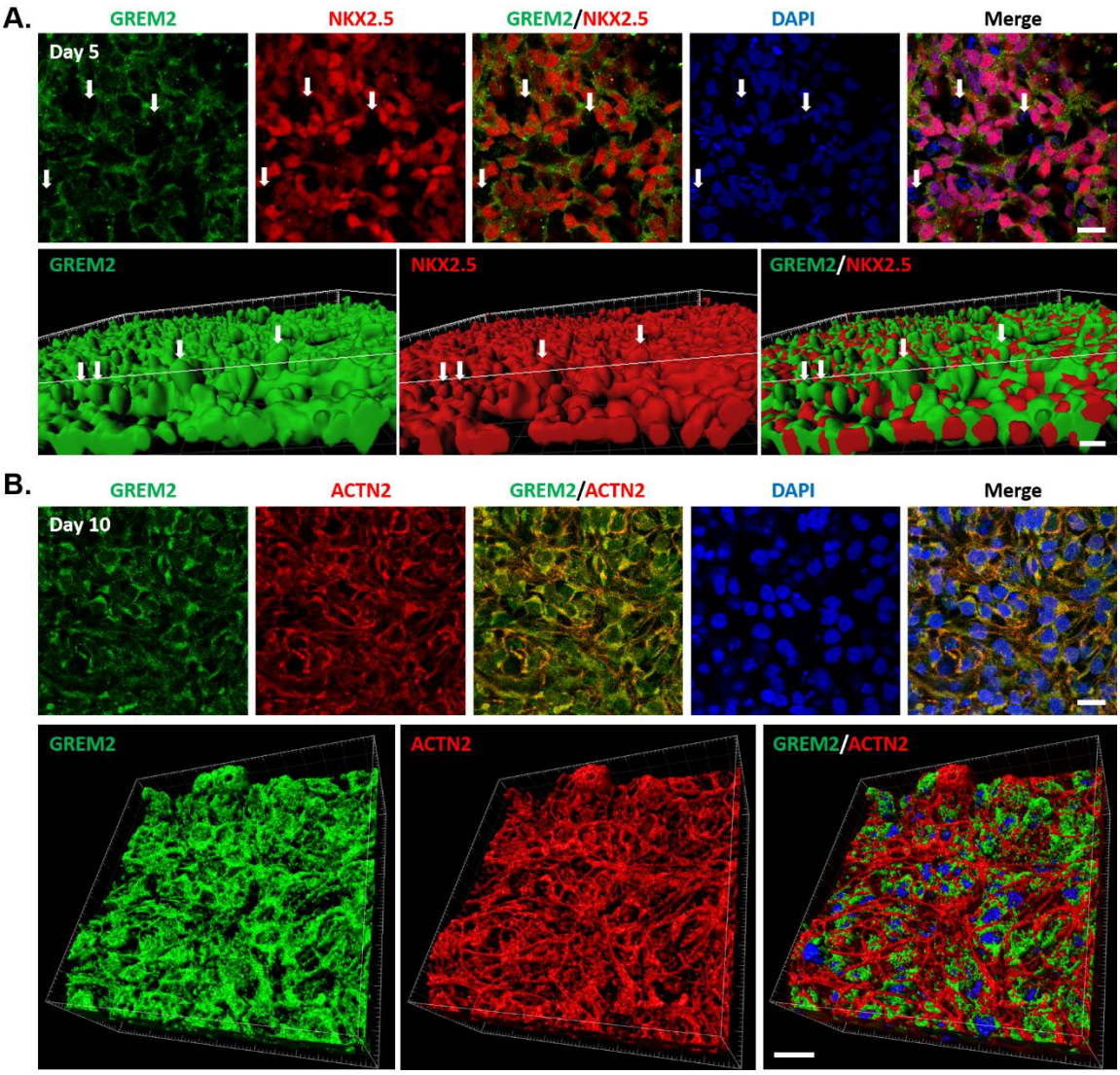


Figure 11: **GREM2 is expressed in cardiac progenitor cells and cardiomyocytes.** Immunofluorescence analysis of differentiating human iPS cells. (A) Cardiac progenitor cells at differentiation day 5, marked with NKX2.5 (red), express GREM2 (green). White arrows mark NKX2.5- cells that also lack GREM2 expression (upper panels). Scale bar, 30 μm. 3-D reconstruction using z-stack confocal microscopy images illustrates GREM2 expression in NKX2.5+ cells (lower panels). Scale bar, 30 μm. (B) Differentiated cardiomyocytes at differentiation day 10, marked with α-ACTININ (red) express GREM2 (green; upper panels). Scale bar, 30 μm. 3-D reconstruction of confocal microscopy images (lower panels) shows GREM2 expression in differentiated cardiomyocytes with sarcomeric structures. Scale bar, 20 μm. DAPI staining (blue) marks cell nuclei. Abbreviations: ACTN2: α-ACTININ.

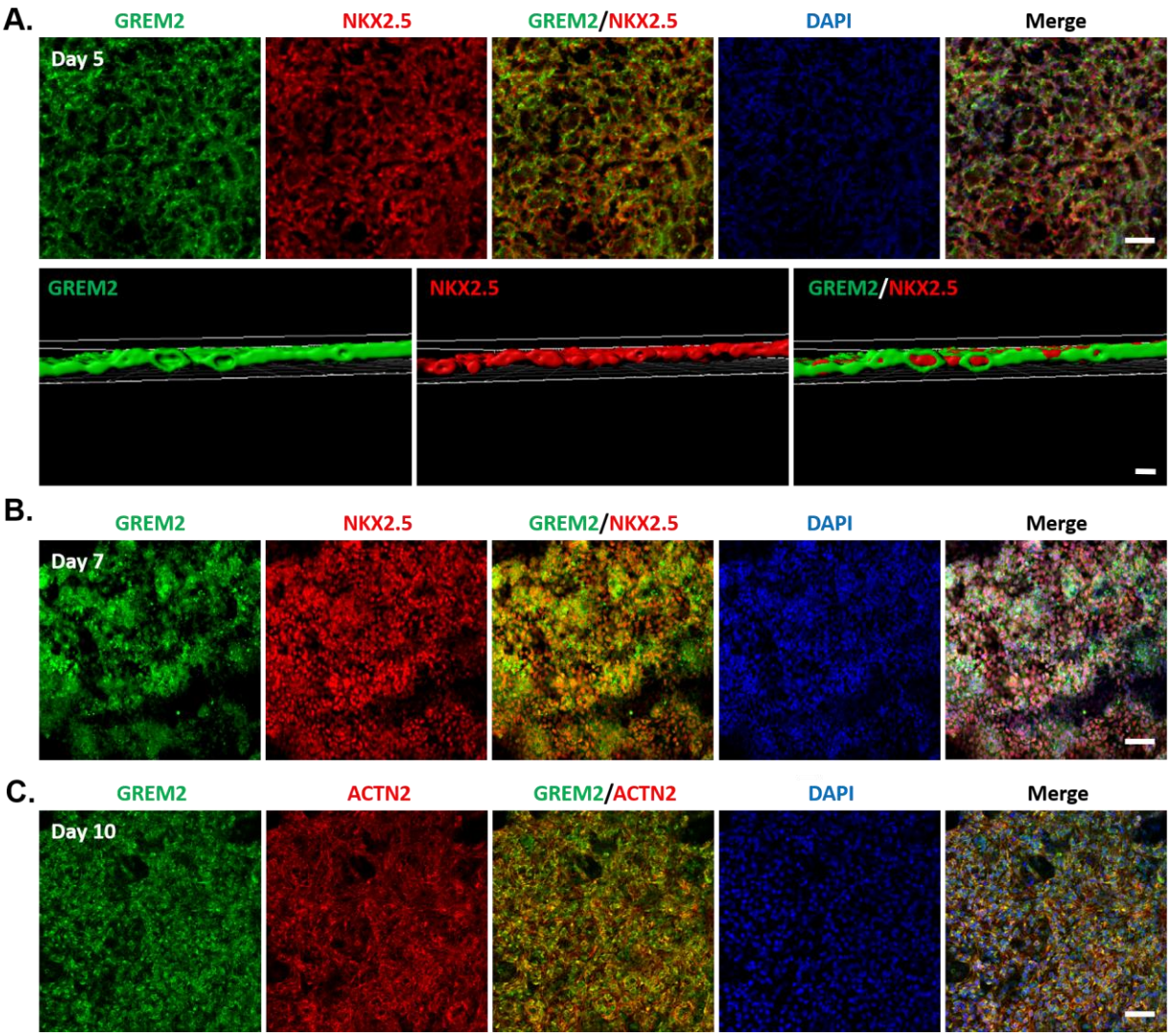


Figure 12: **Low magnification (20X) images show extent of GREM2 expression in hiPSCs.** Immunofluorescence analysis of differentiating human DF 19-9-11 iPS cells. (A) Low magnification images of iPS cells at differentiation day 5 using the "Matrix Sandwich" method showing GREM2 (green) expression in $NKX2.5^{+}$ (red) cardiac progenitor cells (top panels). 3-D reconstruction of confocal microscopy images (lower panels) shows GREM2 expression in the cytoplasm and pericellular areas of $NKX2.5^{+}$ cardiac progenitor cells. Scale bar, top 100 μm; bottom 50 μm. (B) Low magnification images of iPS cells at differentiation day 7 using the "Matrix Sandwich" method showing GREM2 expression persists in differentiating $NKX2.5^{+}$ cardiac cells. Scale bar, 100 μm. (C) Low magnification images of iPS cells at differentiation day 10 using the "Matrix Sandwich" method shows the extent of GREM2 expression in α-ACTININ^{+} cardiomyocytes. Scale bar, 100 μm. DAPI staining (blue) marks cell nuclei.

Summary

Pluripotent stem cells follow a well ordered progression of transition states from their naïve pluripotent state towards cardiomyocytes. This process can be tracked using marker genes and proteins that are characteristic of distinct functional cell types.

The immunofluorescence data described above indicate that GREM2 is present in the cytoplasm of cardiac progenitor cells and maturing cardiomyocytes. GREM2 is a secreted protein (Hsu et al., 1998). It is possible that the GREM2 labeling observed in the cells reflects production of GREM2 within the cytoplasm that is being trafficked to the membrane for secretion. Presence of secreted protein is difficult to detect using immunofluorescence techniques. Future experiments involving crosslinking and then immunolabeling of differentiating cultured hiPSCs could provide clarification.

Using human pluripotent stem cells I have shown that GREM2 is expressed during human cardiac differentiation. This expression begins as cardiac progenitor cells are being specified and continues through cardiac differentiation as cells transition to functional cardiac myocytes. This expression pattern is not unique to a single cell line nor is it unique to a single differentiation protocol. The consistent nature of GREM2 expression among protocols and the timing of its expression during cardiogenesis suggest that GREM2 function is conserved and that it plays a role in human cardiac differentiation.

Chapter 5

BMP Signaling Inhibition is Required for Cardiomyocyte Differentiation

Introduction

BMP2 is a prototypical dimeric BMP ligand and initiates canonical BMP signaling via high affinity binding to BMPRI receptors via a so called “wrist” epitope, a central alpha helical arrangement within the BMP2 homodimer (Kirsch et al., 2000). Once bound to BMPRI the BMPRI:BMP2 complex is then able to bind BMPRII via a “knuckle” region, a smaller, lower affinity epitope comprised of five beta sheets (Kirsch et al., 2000).

BMP2 is regulated by modulator proteins that directly bind the BMP2 homodimer and interfere with its ability to bind BMPRI and/or BMPRII (Keller et al., 2004). These proteins include Noggin and members of the CAN/DAN family including GREM2 (Nolan et al., 2016). These modulator proteins bind with high affinity directly to the wrist and/or knuckle regions of BMP homodimers thereby blocking the ability of BMP ligands to interact with either of their BMPR partners (Keller et al., 2004; Zhu et al., 2006).

L51R is a mutant BMP2 ligand that has a substitution of arginine for leucine at position 51 which renders it unable to bind BMPRs and activate BMP signaling but retains its ability to bind BMP modulators such as Noggin (Albers et al., 2012). Addition of excess amounts of L51R to cultured cells effectively sequesters BMP antagonists by

competing for binding with endogenous, wild-type BMP molecules (Albers et al., 2012). This makes it an effective tool for studying the role of BMP antagonism *in vitro*.

GREM2 is known to inhibit canonical BMP signaling, and the spatial and temporal expression data described above suggest it may play a role in cardiac differentiation. These data also show that the BMP inhibitor Noggin is expressed during cardiomyocyte differentiation. These data suggest that BMP inhibition may play a role in human cardiac differentiation, though it is not known if BMP inhibition is necessary for proper human cardiomyocyte differentiation. To investigate the role of BMP inhibition in human cardiac differentiation I tested the ability of the L51P mutant to interfere with GREM2-mediated inhibition of BMP signaling. L51P was then used to treat differentiating human induced pluripotent stem cells to determine if BMP inhibition is required for proper human cardiomyocyte differentiation.

Materials and Methods

Human pluripotent stem cell culture

Human induced pluripotent stem cell lines iMR90 and DF 19-9-11 from WiCell were cultured under feeder free conditions as described in Chapter 4.

iPS cell differentiation

Human iPS cells were differentiated using the “Matrix Sandwich” method as described in Chapter 4. Cells were treated with L51P decoy protein starting at day 4 of

differentiation. The L51P decoy was provided by our collaborators in the lab of Dr. Thom Thompson at the University of Cincinatti.

Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR)

Cells were collected from each well using Tryp-LE Select (Thermo Fisher Scientific) and centrifuged at 200 x g for 5 min to pellet. Cell pellets were lysed using RLT buffer and RNA was isolated using the RNeasy Mini Kit following the manufacturer's instructions (Qiagen). cDNA was generated by reverse transcription of 1-3 µg of RNA as I have previously reported (Tanwar et al., 2014). cDNA samples were amplified using GoTaq qPCR master mix (Promega) in a Bio-Rad CFX thermocycler. Relative gene expression levels were calculated using the delta-delta Ct method (Beck et al., 2008; Livak and Schmittgen, 2001). Relative primer efficiencies were determined using the Real-time PCR Miner algorithm and confirmed experimentally using the slope of the standard curve from plotting log(DNA copy number) vs. Ct value (Zhao and Fernald, 2005). Amplification primer sequences are reported in Table 1.

Cell quantification

Cells were dissociated into single cell suspensions using Tryp-LE Express (Life Technologies), stained with trypan blue (diluted 1:2 in PBS or cell culture media) to exclude dead cells and quantified using a Bio-Rad TC-10 automated cell counter. For DAPI stained cells, cell numbers were quantified by dividing 3 fields of view from three independent wells per condition into quadrants and manually counting the number of nuclei visible in each quadrant.

Luciferase assays

CGR8 mouse embryonic stem (ES) cells were transfected with the BRE2-Luc reporter construct with luciferase expression under the control of two canonical BMP signaling responsive elements of the *Id2* gene (Monteiro et al., 2004; Tanwar et al., 2014). Cells were treated for 8 hrs with 40 ng/ml BMP4, 50 ng/ml GREM2, and/or 300 ng/ml BMP decoy protein L51P (Albers et al., 2012). Firefly luciferase activity was normalized to Renilla luciferase activity to account for transfection efficiency.

Results

Titration of GREM2 concentrations for effective inhibition of canonical BMP signaling

The GREM2 expression data described above suggest that BMP signaling antagonism is necessary for cardiomyocyte differentiation. To test this possibility, I treated differentiating iPS cells with a L51P mutant of BMP2 that was shown to bind the BMP antagonist NOGGIN, but unable to al bind BMPRI and activate BMP signaling, thus acting as a BMP ligand decoy (Albers et., 2012). To test the ability of the BMP decoy to block GREM2, I first transfected CGR8 mouse Embryonic Stem (ES) cells with a plasmid containing the firefly *luciferase* gene under the control of the BMP-signaling response elements from the *Id2* gene promoter [BRE_2-Luc, (Monteiro et al., 2004; Tanwar et al., 2014)]. Optimal concentrations for effective BMP signaling inhibition by

GREM2 were first determined by titrating increasing concentrations of GREM2 in cells exposed to a constant amount of BMP4 (Fig. 13).

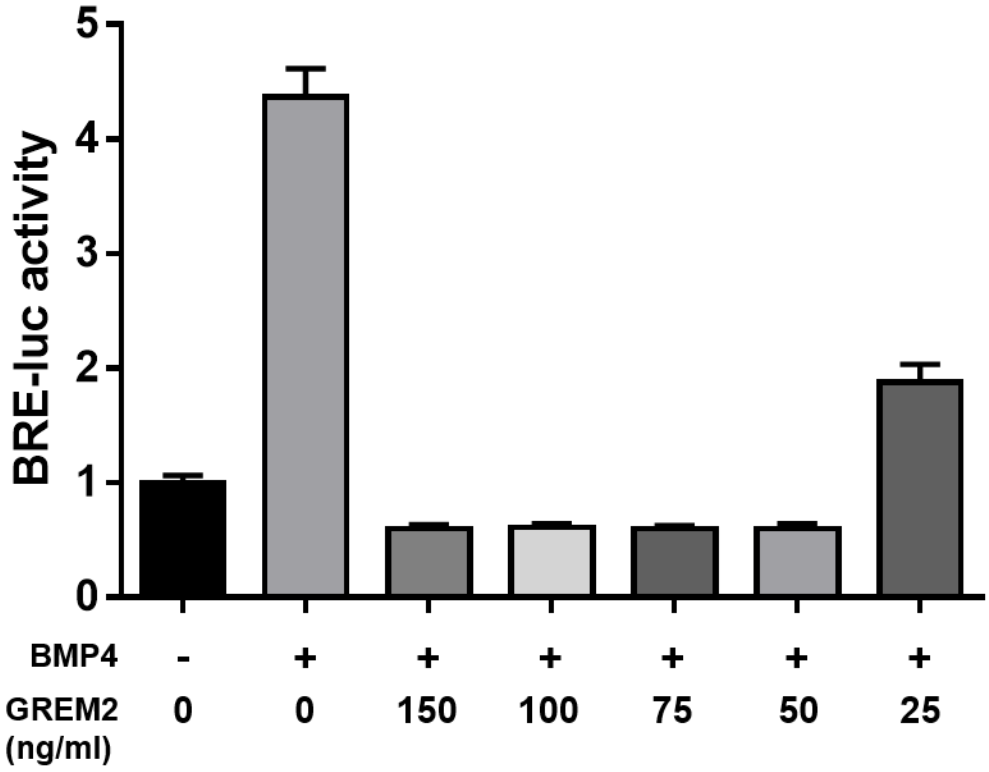


Figure 13: **GREM2 protein titration of BMP signaling inhibition.** Mouse embryonic stem cells were transfected with the BRE2-Luc reporter construct and treated for 8 hrs with 40 ng/ml BMP4 and decreasing concentrations of GREM2 as indicated. 50 ng/ml (or higher concentrations) of GREM2 completely inhibited canonical BMP signaling activated by BMP4. N = 3.

BMP decoy L51P effectively blocks GREM2 mediated inhibition of BMP signaling

The cells were then treated with BMP4, GREM2, and/or the BMP decoy. The results show that BMP4 induces canonical BMP (BRE_2-Luc) signaling, whereas the BMP decoy alone does not signal nor does interfere with BMP4 signaling activation

(Fig. 14). GREM2 inhibits BMP4 activity, where GREM2 alone does not induce BRE_2-Luc activity, as expected. Moreover, co-incubation of BMP4, GREM2 and the BMP decoy restored canonical BMP signaling, indicating that the BMP decoy effectively blocks the inhibitory effect of GREM2 (Fig. 14).

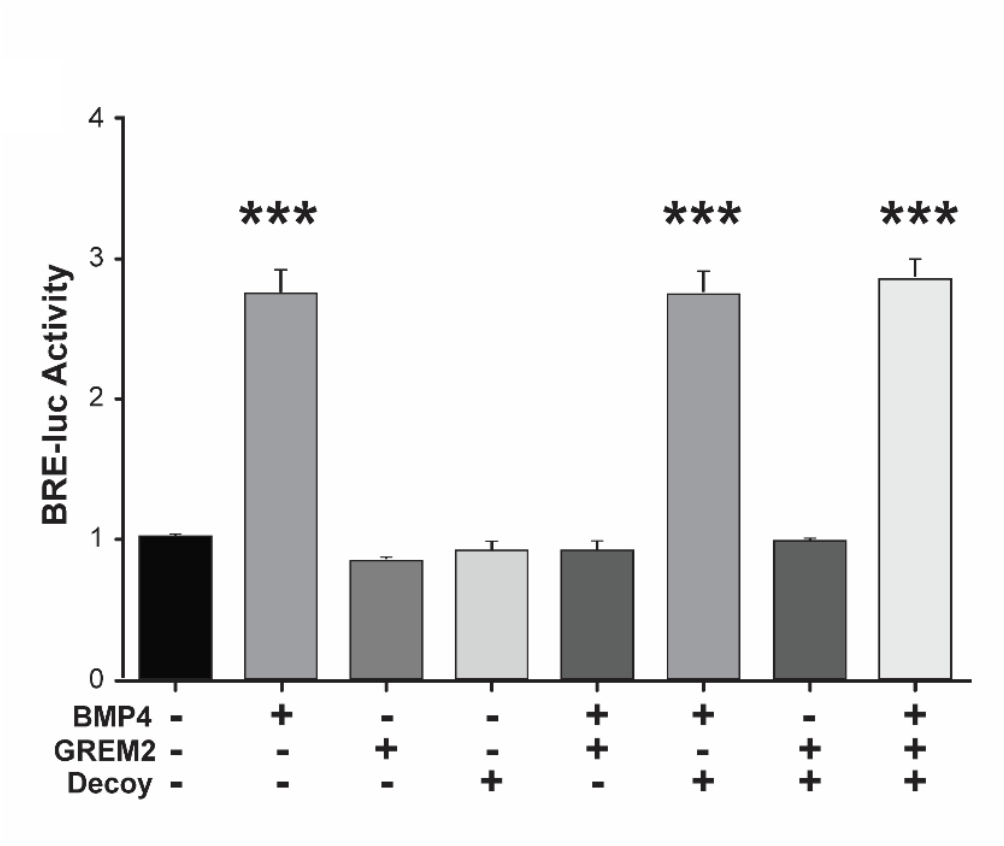


Figure 14: **BMP decoys block the inhibitory effects of GREM2**. Mouse embryonic stem (ES) cells were transfected with the BRE2-Luc reporter construct with luciferase expression under the control of two canonical BMP signaling responsive elements of the Id2 gene. Cells were treated for 8 hrs with BMP4, [GREM2], and the [L51P] BMP2 decoy in various combinations as indicated. In the presence of the BMP decoy, GREM2 inhibition of BMP signaling is aborted. One-way ANOVA with Dunnett's test was performed to compare the experimental groups to the no treatment control group. N = 3 replicates per condition, ***P < 0.001. Error bars represent std dev.

BMP inhibition is required for human pluripotent stem cell cardiac differentiation

Treatment of differentiating human pluripotent stem cells with the BMP decoy protein starting at day 4 of differentiation, when cardiac progenitor cells (CPCs) first appear and GREM2 expression is induced, caused a significant reduction in cardiac gene expression levels and reduced the percentage of contracting cells in culture (Fig. 15A and B respectively).

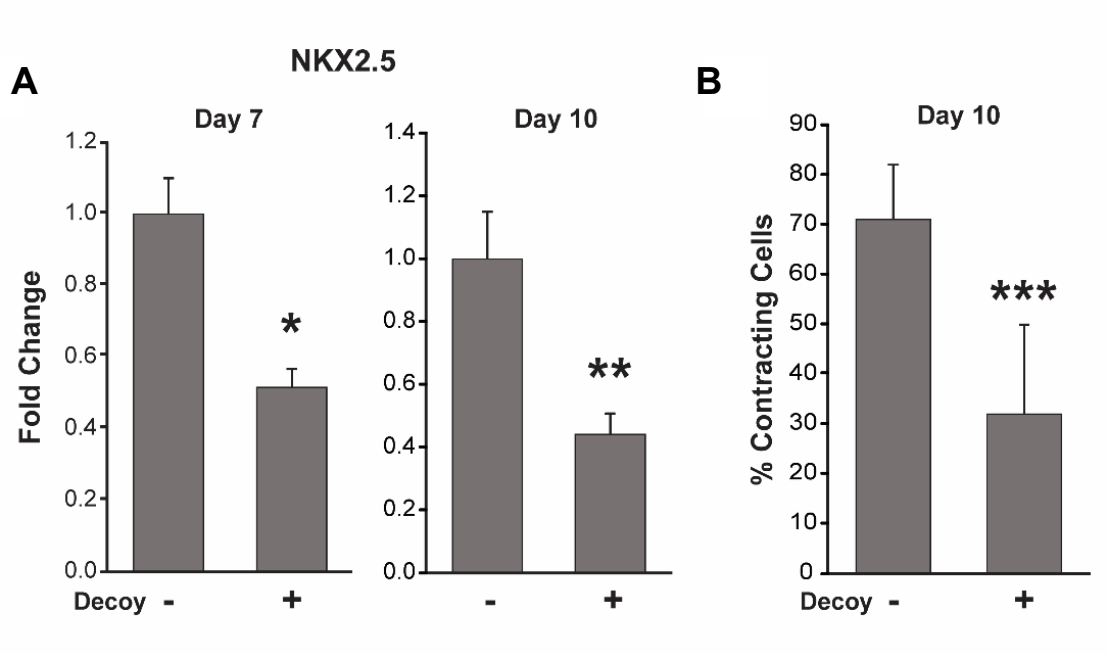


Figure 15: **BMP ligand antagonism is required for cardiac differentiation of human iPS cells.** (A) RT-qPCR gene expression analysis of RNA samples isolated from DF 19-9-11 iPS cells at day 7 and 10 of differentiation with or without BMP decoy treatment shows a reduction in cardiac gene expression in samples treated with the BMP decoy. N=3 replicates per condition. Student's t test. *P < 0.05; **P < 0.01. (B) iPS cells treated with the BMP decoy BMP have a significant reduction in the percentage of contracting cells at differentiation day 10. N=3 replicates per condition. Student's t test. ***P < 0.001. Error bars represent standard deviation.

Summary

Here I used the BMP mutant L51P to investigate the importance of BMP signaling inhibition in the cardiac differentiation process. I showed that when GREM2 is added in combination with an excess of L51P to BMP reporter stem cells then GREM2 is unable to effectively block BMP signaling. This was also observed for the BMP antagonist Noggin, indicating that an excess of L51P can effectively sequester GREM2 and other BMP antagonists (Albers et al., 2012).

I further showed that when L51P was added in excess to differentiating human pluripotent stem cells it was able to reduce the total expression of the cardiac gene NKX2.5 during early and late cardiac differentiation time points (days 7 and 10 respectively). Adding the BMP inhibitor sequestration mutant L51P to the differentiating cells also reduced the total percentage of cardiomyocytes in culture when compared to untreated controls. This reduction was consistent and significant. These data suggest that BMP inhibitors such as GREM2 are required for cardiac differentiation in human pluripotent stem cells.

These results indicate that BMP inhibition is required and that GREM2 mediated inhibition may be necessary for proper cardiac function. However, the system described cannot distinguish between GREM2 and other inhibitors. Based on the qPCR data described in chapter 4, Noggin is the only other inhibitor tested whose expression is timed in such a way as to be involved in the cardiac differentiation process (Fig. 8). This would be consistent with knock-out studies in mice showing that Noggin is required for proper cardiac differentiation (Choi et al., 2007).

Future studies that directly test the necessity of GREM2 and Noggin for proper human cardiac differentiation are required. Emerging gene targeting strategies such as CRISPR have shown promise for conducting loss of function studies in human pluripotent stem cells (Hockemeyer and Jaenisch, 2016). Neutralizing antibodies that recognize and sequester GREM2 could also be used to directly test the necessity of GREM2 for cardiac differentiation. Among the currently available anitbodies tested for this purpose, none were able to neutralize GREM2.

Another way to directly test the effect of GREM2 on cardiac differentiation is to use gain of function assays. Treating differentiating cells with exogenous GREM2 at critical time points will reveal whether GREM2 is sufficient to affect human cardiac differentiation. This approach is described in the following chapter.

Chapter 6

GREM2 Enhances Cardiac Differentiation of Human Induced Pluripotent Stem Cells

Introduction

So far I have shown that GREM2 is expressed during cardiac differentiation as cardiac progenitor cells are forming and then continues as progenitor cells differentiate to mature cardiac myocytes. The data already described above suggest that GREM2 and Noggin may play a critical role in the human cardiomyocyte differentiation process. However a more direct examination of the role of these two inhibitors is required.

In order to test the hypothesis that GREM2 is able to enhance cardiac differentiation, I differentiated human induced pluripotent stem cells and tested the effect of purified GREM2 protein starting at differentiation day 3, as cardiac progenitor cells are beginning to form and when endogenous GREM2 expression is detected. Specifically, I examined the effect of GREM2 addition on the amounts of cardiomyocytes present in culture, both in total and as a percentage, and the expression of cardiac specific genes. GREM2 mutants with variable BMP binding affinities were also used to investigate whether GREM2-BMP interaction is required for any cardiogenic effect seen. I further tested the effect of GREM2 treatment in an independent human pluripotent stem cell line to verify that the effects of GREM2

treatment were not cell line dependent but applied more generally to cardiac differentiation.

Materials and Methods

Human pluripotent stem cell culture

Human induced pluripotent stem cell lines iMR90 and DF 19-9-11 from WiCell were cultured under feeder free conditions in mTeSR1 or Essential 8 (E8) media (Stem Cell Technologies) as already described in Chapter 3.

iPS cell differentiation

Human iPS cells were differentiated using the “Matrix Sandwich” method as described in chapter 3. Cells treated with GREM2 received 1 ml/well of RPMI 1640 media with B27 minus insulin supplemented with 150 ng/ml GREM2 exactly 48 hrs after adding Day 1 media (day 3). At day 5, GREM2-treated wells received basal differentiation medium with 150 ng/ml of GREM2. Media in all wells was replaced daily. Cells were treated with similar fashion with 50 ng/ml NOGGIN or 1.5 µg/ml DAN, based on their specific activities (R&D Systems). GREM2 wild-type protein and mutated versions of GREM2 were synthesized, purified and measured for activity as previously described (Kattamuri et al., 2012b, 2012a; Nolan et al., 2013).

Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR)

Cells were collected from each well using Tryp-LE Select (Thermo Fisher Scientific) and centrifuged at 200 x g for 5 min to pellet. Cell pellets were lysed using RLT buffer and

RNA was isolated using the RNeasy Mini Kit following the manufacturer's instructions (Qiagen). cDNA was generated by reverse transcription of 1-3 µg of RNA as I have previously reported (Tanwar et al., 2014). cDNA samples were amplified using GoTaq qPCR master mix (Promega) in a Bio-Rad CFX thermocycler. Relative gene expression levels were calculated using the delta-delta Ct method (Beck et al., 2008; Livak and Schmittgen, 2001). Relative primer efficiencies were determined using the Real-time PCR Miner algorithm and confirmed experimentally using the slope of the standard curve from plotting log(DNA copy number) vs. Ct value (Zhao and Fernald, 2005). Amplification primer sequences are reported in Supplementary Table S1.

Cell quantification

Cells were dissociated into single cell suspensions using Tryp-LE Express (Life Technologies), stained with trypan blue (diluted 1:2 in PBS or cell culture media) to exclude dead cells and quantified using a Bio-Rad TC-10 automated cell counter. For DAPI stained cells, cell numbers were quantified by dividing 3 fields of view from three independent wells per condition into quadrants and manually counting the number of nuclei visible in each quadrant.

Flow cytometry

Differentiated human iPS cells were washed once with DPBS minus Mg^{2+} and Ca^{2+}, incubated with 1 ml/well Accutase at room temperature for 15 mins, and triturated to break up into single cells. Accutase was diluted by adding two volumes of late differentiation medium per well and contents were transferred to 15 ml conical tubes

through mesh strainer caps to remove large cell clumps. Prior to centrifugation, and after visual confirmation of complete cell detachment and single cell dissociation with Accutase, the total cell number per well was quantified using the Bio-Rad TC-10 automated cell counter as described above. Cell suspensions were then centrifuged at 200 x g for 5 min to pellet cells. Cell pellets were rinsed 1 x time with DPBS minus Mg^{2+} and Ca^{2+} and re-pelleted. Washed pellets were re-suspended in 500 µl Fix/Perm buffer (fixation/permeabilization solution, BD Biosciences), transferred to 5 ml round bottom flow cytometry tubes (BD Biosciences), and incubated for 40 min on ice. Cells were then pelleted at 300 x g for 5 min and supernatant was aspirated. Cells were washed 2 x times with Perm/Wash buffer (BD Bioscience) and pelleted by centrifugation at 300 x g for 5 min. Cells were blocked by adding 100 µl blocking buffer (BD Bioscience) to each tube and incubating on ice with gentle rocking for 30 min. Primary antibodies recognizing NKX2.5 (Abcam, ab91196) and MYH6 (Abcam, ab50967) at a 1:100 dilution were added directly to the cell suspensions in each tube and incubated for two hrs on ice with gentle rocking. To wash out primary antibodies, 3 ml of perm/wash buffer was added to each tube and then centrifuged at 300 x g for 5 min. Supernatant was aspirated and cells were washed with 2 ml/tube fresh Perm/Wash solution and centrifuged at 300 x g for 5 min. For secondary antibody incubation, cell pellets were suspended in 100 µl blocking buffer with secondary antibodies at 1:400 dilutions. Cells were incubated with rocking for 30 min on ice and then washed twice with perm/wash buffer. Cells were then suspended in Flow Assay Buffer (BD Biosciences) and the percentage of $NKX2.5^{+}$ and $MYH6^{+}$ cells was determined using a FACS Aria flow cytometer and analyzed using FACS Diva (BD Bioscience) or FlowJo (FlowJo, LLC)

software in the Vanderbilt University Shared Flow Cytometry Resource Laboratory. The total number of cardiomyocytes per well was calculated by multiplying the total cell number per well (as described above) by the percentage of $NKX2.5^+$ or $MYH6^+$ cells as determined by flow cytometry.

Results

hiPSC cardiac differentiation protocol for GREM2 treatment

The results described in chapter 4 suggest inhibition of BMP signaling is necessary for cardiomyocyte differentiation of human iPS cells. To test whether GREM2 can further enhance human cardiomyocyte differentiation, I added GREM2 protein to differentiating iPS cells from day 3 onwards, when cardiogenic mesoderm appears and endogenous *GREM2* expression begins to rise. The “Matrix Sandwich” protocol (SP) served as a positive control, whereas a modified, truncated, “Matrix Sandwich” differentiation protocol (MP) without any factor addition after mesoderm induction at day 3 served as the baseline or negative control (Fig. 16).

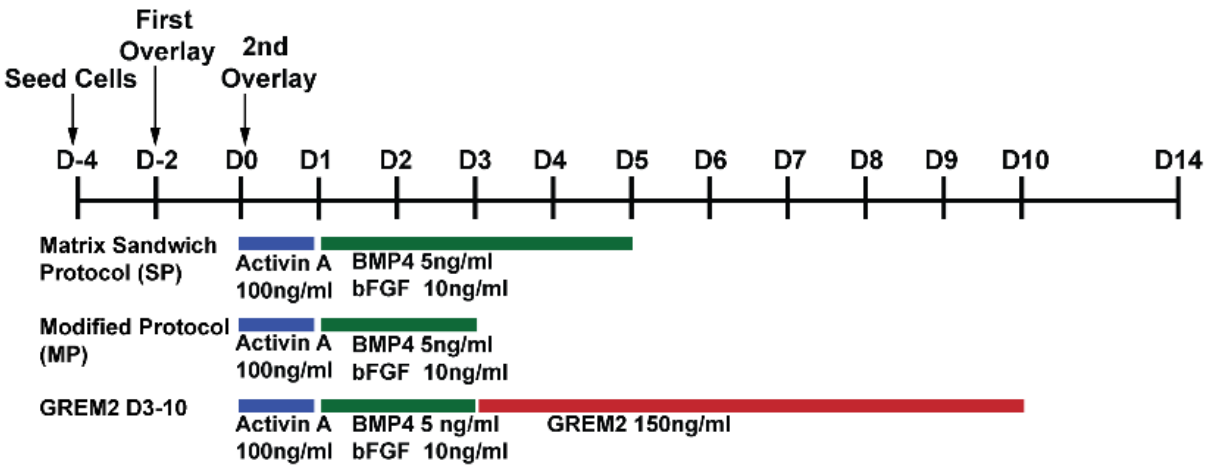


Figure 16: **Cardiac differentiation protocols for testing the effects of Grem2 treatment on differentiating human pluripotent stem cells.** Schematic diagram showing treatment schedule and conditions of the three differentiation protocols using DF 19-9-11 iPS cells. D -4 through D14 represent differentiation day minus 4 through day 14 respectively. Addition of growth factors and treatment duration are indicated below the time line.

GREM2 treatment accelerates the appearance of cardiomyocytes in cultured hiPSCs

Visual comparison of culture revealed that GREM2 protein addition accelerated the appearance of contracting cells, indicating the presence of cardiomyocytes at day 7, which is 1 day earlier than the “Matrix Sandwich” method and 3 days earlier than the negative control (Fig. 17A). In addition, GREM2 also increased the overall areas of contracting cells, suggesting further expansion of the cardiac lineage (Fig. 17B).

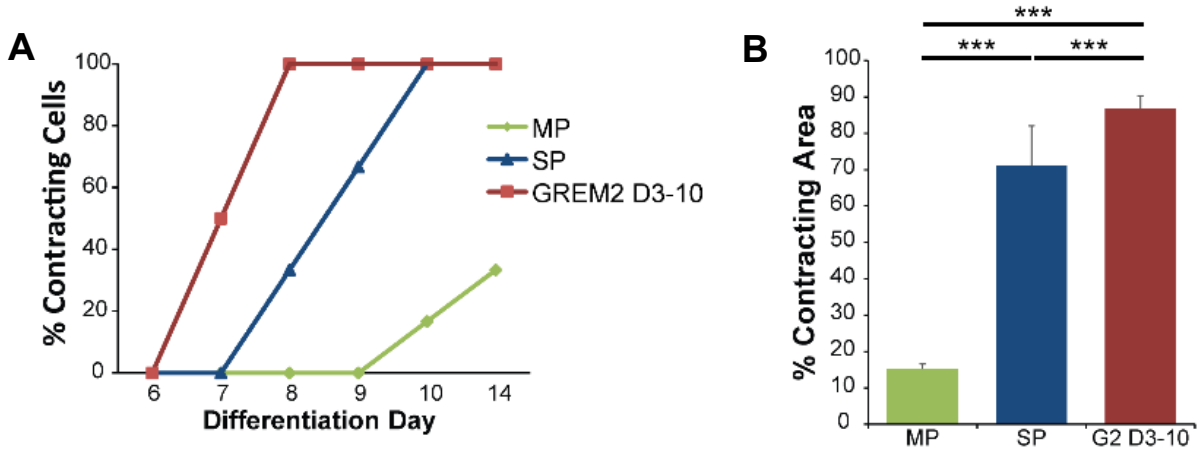


Figure 17**: Grem2 accelerates the appearance of spontaneously contracting cells and increases % of well with contracting cells.** (A) Differentiating iPS cells treated with GREM2 (GREM2 D3-10) start contracting earlier than the "Matrix Sandwich" Protocol (SP) or the modified protocol (MP). (B) Percentage of contracting areas in each well was measured on differentiation day 14 (D14) for each condition. GREM2 treated wells had the highest percentage of contracting areas among the conditions tested, suggesting that GREM2 treatment is able to increase cardiac potential in differentiating iPS cells. N = 9 replicates per condition. Student's t test was used to compare conditions as indicated. ***P < 0.001.

GREM2 treatment causes robust induction of cardiac genes

In agreement with the visual observations, analysis of RNA samples prepared at day 10 and 14 of differentiation showed that addition of GREM2 leads to a further increase in cardiac-specific gene expression levels compared to the "Matrix Sandwich" method (Fig. 18). NKX2.5, MYH6 and TNNT2 were chosen as prototypical markers of cardiac cells. NKX2.5 is a transcription factor whose expression marks both committed cardiac progenitor cells and differentiated cardiomyocytes. MYH6 and TNNT2 code for cardiac contractile proteins and mark more mature, functional cardiomyocytes. These were chosen to gauge increases in the functional genes MYH6 and TNNT2 in samples

treated with GREM2. Gene expression response to GREM2 treatment was quite robust, with increases as high as 1600-fold compared to baseline and 25-fold versus the optimized Matrix Sandwich method in the case of NKX2.5. Interestingly, response to GREM2 treatment among the genes tested was not uniform, with NKX2.5 and MYH6 being more sensitive to GREM2 treatment than TNNT2. This suggests that GREM2 increases the number of cardiomyocytes but also the expression levels of genes such as NKX2.5 and MYH6 at various fold.

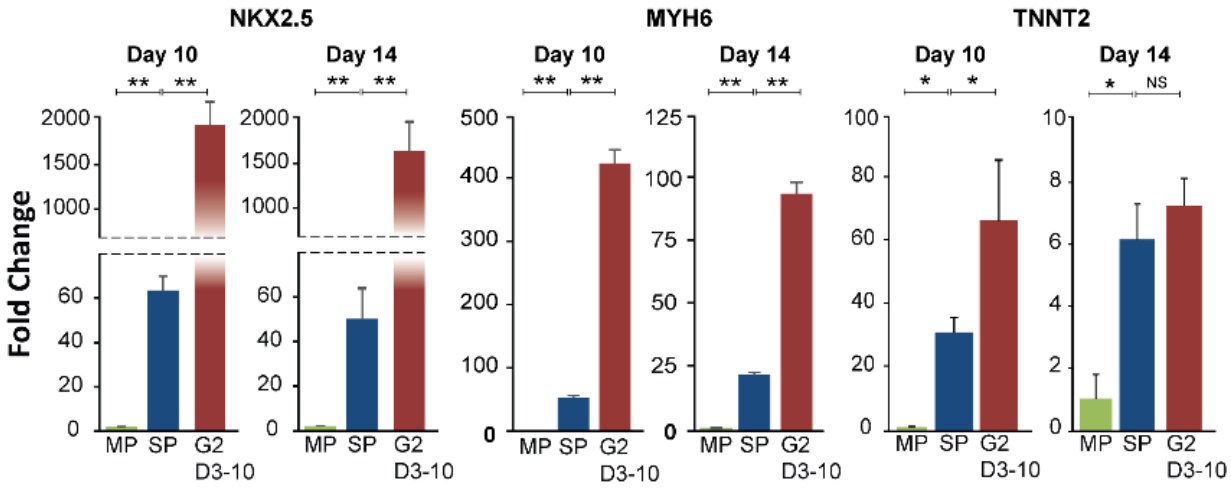


Figure 18: **Grem2 treatment increases the expression of cardiac genes.** RT-qPCR analysis of cardiac genes shows that GREM2 treatment increases expression of the early cardiac gene *NKX2.5* and the late cardiac genes *MYH6* and *TNNT2*. N = 3 replicates per condition. Student's *t* test was used to compare conditions as indicated. $*P < 0.05$; $**P < 0.01$.

GREM2 increases total amount of cardiomyocytes in culture

To quantify the long-term effects of GREM2 on cardiomyocyte yields, I performed flow cytometry analysis at differentiation day 30 using antibodies recognizing the cardiac-specific myosin heavy chain MYH6. I also used antibodies recognizing NKX2.5,

the expression of which is maintained in mature CMs, thus providing an independent marker to quantify cardiac cells. The data showed that GREM2 treatment gives rise to increased numbers of cardiomyocytes as compared to the “Matrix Sandwich” method (Fig. 19). Specifically, GREM2 treatment increased the percentage of $MYH6^+$ cells from ~83% in the optimal Sandwich Protocol (SP) to ~93% and approximately doubled the total number of cells, thus increasing the total number of CMs/well as shown in Fig. 19. Similarly, the percentage of $NKX2.5^+$ cells increased from ~70% to ~88%.

Two populations of high and low $NKX2.5^+$ expressing cells were observed in the flow cytometry experiments. Both of these populations were increased in GREM2 treated samples compared to the standard protocol. However, when total cardiomyocytes were calculated it was the NKX2.5 high population that had the greatest proportional increase (5-6 fold vs 2 fold for the NKX2.5 low population). Although the significance of this observation is currently unknown, these results suggest that GREM2 treatment affects both the quantity and quality of human iPSC-derived cardiomyocytes.

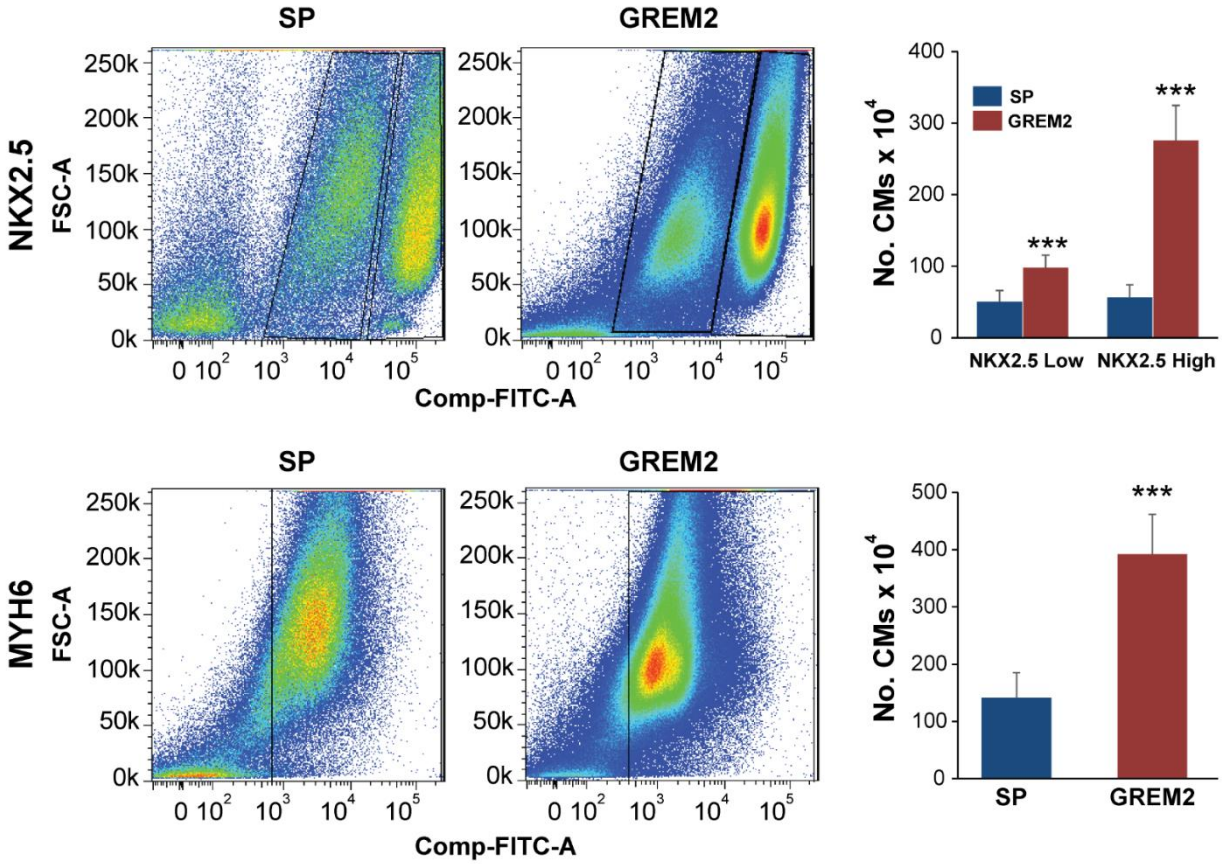


Figure 19: **Grem2 treatment increases the total number of cardiac cells in culture.** Flow cytometry of iPS cell-derived cardiomyocytes at differentiation day 30 using antibodies recognizing NKX2.5 and MYH6. GREM2 treatment increases the total number of cardiomyocytes compared to the "Matrix Sandwich" (SP) protocol. Cardiomyocytes were observed as both high and low NKX2.5+ populations. Cardiomyocytes with high NKX2.5 expression are particularly enriched by GREM2. Quantification of the flow cytometry data is shown to the right. N = 8 replicates. Student's t test. ***P < 0.001.

GREM2-BMP binding is required for cardiogenic effect of GREM2

GREM2 is known to interfere with BMP signaling by binding to BMP ligands directly (Nolan et al., 2013, 2016). I hypothesized that BMP binding may be important for the cardiogenic effect of GREM2. To test this hypothesis I took advantage of a series of engineered GREM2 mutants that were developed in the lab of our collaborator Dr.

Tom Thompson at the University of Cincinnati (Nolan et al., 2013). Each of the mutants contains an alanine substitution in the BMP ligand binding region that causes retention (F122A) or loss (F117A) of BMP binding activity as measured by surface plasmon resonance assays and cell based BMP-Luciferase reporter assays (Nolan et al., 2013). I then treated differentiating hiPS cells with these mutants and wild type GREM2 starting at day 4 of differentiation. qPCR analysis of cardiac genes showed that cells treated with the non-binding mutant did not show an induction of cardiac genes compared to control. Samples treated with the BMP binding mutant F122 showed an induction of cardiac genes comparable or greater than that observed with wild type GREM2 treatment. These data show that BMP binding is required for GREM2 induced cardiogenic differentiation (Fig. 20).

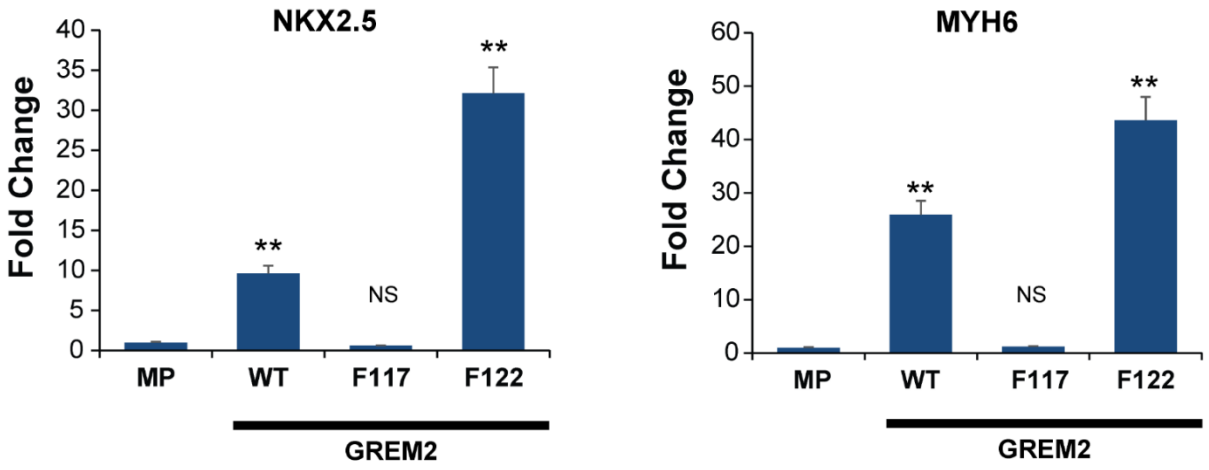


Figure 20: **Treatment with Grem2 mutants reveals BMP binding is required for Grem2 cardiogenic effect.** RT-qPCR analysis of cardiac genes in differentiated iPC cells at differentiation day D10. Cells were treated with 100 ng/ml of wild-type GREM2 (WT), a non-BMP binding GREM2 mutant (F117A), and a strongly-binding mutant (F122A) during differentiation days 5-10. Both the WT and the F122A mutant induce cardiac genes. The F117A mutant was similar to non-treated controls (MP). N = 3 replicates per condition. Student's t test, **P < 0.01. NS: not significant.

Cardiogenic effect of GREM2 is not cell line dependent

As shown above, GREM2 treatment was sufficient to induce cardiogenesis in differentiating DF 19-9-11 hiPS cells. Due to interline variation among different pluripotent stem cell lines it is possible that the GREM2 effect is specific to the DF19-9-11 cell line and not a universal property of GREM2 itself. To determine whether this effect was cell line independent I tested the effect of GREM2 treatment using a separate, independently derived, and well-characterized stem cell line, iMR90 C-4. The positive effect of GREM2 was also evident using the independent iMR90 C-4 hiPS cell line, indicating that the pro-cardiogenic properties of GREM2 are not limited to a single pluripotent stem cell line (Fig. 21).

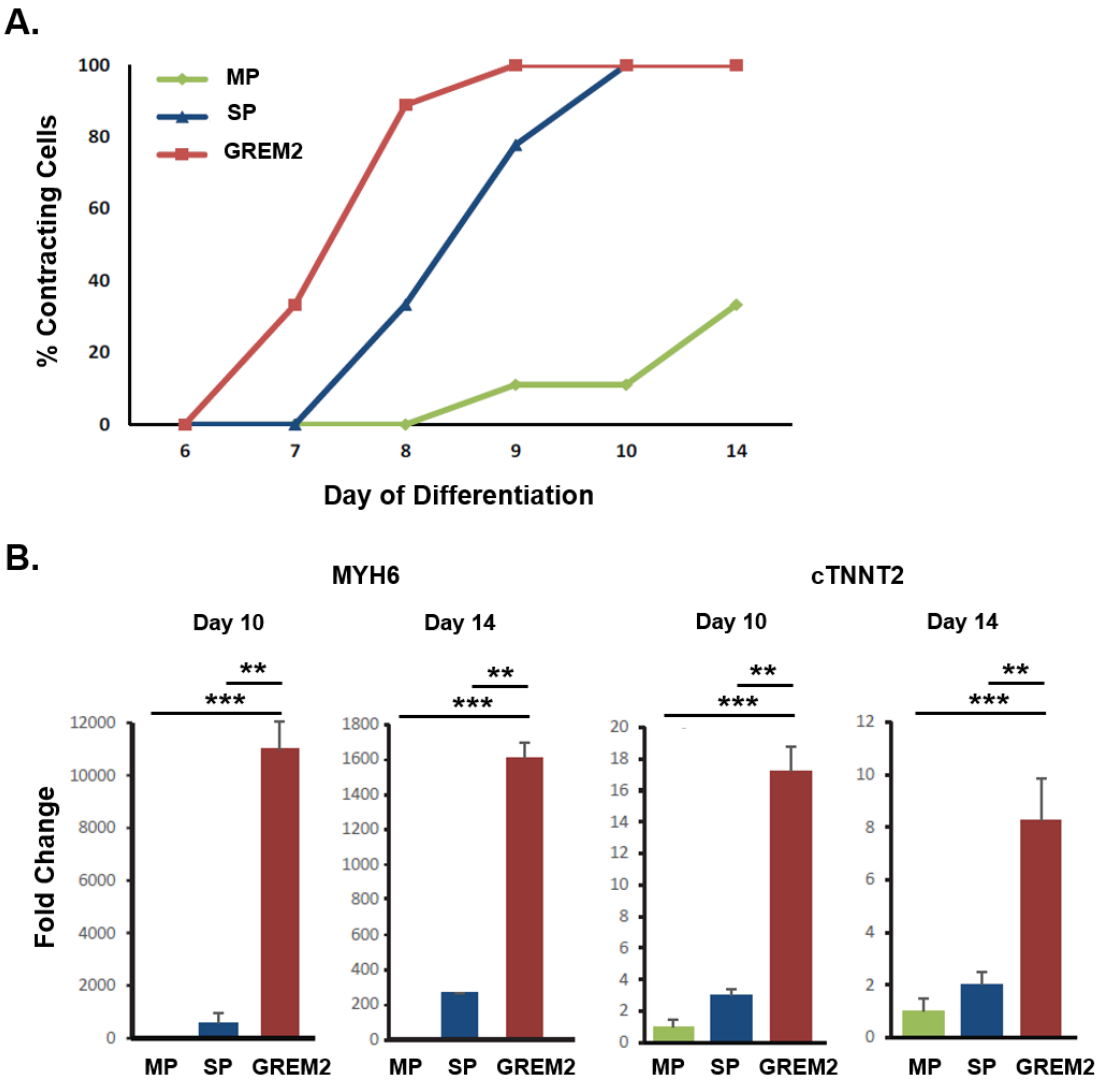


Figure 21: **GREM2 induces differentiation in an independent human iPS cell line.** (A) GREM2 treatment of the human iPS cell line (iM90 C4) during differentiation days 5-10 accelerated the appearance of contacting cells in a similar fashion to DF 19-9-11 iPS cells described in Fig. 4. GREM2-treated cultures presented contracting cells a day earlier than the "Matrix Sandwich" protocol (SP) or the modified baseline protocol (MP). (B) RT-qPCR analysis of cardiac genes shows that GREM2 treatment increases expression of the cardiac genes MYH6 and TNNT2. N=3 replicates per condition. One-way ANOVA with Tukey's HSD test. **P <0.01; ***P < 0.001.

Taken together, these data demonstrate that GREM2 protein addition during cardiac differentiation accelerates and enhances the cardiogenic potential of differentiating human iPS cells.

Summary

In this chapter I showed that using a modified version of the matrix sandwich protocol provided a suitable control condition wherein mesodermal cells were produced and to which GREM2 could be added in order to test the effects of GREM2 on cardiac differentiation. My results show that adding GREM2 to this baseline differentiation protocol was able to accelerate the appearance of cardiomyocytes and increase the total numbers and percentage of cardiomyocytes in culture when compared with the previously reported and optimized matrix sandwich protocol (Zhang et al., 2012). GREM2 treatment was also able to strongly induce cardiac specific genes at early and late time points in differentiating human stem cells. GREM2 mutants that were unable to bind BMP ligands were also unable to robustly induce cardiogenesis while mutants that retained their BMP ligand binding were able to induce cardiac differentiation. These effects were observed in both the DF 19-9-11 human pluripotent stem cell line and the IMR90-C4 hiPS cell line, suggesting that GREM2 plays a fundamental cardiogenic role in human cardiomyocyte differentiation. Taken together all of these data show that GREM2 is a cardiogenic molecule and that its cardiogenic properties require binding to BMP ligands.

GREM2 treatment was sufficient to accelerate the appearance of contracting cells, increase the amounts of cardiac myocytes, and induce the expression of cardiac genes when compared with controls. This acceleration and increase in cardiogenesis is similar to that observed in mouse ES cells as discussed in chapter 3. Interestingly, no atrial cardiomyocyte subtype bias was observed in GREM2 treated hiPSC-derived cardiomyocytes. This is in contrast to the effects observed in mouse ES cells, which were selectively biased towards cardiomyocytes with functional properties characteristic of atrial cardiomyocytes. This difference may be due to differences in the role played by GREM2 among species, or could be due to the timing of addition of GREM2 or of the time points at which the cells were collected for observation. The effects of GREM2 on hiPSCs also share some similarity to that observed in the gain of function studies in zebrafish embryos as discussed in chapter 3. Injection of GREM2 was able to increase the amount of cardiac tissue produced in embryos and caused formation of cardiomyocytes in ectopic areas. These studies also showed a preferential bias of GREM2 towards producing atrial cardiomyocytes and tissues. This difference may indicate that GREM2 function in subtype selection is conserved in zebrafish and mouse but diverges in humans, or it could be that in order to observe a GREM2 effect on cardiomyocyte subtypes in human iPSCs it will be necessary to try a greater range of treatment time points to find the proper temporal context.

Chapter 7

BMP Signaling Inhibition Promotes Proliferation of Cardiac Progenitor Cells

Introduction

During human pluripotent stem cell cardiac differentiation, cardiac progenitor cells form from mesodermal cells starting around day 3 or 4. At this stage, expression of the mesodermal marker T BRY sharply decreases while expression of cardiovascular progenitor markers such as KDR and NK2.5 starts to rise. During this period of cardiac progenitor cell specification, cardiac progenitor cell populations also undergo expansion, presumably recapitulating the expansion of cardiac progenitors observed *in vivo* to satisfy the cell quantity requirements of a functional myocardium (Kuhn and Wu, 2010).

Cardiac progenitor populations expand as the result of either specification of non-cardiac progenitor cells to become cardiac progenitor cells, or by direct proliferation of already specified cardiac progenitor cells (Birket et al., 2015; Kuhn and Wu, 2010). Factors that affect either of these processes can be used to affect total numbers of cardiac progenitor cells in culture (Palpant et al., 2017).

As already discussed, BMP signaling plays an important role in pluripotent stem cell differentiation. The expression data in chapter 4 show that genes for BMP molecules are being actively transcribed during the cardiac progenitor cell stage in the human pluripotent stem cell lines and differentiation protocols tested. In chapter 5, I showed that BMP inhibition by GREM2 was required for cardiac differentiation. I found

that GREM2 treatment during cardiac differentiation was able to increase the total numbers of cardiac cells and that this BMP ligand binding was required for this effect. So far however, it is unknown whether GREM2 and BMP inhibition are affecting cardiac progenitor cell specification, proliferation, and/or the transition of progenitor cells to cardiac myocytes.

Here, pluripotent stem cells and the BMP inhibitors GREM2, NOGGIN, and DAN were used to distinguish among these possibilities.

Materials and Methods

Human pluripotent stem cell culture

Human induced pluripotent stem cell lines iMR90 and DF 19-9-11 from WiCell were cultured under feeder free conditions in mTeSR1 or Essential 8 (E8) media (Stem Cell Technologies) as described in chapter 4.

iPS cell differentiation

Human iPS cells were differentiated using the “Matrix Sandwich” method as already described in chapter 4. As in chapter 6 at day 5, GREM2-treated wells received basal differentiation medium with 150 ng/ml of GREM2. Media in all wells was replaced daily. Additionally, cells were treated in a similar fashion with 50 ng/ml NOGGIN or 1.5 µg/ml DAN, based on their specific activities (R&D Systems).

Immunofluorescence

Cells were seeded onto Matrigel-coated (8.7 µg/cm2) 12-well plastic culture plates (Thermo Fisher Scientific) at a density of 500,000 cells per well and differentiated as described above for the “Matrix Sandwich” method. Cells were fixed at the desired time points (differentiation days 4, 5, 6 and 10) by rinsing with 500 µl DPBS and incubating in 4 % PFA in PBS at 4oC for 5 mins. The PFA solution was then aspirated and cells were rinsed with 1X PBS 5 times. Fixed cells were permeabilized by incubating with 400 µl permeabilization buffer (0.2 % Triton X-100 in 1X PBS) in each well at room temperature for 1 hr. Nonspecific binding was then blocked using 400 µl/well of blocking solution (5 % non-fat dry milk in permeabilization buffer) for 2 hrs at room temperature with gentle rocking. For all subsequent steps, a minimum of 350 µl of solution was added to each well. Cells were then washed 3 x 5 min with 400 µl/well of 1X PBS at room temperature. After washing, cells were incubated with primary antibodies in incubation buffer (0.1 % Triton X-100, 1 % BSA in 1X PBS) overnight at 4°C with gentle rocking. Next, cells were washed 3 x 3 min each, with PBST (0.2 % Tween-20 in 1X PBS) followed by 3 x 3 min each with 1X PBS. Cells were then incubated with secondary antibodies diluted in incubation buffer for 1 hr at room temperature in the dark. Finally, cells were then washed 2 times, 3 min each, with 1X PBS and stored in 400 µL 1X PBS for imaging.

Imaging was done using a Nikon C2 Confocal, Leica DM IRB Inverted Microscope or a Zeiss Laser Scanning Microscope (LSM 880). Image analysis and volume rendering were done using NIS Elements, Zen, ImageJ/FIJI, and Imaris software suites. Primary

antibodies recognizing phospho-HISTONE H3 (Santa Cruz Biotechnology, Cat# sc-8656-R, 1:500), NKX2.5 (Santa Cruz Biotechnology, sc-8697, 1:50), GREM2 (ProteinTech, 13892-1-AP, 1:100), and α-ACTININ (Sigma, A7811, 1:500) were applied. I have also tested anti-GREM2 rabbit polyclonal antibodies from GeneTex (GTX108414), Abcam (ab102563) and R&D Systems (AF2069) at 1:100 dilution. The fluorescent dye 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) was added during the final 15 mins of secondary incubation at a 1:10,000 dilution to stain nuclei.

Western blotting

Protein samples from differentiated hiPS cells (DF 19-9-11, WiCell) were isolated using the RIPA buffer, including the Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail 2 and 3 (Sigma) diluted at 1:100. Cell lysates were shaken at 4^{o}C for 30 min and centrifuged at 12,000 rpm at 4^{o}C for 15 min. Protein concentration in supernatants was measured using the Pierce BCA protein assay (Thermo Fisher Scientific). 15 µg of protein (adjusted to 7.5 µl with distilled water) was then mixed with 7.5 µl of 2X Laemmli Sample Buffer (Bio-Rad) containing β-Mercaptoethanol (Sigma). Proteins were denatured at 95^{o}C for 5 min, then placed on ice for another 5 min. Electrophoresis was run using sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) with 1X MOPS SDS running buffer (Life Technologies). Size fractioned proteins were transferred to nitrocellulose membranes (Bio-Rad) using the semidry system. Membranes were blocked with 5% dry milk or 5% BSA (Sigma) in Tris-buffered saline (Corning) containing 1 % Tween-20 (Sigma) (1X TBST) for 1 hr at room temperature or 4^{o}C, respectively. Next, membranes were incubated overnight with antibodies recognizing

NKX2.5 (Santa Cruz Biotechnology, sc-8697) diluted at 1:500 and β-ACTIN (Sigma Aldrich, A1978) diluted at 1:5,000 in 5% BSA in 1X TBST. Next day, membranes were washed 3 times, 5 min each with TBST and incubated for 2 hrs at room temperature with peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) diluted 1:5,000 in 5% dry milk-TBST. Three 5 min washes were performed to wash away unbound secondary antibodies. Signals were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and images were taken with ChemiDoc Touch System (Bio-Rad). Protein band intensities were quantified using ImageJ.

Results

GREM2 Promotes Proliferation of Cardiac Progenitor Cells

Western blotting analysis of NKX2.5 protein, showed that GREM2 does not affect the initial induction of early cardiac genes (Fig. 22).

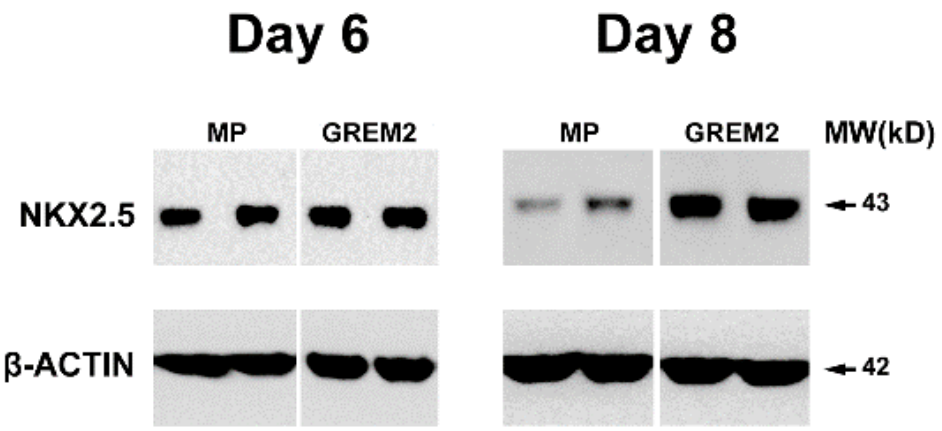


Figure 22: **GREM2 does not affect specification of cardiac progenitor cells and early expression of cardiac marker NKX2.5.** Western blotting analysis at differentiation days 6 and 8 of iPS cell differentiation with an anti-NKX2.5 antibody shows similar NKX2.5 protein levels at day 6 between GREM2-treated samples and baseline (MP) controls but increased NKX2.5 protein by GREM2 treatment at day 8. β-ACTIN served as loading control.

Instead, the GREM2 expression during the initial appearance of cardiac progenitor cells (CPCs) and its stimulatory effect on cardiac differentiation raised the possibility that GREM2 promotes proliferation of CPCs. To directly test this possibility, I stained GREM2-treated differentiating iPS cells with antibodies recognizing the phosphorylated form of HISTONE H3 (pHH3) that is specifically phosphorylated during the mitosis and meiosis phases of cell division, thereby marking proliferating cells. The "Matrix Sandwich" (SP) and baseline (MP) protocols as outlined above in Fig. 16 served as positive and negative controls, respectively.

The results demonstrate that addition of GREM2 protein at day 3 leads to a 5-6 fold increase in $pHH3^{+}$ cells at day 4 (Fig. 23 and Fig. 24). At this stage, pHH3 colocalizes with cell colonies expressing the early cardiovascular progenitor-specific membrane receptor KDR (or VEGFR2) (Yang et al., 2008b). Notably, KDR expression

diminishes in SP cultures compared to MP and even more in GREM2-treated cells, likely due to acceleration of cardiac differentiation and loss of vascular markers, which is consistent with the timing of appearance of contracting cells under the different protocols at later stages (Fig. 17).

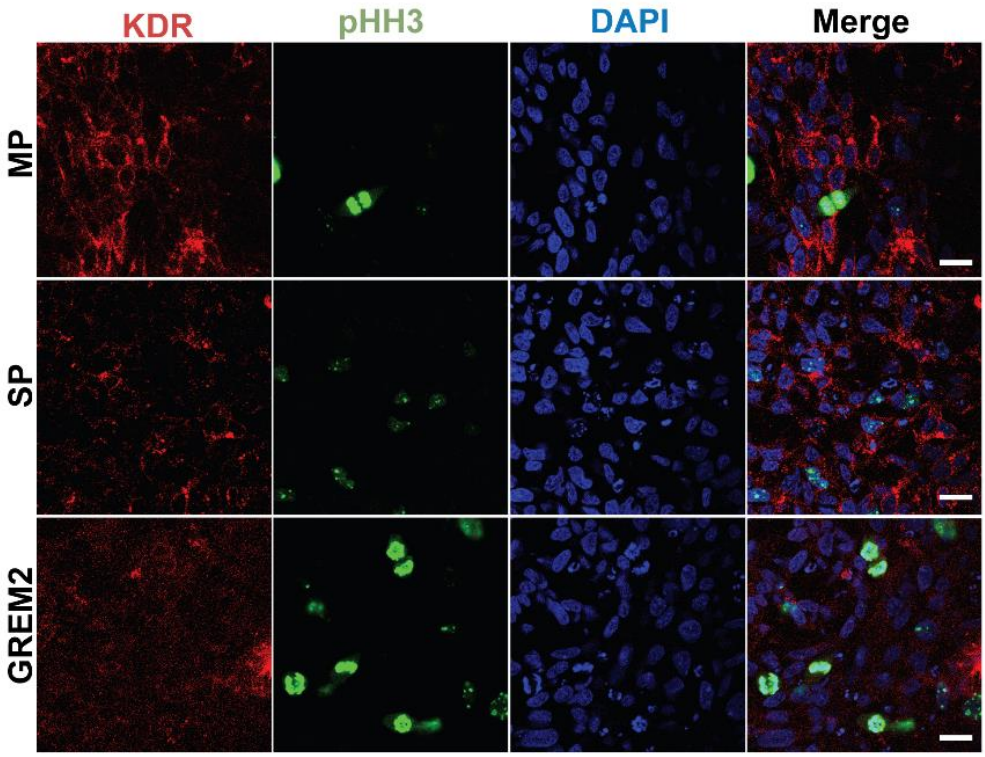


Figure 23**: GREM2 promotes proliferation of cardiovascular progenitor cells.** Immuno-fluorescence staining of cardiac progenitor cells during iPS cell differentiation. Cells were treated with GREM2 and compared to "Matrix Sandwich" (SP) and baseline negative controls (MP) as described in Fig. 11. Representative images of cell cultures stained at differentiation day 4 with the cardiovascular progenitor marker KDR (red) and the active proliferation marker pHH3 (green) show that GREM2 treatment increases the total number of proliferative cells. Scale bars, 30 µm. DAPI staining (blue) marks cell nuclei. Abbreviations: pHH3: phospho-HISTONE H3

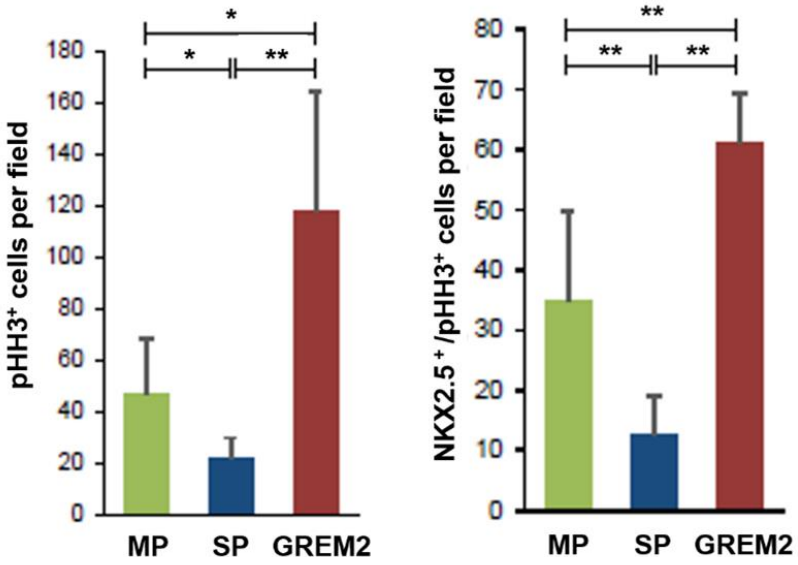


Figure 24**: Grem2 increases the total number of proliferative cells in differentiating human iPS cells.** Left, Quantification of total numbers of pHH3 positive cells in immunostained iPS cells at differentiation day 5 shows a higher number in GREM2-treated wells. Right, Quantification of total numbers of pHH3/NKX2.5 double positive cells in immunostained iPS cells at differentiation day 5 shows that in GREM2-treated wells there are a higher number of double labeled cells, suggesting GREM2 treatment increases the amount of proliferating cardiac progenitor cells. N = 9 replicates per condition. One-Way ANOVA with Tukey's HSD test. *P <0.05; **P < 0.01.

At day 5, pHH3 is specifically found in the nuclei of NKX2.5$^+$ CPCs (Fig. 20). The immunofluorescence analysis results show that GREM2 promotes proliferation of CPCs. Interestingly, the "Matrix Sandwich" protocol, which requires BMP4 addition at differentiation days 3-5, initially generates less proliferating CPCs than the other conditions (Fig. 23 - 25). However, the "Matrix Sandwich" protocol eventually promotes cardiomyocyte differentiation after BMP4 removal at day 5, but not to the same extent as addition of GREM2.

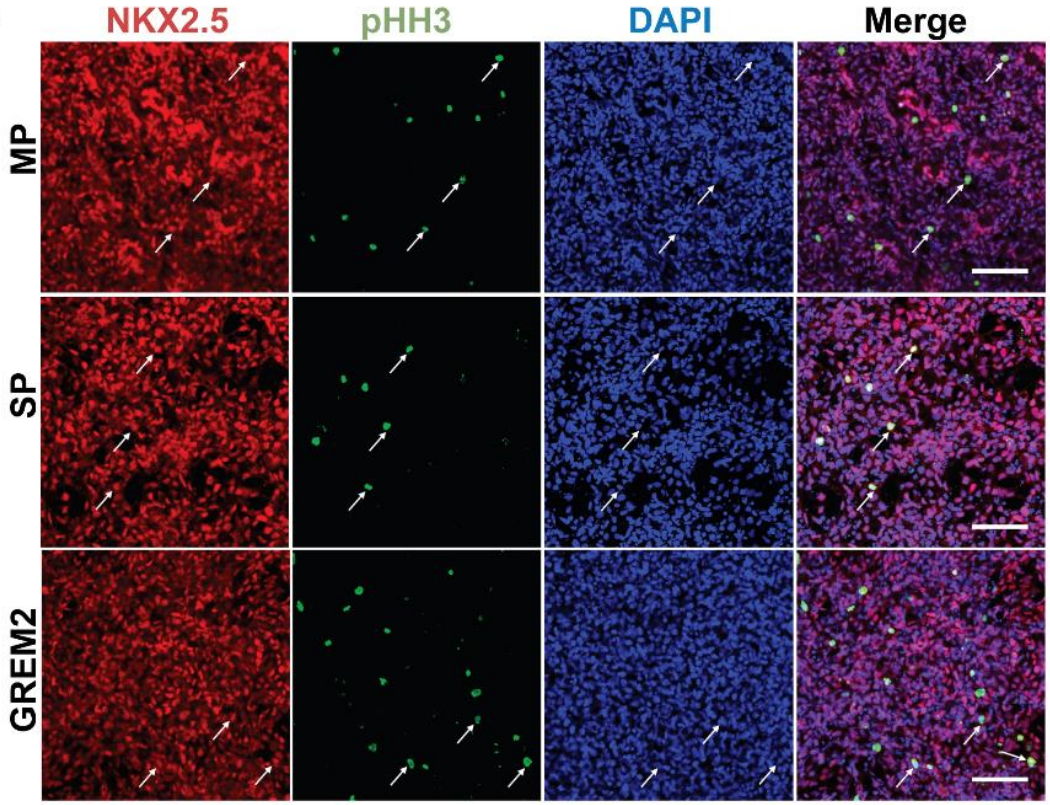


Figure 25: **GREM2 promotes proliferation of cardiac progenitor cells.** Staining at day 5 with the cardiac progenitor cell marker NKX2.5 (red) shows co-localization with pHH3 (green), indicating increased proliferation of cardiac progenitor cells in GREM2-treated cultures of differentiating iPS cells. Representative double-positive cells are marked by arrows. Scale bars, 100 µm. DAPI staining (blue) marks cell nuclei. Abbreviations: pHH3: phospho-HISTONE H3

BMP inhibition promotes proliferation of cardiac progenitor cells

The results described in the previous sections show that GREM2 promotes proliferation of CPCs. To test whether inhibition of BMP is sufficient to also stimulate cardiac differentiation, I compared the effects of GREM2 to two additional, distinct BMP antagonists, namely NOGGIN and DAN (Fig. 26).

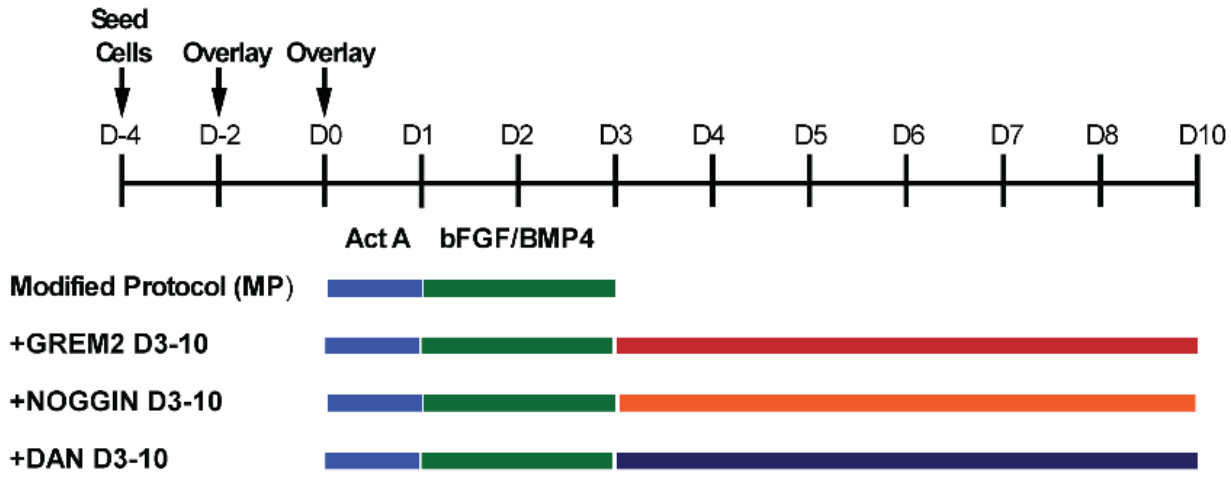


Figure 26: **Protocols for testing effects of BMP antagonists on cardiac differentiation.** Schematic diagram showing treatment schedule and conditions of iPS cell differentiation using DF 19-9-11 human iPS cells. D-4 through D10 represent differentiation day minus 4 through day 10 respectively. Addition of various BMP antagonists is indicated on the left.

To test whether the selected BMP antagonists could inhibit BMP signaling in differentiating hiPS cells RNA was collected from cells treated with each antagonist and analyzed for activation of the canonical BMP signaling target gene *ID2*. All three BMP antagonists led to suppression of the canonical BMP signaling target gene *ID2*, respectively, showing they function as expected (Fig. 27A). Treatment with any of the three BMP antagonists led to a significant increase in overall cell numbers (Fig. 27B).

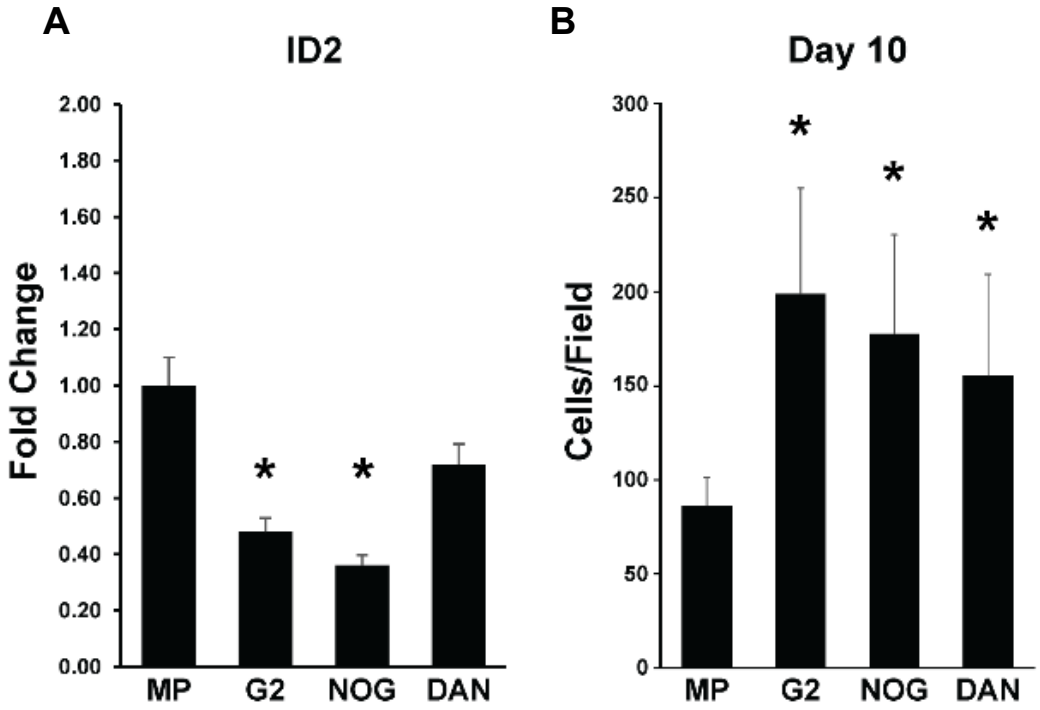


Figure 27**: BMP inhibition causes an increase in the total number of cells per well.** (A) RT-qPCR analysis of the canonical BMP signaling target gene ID2 at differentiation day 5. All 3 BMP antagonists, GREM2 (G2), NOGGIN (NOG), and DAN suppress ID2 expression. N = 3 replicates per condition. *P <0.05; One-way ANOVA with Tukey's HSD test. (B) Total cell numbers were quantified using the live nuclear stain Nuc Blue at day 10 of differentiation. All 3 BMP antagonists increase the total numbers of cells per well. N = 6 replicates per condition. *P <0.05. One-way ANOVA with Tukey's HSD test.

Co-staining of differentiating iPS cells at day 6 with antibodies recognizing NKX2.5 and pHH3 showed that GREM2-treated cells had higher proliferation rates than baseline. NOGGIN and DAN also increased CPC proliferation, compared to baseline (Fig. 28). Of note, there were comparable numbers of $NKX2.5^{+}$ cells at day 6 in control and BMP antagonist-treated differentiating iPS cells, further confirming that GREM2 and BMP signaling antagonists do not abort the specification of CPCs.

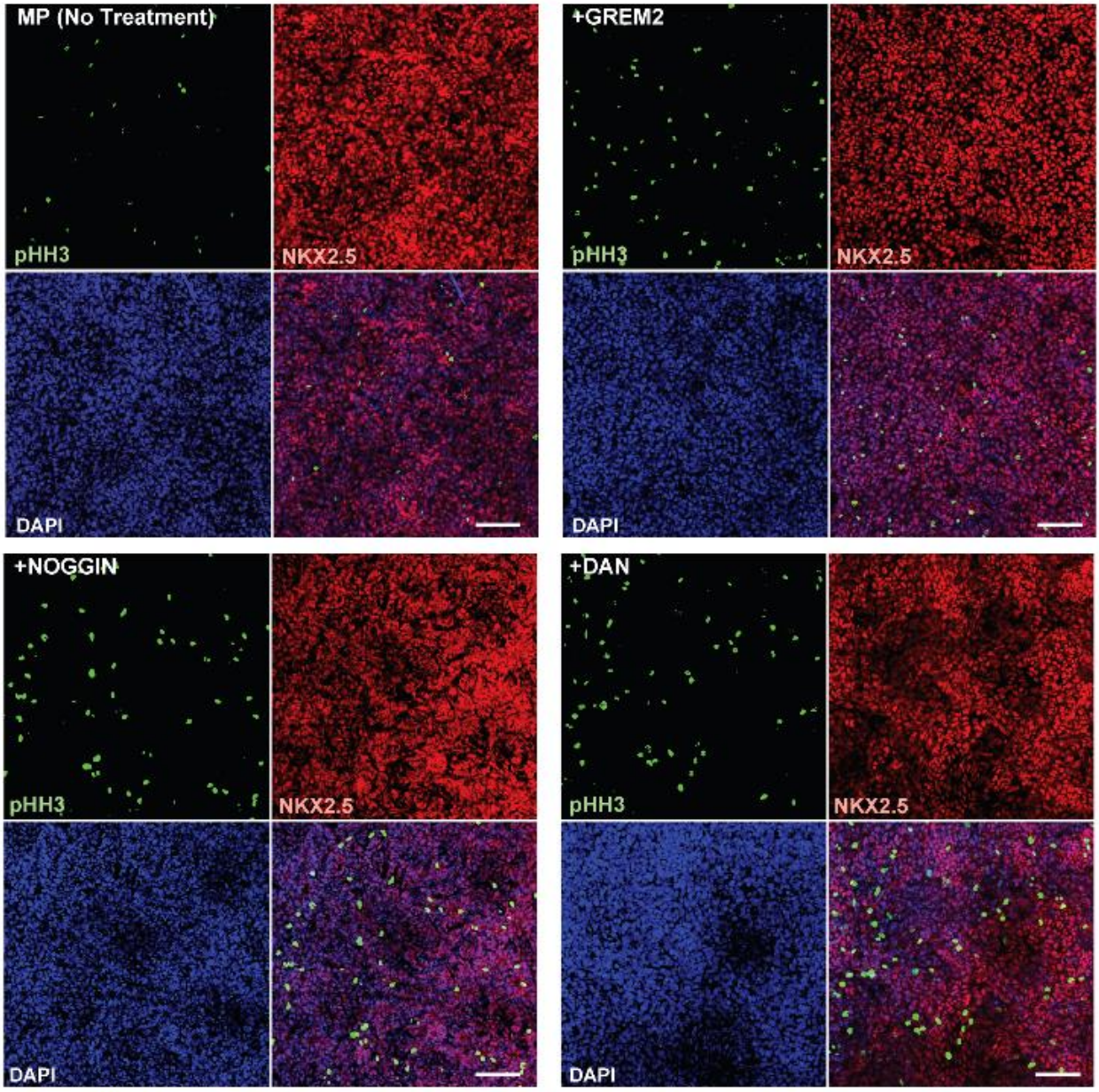


Figure 28**: BMP inhibition promotes proliferation of cardiac progenitor cells.** Immunostaining of cardiac progenitor cells at day 6 of iPS cell differentiation with the proliferation marker pHH3 (green) and the cardiac progenitor cell marker NKX2.5 (red). DAPI staining (blue) marks cell nuclei. Cells were treated with the BMP inhibitors GREM2, NOGGIN and DAN as indicated in panel A. All three BMP inhibitors induce proliferation of cardiac progenitor cells. Scale bar, 100 μm.

BMP inhibition is not sufficient to induce cardiac differentiation

To test whether BMP signaling inhibition is sufficient to promote cardiomyocyte differentiation, I isolated RNA samples at day 10 of differentiation from cells treated as shown in Fig. 26. Quantitative PCR analysis showed that GREM2 led to induction of cardiac-specific gene markers characteristic of contracting CMs such as MYH6 and TNNT2, but NOGGIN and DAN had minimal or no effect (Fig. 29). Therefore, although all three BMP antagonists enhanced proliferation of NKX2.5^{+} cells, induction of cardiomyocyte (CM) differentiation was a characteristic specific to GREM2.

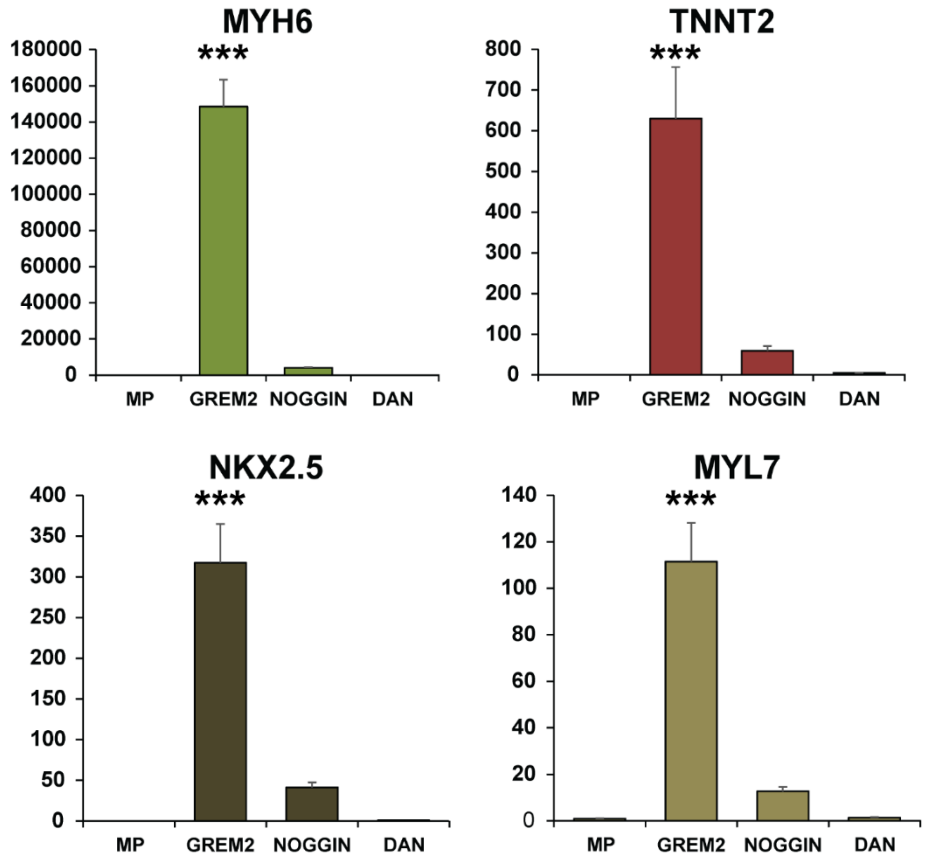


Figure 29: **BMP inhibition is not sufficient to induce cardiac-specific genes.** RT-qPCR analysis of cardiac genes MYH6, NKX2.5, TNNT2 and MYL7 at day 10 of differentiation show an increase in cardiomyocyte gene induction at later time points in GREM2-treated iPS cells while a similar increase in gene induction was not observed for the other BMP antagonists. N = 3 replicates per condition. ***P < 0.001. One-way ANOVA with Tukey's HSD test.

Summary

In this chapter I have examined the effect of BMP inhibition on cardiac progenitor cell specification, proliferation, and differentiation to cardiomyocytes. The results show that treatment of differentiating human stem cells with GREM2 as cardiac progenitor cells are forming increases the total numbers of cells in culture by inducing proliferation of cardiac progenitor cells. GREM2 treatment did not affect specification of cardiac progenitor cells but instead acts on previously specified cardiac progenitors. Treatment of differentiating cells with other BMP antagonists showed that proliferation of cardiac progenitor cells was a common effect of various BMP antagonists, suggesting that cardiac progenitor cell expansion is not unique to GREM2.

There are reports in the literature of using BMP inhibitors to increase the cardiogenic potential of pluripotent stem cells (Yuasa et al., 2005; Hao et al., 2008). These reports indicate an increase of differentiated, functional cardiomyocytes after treatment with inhibitors such as Noggin or the small molecule BMP inhibitor dorsomorphin (Yuasa et al., 2005; Hao et al., 2008). Expansion of cardiac progenitor cell populations could partially be responsible for an increase in cardiomyocytes as reported, however the subsequent cardiomyocyte differentiation of these cells reported by Yuasa and Hao contradicts what was observed in my hiPSC experiments. This is likely due to timing of treatment. In both experimental protocols by Yuasa and Hao the pluripotent stem cells were treated prior to the start of differentiation. Thus adding BMP inhibitors may have had a priming effect on pre-differentiated stem cells. These methods also used the embryoid body method, which may have provided the additional

cellular signaling clues required for proper differentiation of increased numbers of cardiac progenitor cells.

GREM2 was the only BMP inhibitor tested that was able to promote the differentiation of cardiac progenitor cells to cardiomyocytes. This unique property of GREM2 is likely due to its unique structural and binding properties when compared to DAN and Noggin (Nolan et al., 2016). GREM2 is able to form heterodimers with BMP ligands that then become new functional signaling units (Nolan et al., 2013; Nolan and Thompson, 2014b; Nolan et al., 2016). These new complexes may be able to affect BMP signaling by unique receptor binding paradigms not shared by Noggin and DAN. GREM1, a closely related protein to GREM2 is known to directly interact with additional receptors not traditionally considered part of canonical BMP signaling (Nolan and Thompson, 2014b). Reports in the literature indicate that GREM1 is able to promote angiogenesis via activation of the VEGF receptor VEGFR2 (Mitola et al., 2010). It was further reported that GREM1 could still bind and activate VEGFR2 even in complex with BMP ligands (Mitola et al., 2010). Due to the highly similar structures of GREM1 and GREM2 it is likely that GREM2 may also be able to signal through other pathways by yet unstudied direct receptor interactions.

My data show that the transition from progenitor cell to cardiomyocyte was facilitated by GREM2 treatment but not that of other BMP inhibitors. This points to a unique role for GREM2 at the junction between progenitor cell proliferation and differentiation in human cardiogenesis. At this point, the mechanism behind this unique property of GREM2 remains unexplored.

Chapter 8

GREM2 Specifically Induces Cardiomyocyte Differentiation Through JNK Signaling Activation

Introduction

In chapter 7, I described data to show that GREM2 was able to induce proliferation of cardiac progenitor cells and that this was a property of BMP antagonists in general. I further showed that GREM2 was also able to induce differentiation of cardiac progenitors to cardiomyocytes, which was unique to GREM2 among the BMP antagonists tested. This suggests that GREM2 is able to act through a unique mechanism not shared by the other antagonists.

BMP signaling occurs via canonical, Smad mediated signaling activation or can involve non-canonical JNK-mediated signaling activation (Wang et al., 2014). Previous studies have shown that GREM2 is able to affect cardiac differentiation by selectively activating JNK signaling and that this property is unique to GREM2 (Tanwar et al., 2014). It is possible that this selective activation of JNK signaling also plays a role in GREM2 mediated cardiac differentiation of human pluripotent stem cells. To address this possibility, Western blotting and the use of JNK signaling inhibitors were employed to determine if JNK activation by GREM2 is required for cardiac differentiation of human pluripotent stem cells.

Materials and Methods

Human pluripotent stem cell culture

Human induced pluripotent stem cell lines iMR90 and DF 19-9-11 from WiCell were cultured under feeder free conditions in mTeSR1 or Essential 8 (E8) media (Stem Cell Technologies) as already described in chapter 4.

iPS cell differentiation

Human iPS cells were differentiated using either the “Matrix Sandwich” method, the GiWi method, or the BMP/ACTIVIN A method as described in chapters 5 and 6. Differentiating stem cells were treated starting at days 3 or 5 with 5μM JNKi 6o (Tocris Biosciences) in a similar fashion as described for GREM2.

Western blotting

Protein samples from differentiated hiPS cells (DF 19-9-11, WiCell) were isolated using the RIPA buffer, including the Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail 2 and 3 (Sigma) diluted at 1:100. Cell lysates were shaken at 4^{o}C for 30 min and centrifuged at 12,000 rpm at 4^{o}C for 15 min. Protein concentration in supernatants was measured using the Pierce BCA protein assay (Thermo Fisher Scientific). 15 μg of protein (adjusted to 7.5 μl with distilled water) was then mixed with 7.5 μl of 2X Laemmli Sample Buffer (Bio-Rad) containing β-Mercaptoethanol (Sigma). Proteins were denatured at 95^{o}C for 5 min, then placed on ice for another 5 min. Electrophoresis was run using sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) with 1X MOPS SDS

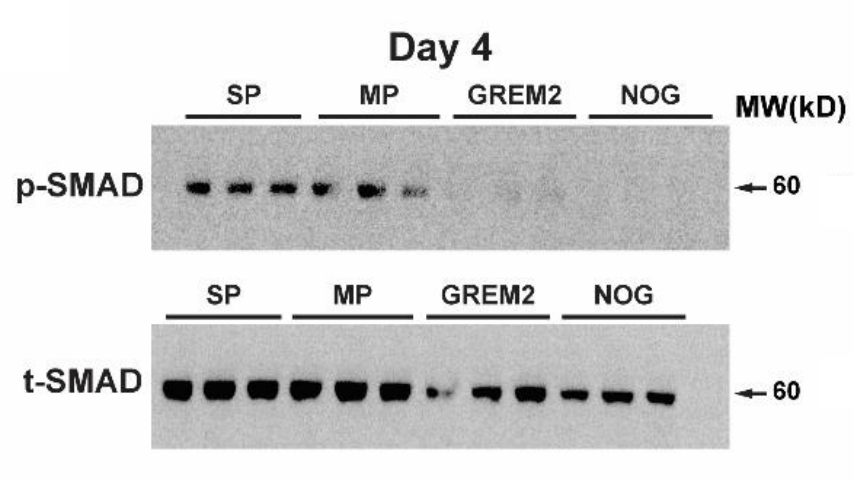
running buffer (Life Technologies). Size fractioned proteins were transferred to nitrocellulose membranes (Bio-Rad) using the semidry system. Membranes were blocked with 5% dry milk or 5% BSA (Sigma) in Tris-buffered saline (Corning) containing 1 % Tween-20 (Sigma) (1X TBST) for 1 hr at room temperature or 4^{o}C, respectively. Next, membranes were incubated overnight with antibodies recognizing phosphorylated SMAD 1/5/9 or total SMAD1 and phosphorylated or total JNK 1&2 (Cell Signaling) diluted at 1:1,000 in 5% BSA in 1X TBST. Next day, membranes were washed 3 times, 5 min each with TBST and incubated for 2 hrs at room temperature with peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) diluted 1:5,000 in 5% dry milk-TBST. Three 5 min washes were performed to wash away unbound secondary antibodies. Signals were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and images were taken with ChemiDoc Touch System (Bio-Rad). Protein band intensities were quantified using ImageJ.

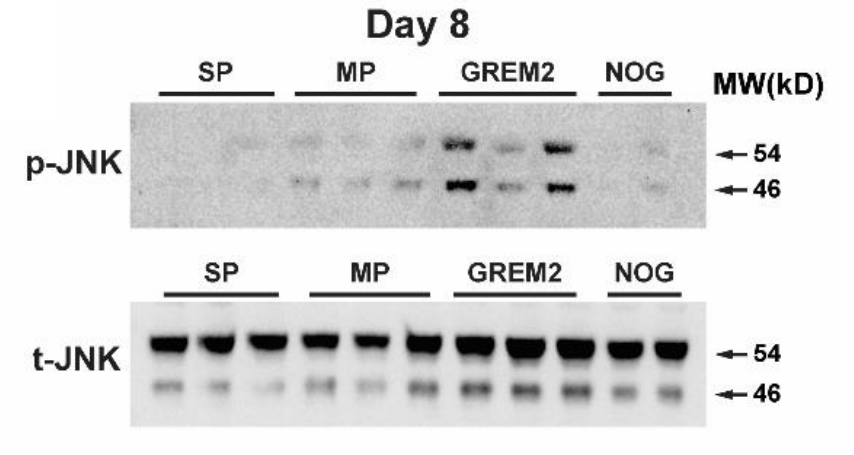
Results

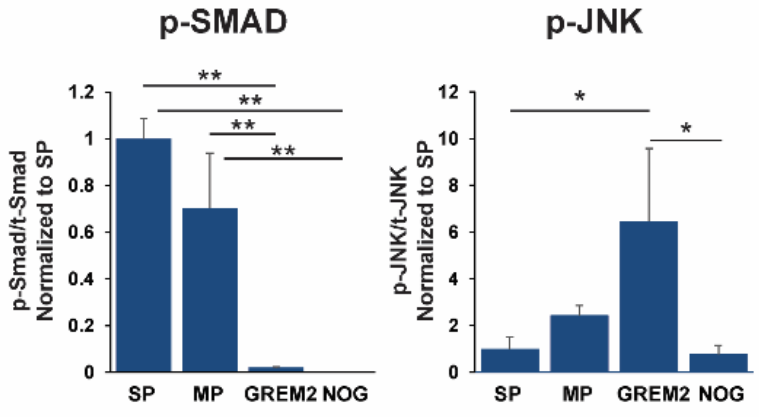
GREM2 activates JNK signaling in differentiating human induced pluripotent stem cells

We have previously shown that GREM2 inhibited canonical BMP, i.e., SMAD1/5/8-mediated signaling shortly after treatment of differentiating mouse ES cells, followed by JNK signaling activation at later stages. Moreover, we found that this dual effect on canonical BMP signaling inhibition and subsequent non-canonical JNK

signaling activation is a unique property of GREM2 compared to Noggin, a typical BMP antagonist that has been well-characterized (Groppe et al., 2002; Zhu et al., 2006; Tanwar et al., 2014). To test whether JNK activation drives cardiogenic differentiation of human iPS cells by GREM2, I prepared protein samples at differentiation days 4, 5, 6, 7 and 8 of GREM2 and NOGGIN treated cells. The "Matrix Sandwich" and baseline protocols served as positive and negative controls, respectively (as described in Fig. 16). Western blotting analysis using antibodies recognizing activated phospho-SMAD1/5/8 showed that both GREM2 and NOGGIN effectively blocked SMAD phosphorylation 24 hrs after treatment (Fig. 29). Phospho-SMAD1/5/8 levels became undetectable in all samples at subsequent time points (not shown). There was no JNK phosphorylation at days 4-7 (not shown). In contrast, Western blotting showed that phospho-JNK1/2 protein was specifically activated by GREM2 at day 8, but not NOGGIN (Fig. 29). Of note, there was no JNK activation by the Matrix Sandwich method at this stage, suggesting either alternative pro-cardiogenic mechanisms are induced by this method, or GREM2 accelerates JNK signaling activation.


Day 4
SP
MP
GREM2
NOG
MW(kD)
p-SMAD
60
SP
MP
GREM2
NOG
t-SMAD
60


Day 8
SP
MP
GREM2
NOG
MW(kD)
p-JNK
54
46
SP
MP
GREM2
NOG
t-JNK
54
46


p-SMAD
p-Smad/t-Smad
Normalized to SP
1.2
1
0.8
0.6
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12
10
8
6
4
2
0
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SP
MP
GREM2
NOG

Figure 30: **GREM2 treatment induces JNK signaling in differentiating hiPS cells.** DF 19-9-11 iPS cells were differentiated according to the Matrix Sandwich method (SP), baseline control method (MP), or treated with GREM2 or NOGGIN (NOG) from day 3 (as described in Fig. 11 and 21). Protein lysates for Western blotting analysis were collected at indicated differentiation time points and analyzed using antibodies recognizing the phosphorylated forms of SMAD1/5/8 [expected Molecular Weight (MW) 60 kD] and JNK1/2 isoforms (46 and 54 kD, respectively) and total SMAD1 and JNK1/2 proteins. Both NOGGIN and GREM2 effectively inhibit SMAD phosphorylation at day 4. In contrast, only GREM2 treatment leads to strong upregulation of JNK phosphorylation at day 6. Graphs on the right represent quantification of phosphorylated SMAD and JNK forms relative to the corresponding total protein **levels.** One-way ANOVA with Tukey's HSD test. *P < 0.05. **P < 0.01.ANOVA with Tukey's HSD test.

JNK signaling activation is required for GREM2 induced human cardiomyocyte differentiation

To test whether JNK activation by GREM2 drives human cardiomyocyte differentiation, I exposed GREM2-treated differentiating iPS cells to the JNK signaling-specific chemical inhibitor TCS JNK 6o (JNKi 6o, (Kauskot et al., 2007; Szczepankiewicz et al., 2006)) or vehicle control. Visual observations and RNA analysis by qPCR showed that JNK signaling inhibition abolished the stimulatory effect of GREM2 on the cardiac lineage expansion (Fig. 31). Of note, JNK inhibition was effective when applied during differentiation of CPCs after day 5.

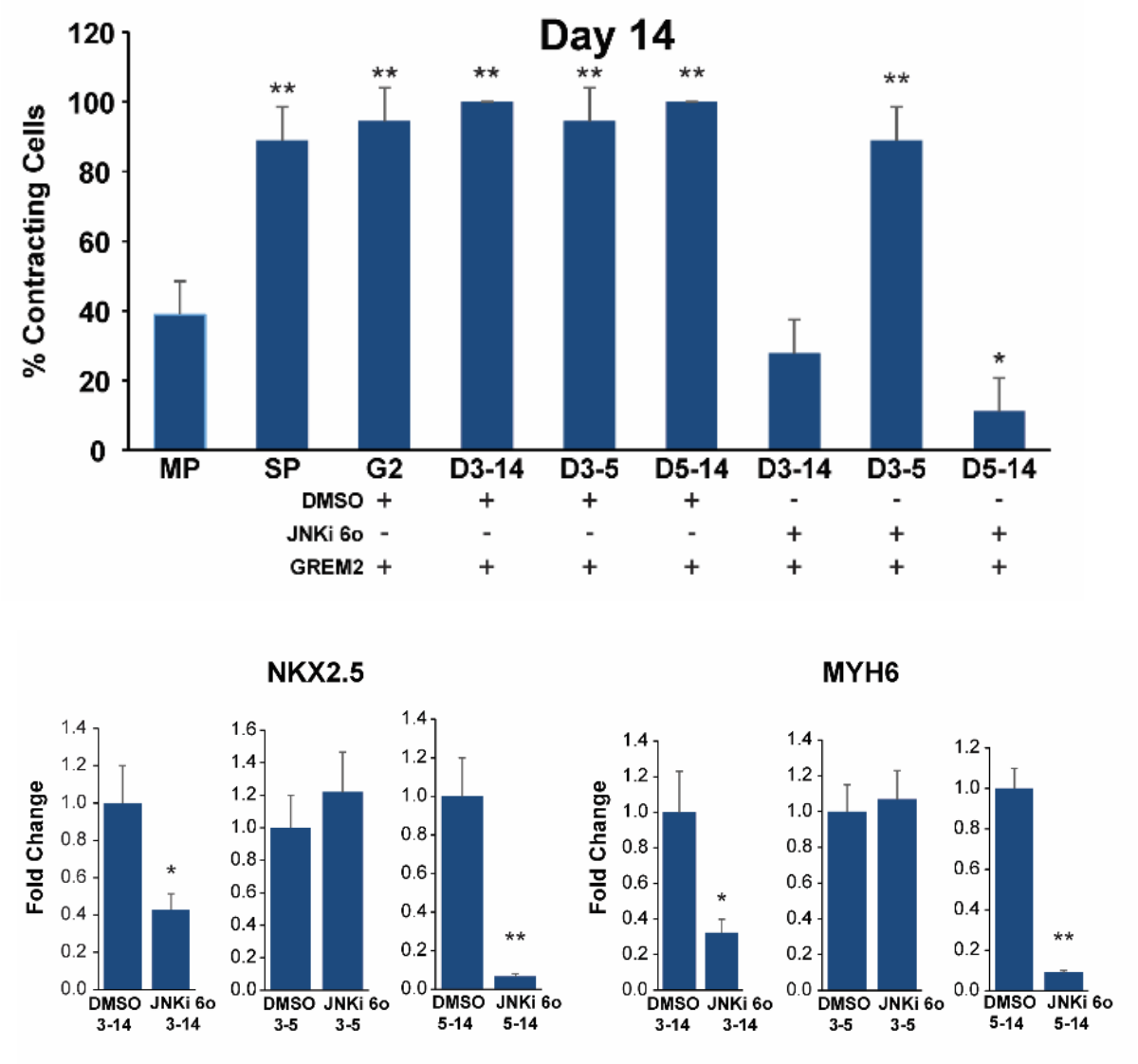

Figure 31: **JNK signaling is required for GREM2-induced cardiac differentiation.** (B) Differentiating DF 19-9-11 iPS cells were treated with GREM2 alone and either with GREM2 and the selective small molecule JNK inhibitor TCS JNK 6o (JNKi 6o) or vehicle as control (DMSO). Addition of JNKi 6o to GREM2-treated wells during the entire cardiac differentiation phase (Days 3-14) or just the late cardiac differentiation phase (Days 5-14) reduced the total percentage of contracting cells in each well. N = 3 replicates per condition. One-way ANOVA with Tukey's HSD test. *P < 0.05; ** P < 0.01. (C) RT-qPCR analysis of cardiac genes NKX2.5 and MYH6 at day 14 of differentiation shows a reduction of cardiac gene expression in cells co-treated with GREM2 and JNKi 6o. N = 3 replicates per condition. Unpaired t-test. *P < 0.05, **P < 0.01.

Summary

The results described in this chapter show that GREM2 treatment of differentiating human pluripotent stem cells is able to induce JNK signaling when compared to controls. Furthermore, this induction is unique to GREM2 and not shared by other BMP antagonists such as NOGGIN. When cells were concurrently treated with GREM2 and a JNK signaling inhibitor during the later stages of differentiation (days 5-7) as progenitor cells are transitioning to cardiomyocytes, the cardiogenic effect of GREM2 was severely diminished.

These data provide additional insight into how GREM2 is able to increase proliferation of cardiac progenitors in common with other BMP antagonists but has the additional property of inducing cardiac differentiation as well. In all cases tested thus far, the ability of GREM2 to modulate both BMP and JNK signaling has proven to be critical for its effects on cardiac differentiation in general (Tanwar et al., 2014).

It is interesting that in this case the effective window for addition of JNK inhibitor to abolish the cardiogenic effects of GREM2 was at the latter stages of differentiation as cardiac progenitor cells are transitioning to cardiomyocytes. This specificity is remarkable in that it presents a new signaling regulation paradigm, wherein a single molecule (in this case GREM2) is able to act as a molecular switch that transitions from BMP inhibition to JNK activation at precisely the right time to facilitate cardiomyocyte differentiation. This possibility is supported by reports in the literature that JNK signaling plays a key role in lineage specific differentiation of pluripotent stem cells (Xu and Davis, 2010)

Chapter 9

Conclusions, Discussion, Significance, and Future Directions

Conclusions

My thesis work has demonstrated that expression of the secreted BMP antagonist GREM2 is induced in pluripotent stem cell-derived human cardiac progenitor cells shortly after the specification of cardiac mesoderm. GREM2 expression is maintained in cardiac progenitor cells throughout their differentiation and persists in differentiated cardiomyocytes. BMP signaling antagonism is required for cardiac lineage development, since BMP ligand decoys that bind antagonists, but do not activate BMP receptors, abort cardiomyocyte differentiation. My results further show that GREM2 increases cardiac gene expression and cardiomyocyte differentiation, a process that depends on JNK signaling activation. Interestingly, although BMP antagonists such as NOGGIN and DAN equally stimulate cardiac progenitor cell proliferation, only GREM2 efficiently promotes differentiation to cardiomyocytes. All together these data show that GREM2 coordinates cardiogenic output in a 2-step mechanism, initially inhibiting canonical BMP signaling to promote CPC proliferation followed by induction of differentiation through JNK signaling activation (Fig. 32).

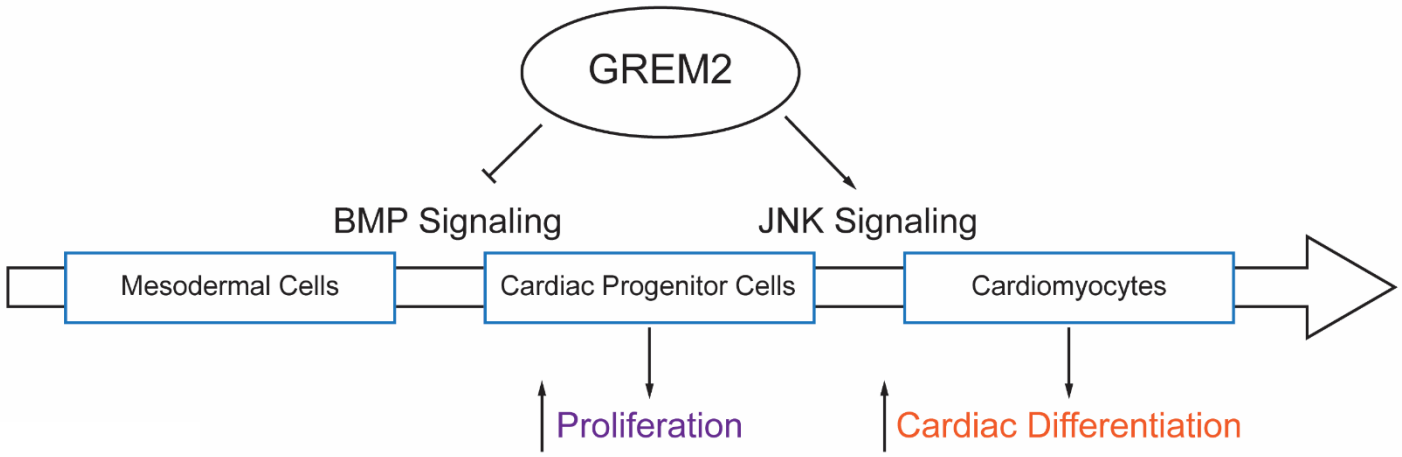

Figure 32: **GREM2 regulates cardiac proliferation and differentiation in human induced pluripotent stem cells.** Schematic diagram of the proposed role of GREM2 in regulating cell signaling during human cardiac differentiation. As cells progress from cardiac progenitors towards more committed cardiac cells GREM2 blocks BMP signaling to induce proliferation of cardiac progenitor cells and activates JNK signaling to promote differentiation.

Discussion

BMP ligands play crucial roles at various stages of cardiac development (van Wijk et al., 2007), starting from the initial cardiogenic specification of mesoderm (Kruithof et al., 2006), to subsequent cardiac tube assembly, asymmetric looping and jogging (Breckenridge et al., 2001), ventricular identity (Marques and Yelon, 2009), outflow track and cushion formation (Ma et al., 2005) and cardiomyocyte differentiation (Pater et al., 2012). Although canonical BMP signaling induces *NKX2.5* gene in the cardiac crescent to generate cardiac progenitor cells, BMP signaling needs to be suppressed to allow proliferation and expansion of cardiac progenitor cells. Interestingly, during mouse secondary heart field development, Nkx2.5 suppresses

BMP signaling by blocking pSmad1, forming a negative regulatory loop that promotes proliferation of cardiac progenitor cells (Kempf et al., 2011). This work shows that this mechanism likely applies to human cardiac progenitor cells as BMP signaling inhibition by either NOGGIN, GREM2, or DAN proteins promotes proliferation of cardiac progenitor cells.

On the other hand, elegant gain and loss-of function studies in mouse embryos have shown that persistent BMP signaling inhibition prevents differentiation of cardiac progenitor cells (Prall et al., 2007). These data confirm that NOGGIN and DAN prevent expression of genes specific to differentiated cardiomyocytes, including sarcomere proteins such as MYH6 and TROPONIN T2. In contrast, GREM2, after the initial stimulation of cardiac progenitor cell proliferation, leads to robust induction of cardiac differentiation. We have previously shown that GREM2 has the unique property among BMP antagonists, after its prescribed canonical BMP signaling inhibition, to subsequently induce JNK signaling activation that is required for cardiac differentiation (Tanwar et al., 2014). These results indicate that this GREM2 property is responsible for promotion of human cardiac cell differentiation as well, since JNK signaling inhibition aborts the pro-cardiogenic effect of GREM2. These findings are consistent with previous reports that have implicated JNK signaling in cardiogenic differentiation of pluripotent stem cells (Kempf et al., 2011; Ou et al., 2016).

Recent crystallographic evidence revealed that GREM2 folds into a unique tertiary shape that has not been described before. Specifically, GREM2 dimerizes in a head-to-tail manner, unlike the head-to-head pairing of NOGGIN (Groppe et al., 2002; Nolan et al., 2013, 2016). This head-to-tail arrangement gives rise to large, constrained and

arching hydrophobic surfaces on the 3-D structure, which precludes GREM2 from wrapping around BMP dimers as NOGGIN does (Groppe et al., 2002; Nolan et al., 2013, 2016). In this work I tested whether this unique structural arrangement is also critical for the function of GREM2 in regulating both proliferation and differentiation of cardiac progenitor cells. Future biochemical analyses may further identify critical structural motifs that could be exploited to design molecules that mimic the biological effects of GREM2, such as the ability to selectively alter its BMP inhibitory and JNK activating properties. Recognizing these mechanisms may offer additional novel insights in the cardiac differentiation process. Moreover, due to the wide interest in regulating BMP signaling in a number of human diseases, numerous BMP signaling inhibitors are being developed for clinical use (Ideno et al., 2009; Kriebitz et al., 2009). Thus, these findings may facilitate future repurposing of these new pharmacological resources for coordinated growth and differentiation of stem cell populations.

It was recently established that during embryonic development in zebrafish, GREM2 first appears in the pharyngeal mesoderm next to the forming heart tube (Müller et al., 2006, 2013). Loss- and gain-of-function approaches demonstrated that GREM2 is necessary for proper cardiac tube jogging and looping, cardiac laterality and cardiomyocyte differentiation by suppression of Smad1/5/8 phosphorylation (Müller et al., 2013). Although, left/right asymmetry and complex morphogenetic processes cannot be replicated in cell culture, our data show that the critical role of GREM2 in cardiomyocyte differentiation has been conserved across species and applies to human pluripotent stem cells. Moreover, it has been shown by this laboratory that GREM2 is critical for atrium formation in zebrafish and promotes differentiation of pluripotent mouse embryonic stem

(ES) cells to atrial-like cardiomyocytes (Tanwar et al., 2014). I did not detect preferential induction of atrial- versus ventricular-specific genes in the examined early stages of GREM2-treated human iPS cells (data not shown). Because human cardiomyocytes take longer to mature in culture than mouse cells (Mummery et al., 2002), it is likely that longer treatment periods might be required to test whether GREM2 can regulate chamber identity in iPS cell-derived cardiomyocytes.

In the adult mouse heart, it was recently discovered that GREM2 expression is highly induced in peri-infarct cardiomyocytes at the end of the inflammatory phase after myocardial infarction. Using genetic gain- and loss-of-GREM2-function mouse models and chemical compounds that inhibit BMPs, it was discovered that GREM2 modulates the magnitude of the inflammatory response and keeps inflammation in check through suppression of canonical BMP signaling (Sanders et al., 2016). GREM2 levels after myocardial infarction correlate with functional recovery, suggesting a new strategy to control inflammation of cardiac tissue after acute ischemic injury and improve cardiac function. It is intriguing that the BMP developmental pathway that initiates cardiac specification is also among the earliest induced after ischemic injury in the adult heart, but assumes a very different role. It is also intriguing that GREM2 induction takes place in peri-infarct cardiomyocytes indicating reactivation of cardiomyocyte-specific expression of GREM2 takes place under pathological conditions. However, unlike in developing cardiomyocytes, GREM2 does not induce cell proliferation in the adult heart. Understanding how this property is lost in adult cardiomyocytes might open the way to unlock cardiac regenerative mechanisms.

Significance

Human pluripotent stem cells hold great promise as a source of cardiac tissues for therapeutic use, as model systems for disease, and for gaining insights into the basic mechanisms of development (Bellin et al., 2012; Passier et al., 2016). To make good on this promise it is necessary to understand the mechanisms that drive cardiac differentiation in differentiating stem cells. Current studies and protocols have built on work in the field that has revealed how to quickly produce beating cells with properties of cardiomyocytes, including spontaneous contractility, the ability to metabolize lactate or pyruvate, and to a limited degree the specialized functions of cardiac subtypes, mostly of the ventricular subtype (Burridge et al., 2012; Mummery et al., 2012). These protocols have been generated at a rapid pace in a race to be first to generate the highest purity, greatest number of cardiomyocytes efficiently and quickly. Still lagging behind are studies that focus purely on the basic mechanisms that underlie cardiac differentiation in human pluripotent stem cells.

This work adds to our knowledge of the basic mechanisms that drive cardiac differentiation in human pluripotent stem cells. As has been reported for many growth factors and small molecules (Yuasa et al., 2005; Laflamme et al., 2007b; Hao et al., 2008; Kattman et al., 2011; Rai et al., 2011; Lian et al., 2013), GREM2 is able to enhance the cardiogenic differentiation of human pluripotent stem cells. However, this work reveals a unique signaling paradigm at play within differentiating stem cells wherein GREM2 sits at the transition point between proliferation of cardiac progenitor

cells and differentiation towards cardiomyocytes. The importance of such a mechanism in biological systems is apparent. As stem cells make the progressive journey from pluripotency to specific somatic cells, they must transition from progenitor populations to increasingly more functional and specialized populations (Birket et al., 2015; Burridge et al., 2012). These transitions must occur at precise points on the temporal and spatial level. To achieve this regulation, growth factor expression and secretion is highly regulated so that growth factor gradients appear and are sequestered at the proper times and places (Bruneau, 2013). GREM2 is able to bind BMP ligands, increase cardiac progenitor cell proliferation, and induce differentiation of cardiac progenitor cells. It accomplishes this by affecting both canonical Smad mediated BMP signaling and non-canonical BMP signaling through the JNK pathway. This reveals a mechanism by which switching from canonical to non-canonical BMP signaling can achieve a regulated transition from proliferating progenitor cells towards differentiated somatic cells.

This work also gives biological context to recent advances in our understanding of the structure and biochemical properties of GREM2 and its unique GREM2:BMP complexes (Kattamuri et al., 2012a; Nolan et al., 2013, 2016). Our work reveals important information about the putative biological roles of these unique GREM2:BMP/receptor complexes. It is likely that the ability of GREM2 to affect proliferation and differentiation relies to some degree on its specific biochemical properties.

This work also has applications in human cardiac development. While not a complete replication of the developing embryo, lacking the complete physiological system with its host of tissues and complex, dynamic cellular signaling interactions,

differentiating human stem cells provide a relatively high fidelity model of human development (Passier et al., 2016). In that case, it is likely that the molecular switch role played by GREM2 in differentiating hu12

man pluripotent stem cells is also important in human cardiac development. Indeed GREM2 mutants have been associated with cardiac disease in a clinical setting, and while the causal connection between GREM2 mutants and cardiac disease is still under investigation, it is possible that these GREM2 mutants fail to properly perform their intended function during development, leading to dysfunctional cardiac tissues (Müller et al., 2013).

Future Directions

This work has revealed a role for GREM2 in regulating human cardiac differentiation. While the context of GREM2 expression and localization was established, and gain of function studies using purified GREM2 protein revealed that GREM2 is sufficient to increase cardiogenesis in differentiating cardiomyocytes, more conclusive loss of function studies must be carried out to determine if GREM2 is specifically necessary for proper cardiac differentiation in human pluripotent stem cells. The work described here shows that sequestration of BMP inhibitors like GREM2 and Noggin reduces cardiac gene expression and decreases the numbers of cardiomyocytes observed in differentiating cultures. Expression data also shows that GREM2 and NOGGIN are the two antagonists expressed during the cardiac differentiation process. However these assays are unable to distinguish between

GREM2 and NOGGIN. Thus future studies that specifically knock out or otherwise neutralize GREM2 and NOGGIN are required to establish the necessity for GREM2 in cardiac differentiation of human pluripotent stem cells. Such efforts would likely employ advanced gene editing techniques such as CRISPR that specifically target GREM2 (Hockemeyer and Jaenisch, 2016). Cell lines with GREM2 removed from the genome or with mutant GREM2 coding sequences substituted for the wild type could be differentiated using the protocols described in this work. Alternatively, with our more detailed understanding of the residues responsible for binding between GREM2 and BMP ligands and how these differ from those found within NOGGIN, engineered BMP proteins similar to L51P but that specifically bind and sequester GREM2 could be used to obtain similar results.

This work shows that GREM2 is able to affect both proliferation of cardiac progenitor cells and differentiation of cardiomyocytes in differentiating human pluripotent stem cells. This molecular switch effect is shown to be unique to GREM2 among other BMP antagonists. However it still remains to be determined if this paradigm is employed by other signaling pathways and their respective ligands.

Also remaining to be explored is the mechanism behind the unique molecular switch effect of GREM2. The current hypothesis is that this unique effect is accomplished via the unique biding properties observed in GREM2:BMP complexes and their interactions with BMP receptors (Nolan et al., 2016). GREM2:BMP complexes are able to form unique polymeric structures and retain some affinity for BMP and other receptors. Each of these possible mechanisms needs to be investigated using

pluripotent stem cell differentiation assays to test the hypothesis that these unique properites indeed play a role in GREM2's molecular switch effect.

As already discussed, in differentiating mouse ES cells and zebrafish, GREM2 was able to selectively affect atrial cardiomyocyte differentiation at the expense of ventricular myocytes. So far this effect has not been observed in differentiating human pluripotent stem cells. This does not rule out a role for GREM2 in affecting the type of cardiac cells generated during human pluripotent stem cell differentiation. Differentiating human pluripotent stem cells are highly sensitive to the concentrations, timing, and duration of treatment with exogenous factors or small molecules (Mummery et al., 2012; Burridge et al., 2012; Lian et al., 2013). It is possible that a thorough investigation of the effect of adjusting treatment duration, concentration, and day of GREM2 treatment will reveal an effect on the subtypes of cardiomyocytes generated from human pluripotent stem cells. RNA seq analysis of the transcriptome of pluripotent stem cells treated with GREM2 vs. controls would also reveal the extent to which GREM2 might affect the properties of the cardiomyocytes generated from GREM2 treated cultures. The RT-qPCR studies reported in this work were able to indicate the degree to which GREM2 treatment affected specific genes chosen for their well characterized ability to mark cardiomyocytes. However the low throughput nature of RT-qPCR makes it impractical to sample all known cardiac genes and their regulatory signaling pathways. RNA seq allows global profiling of all genes being actively transcribed in a cell population (McGettigan, 2013; Mortazavi et al., 2008). Subsequent bioinformatics analysis can then be used to look at how GREM2 treatment is affecting the entire complement of known cardiac and signaling pathway markers.

Appendix A

Table 1 Amplification primer sequences

Gene	Forward	Reverse
ALK3	GGACATTGCTTTGCCATCATA	CAGACCCACTACCAGAACTTT
ALK6	AGTGTCGGGACACTCCCATTC	TGAACCAGCTGGCTTCCTCTGTG
BMP2	ACCCGCTGTCTTCTAGCGT	TTTCAGGCCGAACATGCTGAG
BMP4	GCTGTCAAGAATCATGGACTGTTAT	CCTCATAAATGTTTATACGGTGGAA
BMPR2	AGGTGAAAAGATCAAGAAACGTGTG	TCCAGCGATTCAGTGGAGATG
CERBERUS	CATCCAGGGACTCAGATAGTGA	GCAGGTCTCCCAATGTACTTCA
CHORDIN	CCCAACACATGCTTCTTCGAG	CACCATCAAAATAGCAGCCCT
GAPDH	AAGGTGAAGGTCGGAGTCAAC	GGGGTCATTGATGGCAACAATA
GREM2	ATCCCCTCGCCTTACAAGGA	TCTTGCACCAGTCACTCTTGA
ID2	GCATCCCCCAGAACAAGAAGGTGAG	CGCTTATTCAGCCACACAGTGCTTTG
ISL1	GCGGAGTGTAATCAGTATTTGGA	GCATTTGATCCCGTACAACCT
KDR	GTGATCGGAAATGACACTGGAG	CATGTTGGTCACTAACAGAAGCA
MESP-1	CGCTATATCGGCCACCTGTC	GGCATCCAGGTCTCCAACAG
MYH6	GGGGACAGTGGTAAAAGCAA	TCCCTGCGTTCCACTATCTT
MYL7	GAGGAGAATGGCCAGCAGGAA	GCGAACATCTGCTCCACCTCA
NKX2.5	GCGATTATGCAGCGTGCAATGAGT	AACATAAATACGGGTGGGTGCGTG
NOGGIN	ATCGAACACCCAGACCCTATC	TCTAGCCCTTTGATCTCGCTC
ß-ACTIN	TGAAGTGTGACGTGGACATC	GGAGGAGCAATGATCTTGAT
T BRY	TATGAGCCTCGAATCCACATAGT	CCTCGTTCTGATAAGCAGTCAC
TNNT2	TTCACCAAAGATCTGCTCCTCGCT	TTATTACTGGTGTGGAGTGGGTGTGG

Appendix B

Contributions

The following people contributed to the work described in chapter 3. Vineeta Tanwar, Jeffery B. Bylund, Jianyong Hu, Jingbo Yan, Joel M. Walthall, Amrita Mukherjee, Sabina Kupershmidt, Ela W. Knapik and Antonis K. Hatzopoulos. V.T., J.B. and J.Y, conception and design, collection and data assembly, data analysis and interpretation, manuscript writing; J.H., J.M.W., A.M., W.H.H., W-D.W., F.P. and M.R. data collection, data analysis and interpretation; S.K., data analysis and interpretation; E.W.K and A.K.H., conception and design, data analysis and interpretation, manuscript writing.

The following people contributed to the work described in chapters 4-8. Jeffery B. Bylund, Linh T. Trinh, Cassandra Awgulewitsch, David T. Paik, Christopher Jetter, Rajneesh Jha, Jianhua Zhang, Kristof Nolan, Chunhui Xu, Thomas B. Thompson, Timothy J. Kamp, and Antonis K. Hatzopoulos. J.B. conception and design, collection and data assembly, data analysis and interpretation, manuscript writing; L.T., C.A., and D.P. data collection, data analysis and interpretation, manuscript writing; C.J., R.J., J.Z., K.N., C.X. data collection, data analysis and interpretation; T.T., T.K., conception and design, data analysis and interpretation. A.K.H., conception and design, data analysis and interpretation, manuscript writing.

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