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Glucocorticoid Receptor Directs Cardiogenic Mesoderm Specification in Murine Embryonic Stem Cells by Induction of Cardiogenic Factors in Endoderm

A dissertation submitted in partial satisfaction

of the requirements for the degree

Doctor of Philosophy

in

Molecular Pathology

by

Joaquim Miguel Cabral Teixeira

Committee in charge:

Mark Mercola, Chair
Fred Levine, Co-Chair
Ju Chen
Marcia Dawson
Sylvia Evans
Sanjay Nigam
David Larocca

2011

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The Dissertation of Joaquim Miguel Cabral Teixeira is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2011

DEDICATION

In recognition of her incommensurable love, faith and support, this dissertation is dedicated to my aunt, Ermelinda da Purificação Cabral. This may not be a dissertation on the cure for cancer, as she hoped I would one day find, and even if it was, it would have come too late to help keep her around a while longer. This is, however, a dissertation written in honor of her memory and as a testimony to the thousands of young avid minds molded by her teachings and nurtured by her unabated devotion to education.

Though poorer without you, the world sure is
a better place because of you, Tia Ermelinda.

EPIGRAPH

"My philosophy, like color television, is all there in black and white"

Monty Python

TABLE OF CONTENTS

Signature Page iii

Dedication iv

Epigraph v

Table of Contents vi

Acknowledgements ix

Vita xii

Abstract of the Dissertation xv

Introduction 1
- Cardiovascular Disease and Cell-Based Therapies 1
- Meso-Endoderm Induction 3
- Cardiogenesis 7
- TGF-beta Signaling 12
- Glucocorticoid Receptor 19
- Glucocorticoids 22
- HNF4-alpha 24
- SOX17 31
- CER1, a Nodal-Signaling Inhibitor and Inducer of Cardiogenesis 33
- In This Dissertation 35

Introduction to Chapters 1 and 2 36

Chapter 1 - Glucocorticoid Receptor Directs Cardiogenic Mesoderm Specification In Murine Embryonic Stem Cells By Induction Of Cardiogenic Factors In Endoderm.. 39
- Abstract 39
- Results 41
  - HTS for Small-Molecule Inducers of Cardiomyogenesis 41

Hydrocortisone Induces Cardiomyocyte Differentiation in mESCs .. 41
Dexamethasone Acts Downstream of Smad-2, -3 Phosphorylation 44
Dexamethasone Induces Cardiomyogenesis Through GR ………. 45
Dexamethasone Upregulates Early Endodermal Markers ………... 46
Dexamethasone Does Not Affect Mesoderm:Endoderm Lineage Choice ……….. 47
Dexamethasone Upregulates Endoderm Secreted Cardiac Inducing Factors ………… 48
Dexamethasone Does Not Alter Flk-1/Foxa2 Ratio and Proliferation in Culture……….. 48
Hnf4a Transcriptional Activation Induces Sox17 And Cer1 In mESCs ………… 49

Chapter 2 - Novel Activity Of Dihydropyridines In Mouse And Human Embryonic Stem Cell Differentiation ………… 51

Abstract ………… 51
Results ………… 53
Dihydropyridines Have a Biphasic Role During Mesoderm Differentiation ………… 53
DHP Blocks SBE4-Luc Induction By TGFb-2, Activin A Upstream of Smad Phosphorylation ………….. 54
DHP Inhibits TGFb-Signaling Upstream of Smad-2 Phosphorylation ………… 55
DHP Blocks Lefty-1 Induced by Activin A Though Not by TGFb-2 .. 56

Discussion and Conclusions ……….. 57
Corticosteroids Induce Cardiogenesis in mESCs …………... 57
Dexamethasone Acts Downstream of Smad-2, -3 Phosphorylation 58
Dexamethasone Acts Via GR to Induce Cardiogenesis ……..…… 59
Dexamethasone Does Not Affect Meso-Endodermal Lineage Choice ……….. 60
Dexamethasone Upregulates Endoderm Specific Cardiac Inducing Factors ………… 61
Hnf4a Acts Downstream of GR to Induce Sox17 And Cer1 on mESCs………… 63
DHP TGFb-Signaling Acts at Distinct Times In Differentiation With Contrasting Results ………... 64

DHP and Dexamethasone Differently Modulate Nodal-Signaling to Induce Cardiogenesis 65

Final Remarks 67

Materials and Methods 69

Acknowledgements 72

Appendix 73

References 96

ACKNOWLEDGEMENTS

I would like to thank my mentor, Professor Mark Mercola, for the extraordinary opportunity to join his lab, for his unfazed guidance and mentorship over the years, for his inspiring love of Science and the acquisition of knowledge, his unabated drive to tackle new challenges, and for always encouraging me to seek and find unequivocal answers to the open questions in Cellular and Developmental Biology.

I would like to acknowledge my Committee Members for their patience, understanding, and constructive criticism. It has been a true pleasure discussing my findings with all. For several years, I was particularly fortunate to work with Professor Levine's and Dr. Larocca's groups. I would further like to thank Professor Dawson for the time we spent discussing my project and for helping me to better understand my findings, design improved assays and for Professor Dawson's genuine interest in my work.

I would like to acknowledge former and current members of the "Mercola Clan", as well as the countless research groups on and off campus with which I collaborated over the years: it has been a pleasure working alongside all.

I would like to acknowledge James "Jim" o'Fallon and Kathleen "Kathy" Kazules for their precious help in successfully navigating through paperwork and

departmental procedures. Their contribution to my peace of mind was just as crucial to the completion of this dissertation.

I would like to acknowledge Judie, Josh, Malene, David, Amy, Amanda, Vasco and Paulinha: more than just showing me there is life outside of lab, they are living proof of unconditional friendship.

I would like to acknowledge my family, António, Ana, mana Luísa and baby Nini, for being such uniquely precious and extraordinarily devoted individuals, always giving, always nurturing, always supportive, never demanding and ever enthusiastic about my work. I would also like to acknowledge my grandparents, Avó Céu and my best friend Avô Cabral, whom all their lives worked from dawn to dusk without ever complaining about how tough their life was. Their example has kept me grounded and their legacy serves as a constant reminder of the value of hard work and Family.

Finally, I am deeply grateful to Jaime for her partnership, her companionship, her love, her unstinting support and understanding, and for showing me there is Love outside of lab.

Chapter 1, in part, is currently being prepared for submission for publication of the material. Cabral-Teixeira J, Mercola M. **Glucocorticoid Receptor Directs Cardiogenic Mesoderm Specification in Murine**

Embryonic Stem Cells by Induction of Cardiogenic Factors in Endoderm. The dissertation author was the primary investigator and author of this material.

Chapter 2, in part, is currently being prepared for submission for publication of the material. Willlems E, Cabral-Teixeira J, Lanier M, Cai W, Bushway PJ, Xia Z, Dawson M, Cashman J, and Mercola M. **Novel Activity of Dihydropyridines in Mouse and Human Embryonic Stem Cell Differentiation**. The dissertation author was a contributing investigator and author of this material.

VITA

2002	Degree in Biology, University of Lisbon, Faculty of Sciences, Portugal
2001-2002	Honors Research Project in Cellular and Molecular Biology, Roche Pharma, Penzberg, Germany
2002-2003	Laboratory Manager, The Burnham Institute, La Jolla, California
2011	Doctor of Philosophy in Molecular Pathology, University of California, San Diego

PUBLICATIONS

Wei ZL, Petukhov PA, Bizik F, Cabral-Teixeira J, Mercola M, Volpe EA, Glazer RI, Willson TM, Kozikowski AP (2004). **Isoxazolyl-serine-based Agonists of Peroxisome Proliferator-Activated Receptor*: Design, Synthesis, and Effects on Cardiomyocyte Differentiation*.** J Am Chem Soc. 126(51): 16714-5.

SCIENTIFIC COMMUNICATIONS

2003 High Throughput Microscopy,
The Burnham Institute, La Jolla, California
Cell-Based Assays: Practical Applications to Cardiogenesis and Diabetes
Joaquim Cabral-Teixeira and Mark Mercola

SCIENTIFIC POSTERS

2004 Molecular Pathology Graduate Program Annual Symposium
UCSD, La Jolla, California
Small Molecule Inducers of Cardiomyogenesis
Joaquim Cabral-Teixeira, Yessenia Ibarra, Rosa Guzzo, Ann Foley and Mark Mercola

2006 Burnham Science Network Annual Poster Symposium
The Burnham Institute, La Jolla, California
Small Molecule Inducers of Cardiomyogenesis
Joaquim Cabral-Teixeira, Paul Bushway, Clemencia Pinilla, Richard Houghten, Marcia Dawson and Mark Mercola

2006 Salk Institute Stem Cell Symposium,
La Jolla, California
Small Molecule Inducers of Cardiomyogenesis
Joaquim Cabral-Teixeira, Paul Bushway, Clemencia Pinilla, Richard Houghten, Marcia Dawson and Mark Mercola

2009 Weinstein Cardiovascular Development Conference
San Francisco, California
Discovery of Small Molecules for Myocardial Regeneration
Paul J. Bushway, Erik Willems, Karl Okolotowicz, Marion Lanier, Joaquim Teixeira, Zebin Xia, Masanao Tsuda, Cyndie Gilley, Marcia I. Dawson, Jeff H. Price, Richard Houghten, Clemencia Pinilla, John Cashman and Mark Mercola

2010 Sanford|Burnham Science Network Annual Poster Symposium
San Diego, California

Glucocorticoid Receptor Directs Meso-Endoderm Specification in Murine Embryonic Stem Cells by a GRE-Independent Modulation of TGF-beta Signaling

Joaquim Cabral Teixeira, Mark Mercola

FIELDS OF STUDY

Major Field: Biology

Studies in Cellular and Molecular Biology

Professor Rui Malhó and Dr. Silke Hansen

Studies in Stem Cell and Developmental Biology

Professor Mark Mercola

ABSTRACT OF THE DISSERTATION

Glucocorticoid Receptor Directs Cardiogenic Mesoderm Specification in Murine Embryonic Stem Cells by Induction of Cardiogenic Factors in Endoderm

by

Joaquim Miguel Cabral Teixeira

Doctor of Philosophy in Molecular Pathology

University of California, San Diego, 2011

Professor Mark Mercola, Chair
Professor Fred Levine, Co-Chair

Heart failure is the number one cause of death in the developed world. The progression of the disease leads to the loss of cardiomyocytes that cannot be replaced endogenously. Embryonic stem cells (ESCs) can be differentiated in vitro into cardiomyocytes. The identification of drug-like compounds that can enrich the differentiating ESC cultures with cardiomyocyte progenitors would be beneficial to generate and expand cardiomyocyte cultures for research and

clinical applications, while also serving as probes to dissect the mechanisms that control differentiation.

In order to identify novel signaling proteins and pathways involved in cardiogenesis, we developed a fluorescence-based reporter system to screen chemical libraries for cardiomyocyte-inducing molecules. This was screened against a library of known drugs and small molecule pathway modulators. Active compounds included hydrocortisone and the artificial glucocorticoid Dexamethasone (Dex), which induced 10-fold increase in cardiomyocytes, confirmed by an upregulation of cardiac markers Tbx5, MEF2c, cTnt, alphaMhc, as well as the endothelial cell marker CD31. Ligand activated GR seems to upregulate a cascade of events in *Foxa2*+ definitive endoderm that begins with *Hnf4a* induction and results in Sox17 -> Cer1 upregulation. Cer1 is a known Nodal inhibitor and has been shown to promote cardiogenesis by locally blocking Nodal in committed cardiac progenitors (*Flk1*+, *MesP1*+) and allowing them to proceed with the cardiac program. Dex does so without affecting meso-endoderm lineage choice.

DHP seems to alter the balance between mesoderm and neurectoderm when added early to the cultures, in a time when mesoendoderm specification occurs. Later, once the mesoendoderm has been established, it promotes cardiogenesis by inhibiting Nodal/Activin signaling at the receptor level by a mechanism yet to be elucidated - recent data suggests this might be receptor

type specific. It appears to mimic the effects of the natural cardiac inducer Cer-1 by blocking Nodal/Activin/TGFb- and BMP-signaling both in cardiac progenitors, resulting in an augmentation of the a cardiac restricted *Flk1+, MesP1+* progenitor population and ultimately of cardiomyocytes. Our results should contribute to a better understanding of the important cross-talk between endoderm and mesoderm progenitors in the establishment and expansion of a cardiac progenitor population.

INTRODUCTION

Cardiovascular Disease and Cell-based Therapies

Cardiovascular disease is the primary cause of morbidity and mortality in the developed world: according to World Health Organization (WHO) estimates, in 2005, 17.5 million people died of CVD. This represents 30 percent of all deaths globally, making cardiovascular diseases the leading cause of death in the world, particularly among women (World Health Organization Statistical Information System [WHOSIS], 2009 update; AHA 2010 Update).

Cardiovascular disease often progresses to a state of myocardial ischemia, with loss of contractility and irreversible cardiomyocyte injury (Jennings et al., 1991). Within 24 hours the infarcted myocardium will undergo primary and secondary necrosis, losing as many as one billion cardiomyocytes, followed by acute and chronic inflammation to clear the dead tissue. The wound repair mechanism that initiates this debridement replaces the lost myocardium with dense collagenous scar tissue (Mallory et al., 1939). The pathology leads to a profound decrease in myocardial function in the ischemic region that impairs the essential function of the heart as a biomechanical pump. Depending on location and extent of injury, pathogenesis can progress to heart failure and death.

Current therapies for heart failure are not designed to replace myocytes. The need for heart replacement surpaces the current availability of suitable

organs from donors, and transplantation is further limited by high costs and histocompatibility issues. Clearly there is a need to find alternative therapies to repair the damaged heart.

Remuscularization has been pursued by many groups over the past 15 years. Success in finding a good candidate for cell-based myocardial therapy requires not only that the cell type is capable of differentiating into a fully functional cardiomyocyte, but also that it can physically and electrically integrate into the healthy tissue bordering the damaged region, while responding to the hierarchy of signals that operate the heart in a concerted fassion. Adult muscle cell types, such as skeletal muscle, are unable to form the proper gap junctions with the resident tissue, leading to arrhythmia. Other cell types, such as bone marrow-derived stem cells, resident cardiac stem cells, and endothelial cels, have failed to unequivocally show a significant number of *de novo* cardiomyogenesis, despite clear paracrine beneficial effects.

A billion myocytes can be lost after myocardial infarction. A large source of cells is therefore needed if one is to replace the loss of myocardium with either exogenous cardiomyocytes, or by promoting regeneration within the cardiac muscle. ESCs and the recently developed induced-Pluripotent Stem Cells (iPSCs) are a plausible source of cardiomyocytes for transplantation, as well as models in which to study the biology of resident stem cells in the heart and the signaling molecules that enchance the limited regenerative capacity of the adult

human heart.

In 1998, Thompson et al. first demonstrated the ability to grow and expand human embryonic stem cells (hESC) *in vitro* for an indefinite period of time. Since then, many reports have arised showing ESCs unquestionable ability to differentiate into virtually all cell-types in the human body. Methods to direct the production of cardiomyocytes from human and murine ESCs were among the first to be developed. The most efficient recapitulate the natural developmental processes in the embryo (reviewed in Mercola et al., 2011). Human and mouse ESCs represent a unique oportunity not only to develop cell-based therapies for cardiovascular disease, but also a model in which to study embryonic development in a simplified system, with clear advantages in terms of ease of manipulation and observation. On the other hand, the multipotency of ESCs also makes the fate of these cells challenging to control as they differentiate and mature.

Meso-Endoderm Induction

Axial pattern in the mouse embryo can be aligned with the axes of the blastocyst; however, it is not determined and can be altered, or re-oriented at later times. Axial pattern is determined when the inner cell mass of the blastocyst elongates to form the egg cylinder. At this time, the egg cylinder's proximal-distal axis is apparent by gene expression patterns, including the differential and localized expression of factors like Cerberus1 (Cer1) and Lefty1

in the distal visceral endoderm (DVE), and genes such as Cripto and Brachyury (T/Bra) expressed in the proximal epiblast (Belo et al., 1997; Beddington, 1998; Beddington and Robertson, 1998, 1999; Ding et al., 1998; Yamamoto et al., 2004). With continued growth of the embryo, the DVE shifts position anteriorly to become the anterior visceral endoderm (AVE), re-orienting the proximal-distal axis into an anterior-posterior axis, while the localized expression of the proximal genes in the posterior embryonic pole gives rise to the primitive streak. This movement is dependent on Nodal signaling, which plays an important role both in specifying the DVE as well as to pattern the epiblast. Together with the localized and tightly timed expression of Cer1 and Lefty1, Nodal establishes the anterior-posterior axis and cell movement during gastrulation. The primitive streak becomes localized to the posterior pole of the embryo, as a result of a synergy between Cer1 and Lefty1 to determine the direction of DVE migration. This establishes important asymmetries that will later determine lineage development and cell fate. In the embryo, Nodal expression persists until E7.5 (Collignon et al., 1996; Meno et al., 2001). Both *in vivo* and *in vitro*, up-regulation and subsequent down-regulation of Nodal is necessary for maturation of endoderm and mesoderm to occur.

Gastrulation in higher vertebrates consists of the mesodermal cells undergoing an epithelial to mesenchymal transition and emigrating from a transient structure known as the primitive streak, which begins at the posterior margin of the embryo and extends anteriorly along the middle. Gastrulation was

first noted by Christian Pander (1794-1865), in the early 19th century. Pander soon realized that the three germ layers in the chick embryo had to interact in order for the organs to form properly. Over a century later, British biologist Lewis Wolpert would call gastrulation “the most important time of your life”. In fact, it is during gastrulation that cells from the primitive ectoderm, or epiblast in chicks, receive instructive signals, such as Nodal, from the AVE on what to become. The patterns of gastrulation can vary significantly between animal species. Here, I will focus mostly on mouse development, while noting important aspects of chick, *Xenopus sp.*, and zebrafish development as well.

Gastrulation is the process that gives rise to the three germ layers (endoderm, mesoderm and ectoderm) and positions them relative to one another so that eventually, with body closure, the endodermal organs will form in the center of the body cavity surrounded by mesodermal cells and an outer layer of ectoderm. In amniotes, gastrulation occurs as cells ingress along a furrow known as the primitive streak, a convergence of cells in the midline of the epiblast into a thick and hyper-proliferative structure extending anteriorly from the posterior of the embryo.

As gastrulation ensues, cell fate in the 3 germ layers is being patterned to specify organotypic differentiation. Differentiating cells from the embryonic epiblast ingress through the primitive streak, and undergo an epithelial to mesenchymal transition to delaminate from the epiblast and migrate anteriorly.

Those cells destined to form endoderm will re-epithelialize and displace the extra embryonic endoderm (Poelmann, 1981; Lawson et al., 1986; Tam and Beddington, 1992; Tam et al., 1993; Tam et al., 2004). This new layer of endoderm cells in turn gives rise to the definitive endoderm (Lin et al., 1994) and therefore to organs like the stomach, the liver, and the lungs. Mesoderm cells position themselves between the endoderm precursors and the ectoderm. Blood, heart and skeletal muscle are all embryonic mesoderm derivatives. The most anterior cells to ingress from the node form prechordal mesoderm and a transient structure called the notochord, which persists as the pulpous region of intervertebral discs.

Classic transplantation experiments in the late 19th century showed inductive interactions between the germ layers to pattern the embryo. Later work from as early as 1920's showed that multipotent cardiac progenitors were present throughout the mesoderm, and that heart could be induced in posterior mesoderm transplanted to near the foregut (Stöhr, 1924; Orts-Llorca and Gil, 1965; Jacobson and Duncan, 1968). In the late 1960's, amphibian embryo work initiated by Dutch embryologist Pieter Nieuwkoop (1917-1996) juxtaposed presumptive ectoderm with endoderm, resulting in induction of mesoderm within the ectoderm (Ogi, 1967; Nieuwkoop, 1969a, 1969b; Nieuwkoop and Ubbels, 1972). This work shed light on how the three germ layers reciprocally interact to pattern the early embryo by secreting and responding to inducers. Both inductive and repressive signals come into play at specific times in a succession of

interactions and gradients to promote cardiogenesis. Nieuwkoop showed that endodermal signals could induce mesoderm from ectoderm; this assay was then used to purify TGFb family members responsible.

The intensity, duration and the spatial-temporal activity of inducers and repressors throughout development are important for controlling cell fate. Since Nodals are secreted as pro-proteins, the expression of convertases and their level of expression, together with a graded expression of Nodal inhibitors, can determine the level of signaling in a cell. In fish, high Nodal, for instance, induces Goosecoid (Gsc) and prechordal plate fates (Gritsman et al., 2000), as well as endoderm (Schier et al., 1997; Thisse et al., 2000; Stainier, 2002), whereas T/Bra and notochord progenitors seem to be induced by lower levels of Nodal signaling (Gritsman et al., 2000). In mouse, evidence suggests that high levels of Nodal are required for node and anterior mesendoderm induction, whereas lower levels result in posterior mesoderm formation (Lowe et al., 2001, Norris et al., 2002). Still, we lack precise understanding of how the duration and intensity of Nodal signaling controls cell-fate specification.

Cardiogenesis

The heart is the first organ to form in the developing embryo and lies at the center of the embryo’s first functional unit: the cardiovascular system (Mohun et al., 2003; Foley and Mercola, 2004). In vertebrates, the formation of a functional heart is also one of the most fundamental steps in the developing

embryo: defects in heart development are often embryonic lethal and may affect the normal development of many if not all other structures in the embryo.

As gastrulation progresses and the embryo undergoes dorsoventral and anterioposterior patterning, a group of cells in the anterior lateral plate mesoderm responds to signals from both endoderm and ectoderm, and forms a crescent-shaped structure referred to as cardiac-crescent. In addition, the anterior endoderm secretes Dickkopf-1 (Dkk1), Nodal and Cerberus to induce or stimulate further secretion of cardiac inducers, and in response to canonical Wnt-mediated suppressing signals (Wnt3a and Wnt8) from the nothocord (Nascone and Mercola, 1995; reviewed by Foley and Mercola, 2004 and in Foley et al., 2006). Experimental evidence suggested that inhibition of Wnt signaling in the anterior lateral plate mesoderm allows for the formation of heart tissue (Marvin et al., 2001). Andrew Lassar's group went on to propose a model in which a Wnt activity gradient from the anterior-posterior axis intersects BMP signals from the dorsal-ventral axis in a region where a combination of high BMP and low Wnt activity would induce cardiogenesis. This results in a spatial-temporal combination of both suppressive and inductive factors, which determine where and when the heart primordia will form. The outcome of these opposing signals is the activation of transcription factors responsible for the myocardial gene program (McFadden and Olson, 2002). Reduced Wnt signaling seems to initiate the expression of the homeodomain protein Nkx2.5 and the zinc-finger transcription factor Gata4 in the anterior lateral plate mesoderm. Nkx2.5 and

Gata4 are two important regulators of the cardiac program and are present in the migrating cardiac mesoderm. The lateral plate mesoderm now expressing such factors becomes responsive to BMP and FGF signaling coming from the endoderm and lateral mesoderm, resulting in the sustained expression of Nkx2.5 (Zaffran et al., 2002) in both primary and secondary heart fields, though the proximity to the neural tube and consequent exposure to elevated Wnt signaling might help explain the slight delay in Nkx2.5 expression in the secondary heart field (Raffin et al., 2000; Brown et al., 2004). BMPs and Wnts inhibit cardiogenesis during the time when uncommitted cardiac progenitors adopt a cardiac fate; however, these factors are subsequently needed for terminal differentiation of committed precursors, suggesting that exposure to such molecules must be tightly regulated (Schultheiss et al., 1997). Nkx2.5 is one of the first factors to be expressed in the emerging cardiac regions of the embryo, being present from heart tube morphogenesis to the differentiation into myocardium, mesocardium and pericardium, and is synonym with cardiac progenitor cells (Raffin et al., 2000). Together, Nkx2.5 and Gata4 synergize to activate cardiac gene expression even in non-myocardial cells if ectopically expressed (Bodmer, 1993; Gajewski et al., 1999; Gajewski et al., 2001). These transcription factors are critical for the induction of members of the T-box and Mef2 families, which help instruct the competent mesoderm to become heart. Mef2, Srf, Tbx-2, -5, and -20 follow suit to induce the expression of sarcomeric proteins (Han et al., 1992).

Prior to ventral body wall closure, each of the paired primordia in the splanchnic mesoderm forms a vascular plexus. With body wall closure, the primordia fuse at the ventral midline, eventually resulting in a linear heart tube consisting of cardiac mesoderm cells and endothelial lining. Shortly thereafter, the linear heart tube develops into a single pumping chamber comprised of a myocardial outer layer separated by extracellular matrix from an endocardial inner layer, and anteriorly to posteriorly composed of bulbus cordis, ventricle, atrium and sinus venosus. Subsequently, the first heartbeats occur even though blood circulation will not begin for another day (mouse). The heart will sequentially (1) undergo elongation, (2) loop to the right (involving left-right patterning factors such as Nodal, Lefty2 and Pitx2), (3) incorporate a second group of progenitor cells originated in the pharynx and known as the second heart field, (4) position the atria dorso-anteriorly relative to the ventricle, and (5) undergo septation of the linear tube, separating the right and left chambers of the heart. Each chamber will express different contractile proteins and present a distinguishable gene expression pattern. For instance, the MADS-box transcription factor Mef2c is required for ventricular, but not atrial expansion (Lin et al., 1997) while dHand2, a basic helix-loop-helix transcription factor will selectively promote right ventricle growth (Srivastava, 1997). Illustrative of that are embryos missing the T-box transcription factor Tbx5, which fail to completely develop atria and left ventricle, yet show no right ventricle or outflow tract phenotype (Bruneau et al., 2001). The four-chambered heart will continue to

expand, convolve and mature until shortly after birth (Wilens, 1955; Jacobson, 1961; DeHaan, 1965).

Embryonic Stem Cells (ESCs) spontaneously differentiate *in vitro* to form beating cardiomyocytes expressing cardiac markers. The efficiency of this process and the yield of cardiomyocytes are determined in part by how successfully one is able to replicate *in vitro* specific embryonic differentiation programs during certain time windows. Four main steps are required in order to successfully differentiate ESCs into functional cardiomyocytes (for a review, see Willems et al., 2009 and references within):

1. Primitive streak mesoderm induction (marked by *T/Brachyury*);
2. Patterning of mesoderm into anterior mesoderm or cardiogenic mesoderm (*Goosecoid [Gsc] and Mesoderm posterior-1 [MesP1]*);
3. Induction of cardiac mesoderm (*Nkx2.5 and Mef2c*);
4. Differentiation into maturing cardiomyocytes (*alphaMhc* and *cTnT*).

Mesoderm induction requires a combination of Wnts, Nodal/Activin A, and BMPs. TGFb signaling through Nodal, aside from promoting both endoderm and mesoderm, is also capable of inducing cardiac progenitors (Faure et al., 2000; Perea-Gomez et al., 2002; Yamamoto et al., 2004). Cardiomyocytes are mesoderm derivatives, though specified during early embryogenesis by inductive signals from endoderm that develops in close association with presumptive cardiac mesoderm (Foley et al., 2006). Ablating the anterior endoderm in

Xenopus embryos results in failure to achieve cardiac specification (Nascone and Mercola, 1995).

The molecules involved in cardiac specification show a bimodal effect in mamallian heart induction, as ilustrated by a prolonged exposure of meso-endoderm to high Nodal signaling resulting in foregut endoderm and craniofacial mesoderm. Exposure of *Flk1*$^{+}$, *MesP1*$^{+}$ cardiac progenitors to BMPs and Wnts inhibits their cardiac fate; once committed, however, both signaling pathways need to be activated in order to induce terminal differentiation into functional cardiomyocytes (Schultheiss et al., 1997). Murray's group (1999) showed in zebrafish that exposure of embryos to Wnt signaling before gastrulation resulted in an increase in lateral mesoderm derivatives (*Nkx2.5*+); however, if overexpressed after gastrulation, Wnt reduced the number of cardiac progenitor cells (Ueno et al., 1997). Similar results were obtained in mESCs, with an exposure to Wnt-3a between days 2-5 of differentiation resulting in a 20-fold increase in embryoid body (EB) spontaneous contractions accompanied by the relevant cardiac markers. These examples suggests the necessity for precise dosage and time windows during which each cell population is to be exposed to inducing and inhibitory factors.

TGF-beta Signaling

Nieuwkoop (1973) demonstrated that *Xenopus* animal cap explants, from a tissue that during normal development forms ectoderm derivatives, can be

induced to assume a mesodermal fate when in the presence of vegetal pole cells. Slack and colleagues first identified FGF as a mesoderm inducing factor, by incubating animal caps with purified FGF. Since then, Doug Melton, Jim Smith and colleagues identified TGFb family members as potent mesoderm inducers capable of mimicking the mesoderm-inducing signals from the vegetal pole cells.

The transforming growth factor-beta (TGFb) superfamily is a large family of structurally related proteins involved in many cellular processes in both the embryo and the adult, including, among others, cell growth, differentiation, apoptosis, and homeostasis. The TGFb family of regulatory proteins is present in both invertebrates and vertebrates, and to date includes over 30 distinct genes belonging to four major subfamilies:

1. the Activin/inhibin subfamily;
2. the TGF-b subfamily;
3. the decapentaplegic-Vg-related (DVR) related subfamily (including BMPs and certain growth differentiation factors or GDFs);
4. a group including more divergent members.

Activins are composed of inhibin-beta dimers and were first shown to mimic the ability of vegetal cells to induce mesoderm and endoderm in Xenopus. Importantly, different doses of Activin were subsequently shown to specify different mesodermal and endodermal fates, with high doses inducing endoderm

and anterior mesoderm, and progressively lower doses inducing ventroposterior mesodermal fates. The action of Activin is thought to mimic the natural functions of Nodal. In addition to inducing and patterning mesoderm, Nodal is a critical player in left-right asymmetry. A brief mention is made here to the capability of BMPs of inducing ventral mesoderm during early embryogenesis. Next, we will see how, despite having diverse biological effects, different TGFb family members share intracellular pathways to transmit similar though not identical signals.

In general terms, TGFb signaling occurs through a mechanism by which a secreted homo- and sometimes hetero-dimeric pre-pro-polypeptide undergoes proteolytic cleavage and binds to a hetero-tetrameric cell surface protein complex comprised of two type I (TGFb-RI) and two type II (TGFb-RII) receptors. Upon ligand binding, the type II receptors become phosphorylated and consequently phosphorylate the short GS domain of the type I receptors on serine and threonine residues (Souchelnytskyi et al., 1996). This in turn activates the type I receptor kinases which phosphorylate two Smad proteins, recruited to the complex, at the C-terminal serines. Prior to receptor activation, the N-terminal Mad homology (MH1) domain and the C-terminal MH2 domain of the receptor activated Smads (R-Smads) are physically associated (Miyazawa et al., 2002).

Upon receptor activation and phosphorylation of R-Smads, the interaction between the MH1 and MH2 domains is interrupted and the receptor-activated

Smads form a trimeric complex with the common-mediator Smad (co-Smad; Smad-4 in vertebrates). The effector complex shuttles to the nucleus where it recruits sequence-specific binding partners to co-activate or co-repress gene regulatory sequences, and modulate gene expression by activating or repressing downstream targets. Smad-3 and a partner Smad, known as a Co-Smad, Smad-4, as heterotrimers, can bind directly to Smad Binding Elements (SBE), rich in CAGA repeats, and related DNA sequences (Dennler et al., 1998; Zawel et al., 1998), as well as TGFb Inhibitory Elements (TIE). Smad-2 dimers however, do not directly bind DNA; instead, Smad-2 is able to form trimeric complexes with Smad-3 and/or Smad-4, which in turn are able to bind to the CAGA sequences. BMP-activated R-Smads seem to preferentially bind to and activate GC-rich sequences, though with low-affinity (Ishida et al., 2000; Kusanagi et al., 2001).

The pathways modulated by the TGFb family of proteins have been extensively studied yet remain to be fully characterized. It is thought that BMPs and GDFs act through Smad-1, -5 and -8, whereas TGF-betas, activins, Nodal and myostatin act through Smad-2 and -3 (Derynck and Miyazono, 2008). However, post-translational modifications, such as phosphorylation, ubiquitylation and sumoylation, can determine the stability and availability of the receptors. Many proteins that interact with the receptors can also up-regulate (e.g. SARA facilitates Smad-2 and Smad-3 signaling; Endofin facilitates Smad-1 signaling; Axin facilitates Smad-3 phosphorylation by TGFb-RI; Dab2, found in clathrin-coated vesicles, is required for TGFb-induced Smad signaling; Dok-1 is required

for Activin signaling) or down-regulate (e.g. Smad-6 preferentially inhibits BMP signaling and Smad-7 inhibits both TGFb and BMP signaling) downstream Smad-mediated events (for an extensive review, see Miyazono, 2008; Kang and Derynck, 2009; and Kang et al., 2009). It has recently been shown that TGFb is able to, for instance, activate downstream events through Smad-1 and Smad-5, a mechanism previously thought to be exclusive of BMP signaling (Goumans et al., 2003; Daly et al., 2008; Liu et al., 2009).

TGFb proteins also activate non-Smad mediated pathways such as Erk, p38 and JNK MAP kinases, Rho-like GTP-ases, and the PI3 kinase-Akt-TOR pathway (Derynck and Zhang, 2003; Moustakas and Heldin, 2005).

Different type I receptors can combine in the same receptor complex and result in the dual activation of the Smad-2/Smad-3 pathway and the Smad-1/Smad-5 pathway (Daly et al., 2008). Furthermore, one cannot exclude the possibility that heterodimeric complexes may form between Smad-2 or Smad-3 and Smad-1 or Smad-5, thus resulting in completely different gene expression profiles (Liu et al., 2009). Activin/TGFb and BMP signaling can therefore no longer be considered two separate and independent pathways (Wharton and Derynck, 2009).

The mechanism of internalization of the receptor further seems to be able to regulate the cellular response. In most growth factor-activated tyrosine kinase

receptors, binding of the ligand to the receptor induces or enhances endocytosis of the ligand-receptor complex. Consequently, the intracellular routing of the receptors is able to determine the range and duration of the downstream response. This, however, is not the case for the TGFb family of proteins: here the receptor complexes internalize through clathrin-, caveolin-1-, or lipid-raft-mediated endocytosis (the former associated with SARA- and cPML-mediated Smad activation; the latter two associated with receptor degradation; see Di Guglielm et al., 2003, and Faresse et al., 2008) in a process which is both constitutive and independent of ligand binding. Not surprisingly, there is also evidence suggesting that both ligand-dependent and -independent TGFb receptor recycling can be regulated by clathrin-mediated endocytosis (Mitchell et al., 2004).

At the ligand level, the TGFb family members are generated as latent pre-pro-polypeptides. The active mature peptides are cleaved from the latent forms by cellular proteases. TGFb-1 is predominantly processed by a proprotein convertase, furin, whereas TGFb-2 has a consensus cleavage site for furin and is, therefore, presumed to be cleaved by furin. However, TGFb-2 is often secreted as the latent form, which appears to be inconsistent with its postulated sensitivity to furin. This illustrates the fact that TGFb proteins are secreted as inactive complexes with other proteins, which modulate processing and/or prevent the ligand from binding to the receptor and thus eliciting a cellular response. Activins, for instance, are often secreted along with follistatin, which results in ligand

inactivation (Chang, 2008). TGFb, aside from being non-covalently bound to its prodomain, can in addition form a complex with the inactive TGFb binding protein (Rifkin, 2005). BMP-binding proteins like Chordin, Noggin, and Cerberus help define BMP morphogenic activity by establishing BMP gradients in developing *Xenopus* sp. and *Drosophila* sp. embryos (reviewed in Umulis et al., 2009). Furthermore, the ligands can also present to the receptor as heterodimers and elicit a more potent response than homodimeric ligands (Israel et al., 1996; Shimmi, 2005; Tanaka et al., 2007).

Altogether, these examples illustrate the diversity of responses elicited by the TGFb family of proteins and the intricate mechanisms by which such responses are regulated. In summary, Smad proteins mediate TGFb- and BMP-signaling pathways (Miyazawa et al., 2002). Smads can be placed into three subtypes: receptor-regulated Smads (R-Smads), common-partner Smads (Co-Smads), and inhibitory Smads (I-Smads). R-Smads Smad-2 and Smad-3 are activated by Nodal, Activin, and TGFb type I receptors (i.e. Alk-4, Alk-5, and Alk-7), whereas R-Smads Smad-1, Smad-5, and Smad-8 seem to be activated predominantly by BMP type I receptors (i.e. Alk-3 and Alk-6). Smad-1 and Smad-5 can also be activated by Alk-1 and Alk-2 receptors. To date, only one Co-Smad has been identified in mammals: Smad-4. Finally, Smad-6 and Smad-7 act as I-Smads.

Glucocorticoid Receptor

The TGFb-signaling R-Smad Smad-3 has been shown to physically interact with the glucocorticoid receptor (GR) (Song et al., 1999) independently of receptor-ligand binding or nuclear translocation (Li et al., 2003; Li et al., 2006). Liganded GR:Smad-3 heterodimers are also able to repress TGFb transactivation of downstream events (Song et al., 1999) whereas TGFb-1 increases glucocorticoid binding and signaling in inflammatory cells through a Smad-2, -3- and AP-1–mediated. This reflects not only the ability of the glucocorticoid receptor to interact with and reciprocally modulate TGFb-family signaling molecules, but also how it might play a pivotal role in a wide array of biological processes, from development to reproduction and homeostasis (Mangelsdorf et al., 1995).

The GR, also known as nuclear receptor subfamily 3, group C, member 1 (Nr3c1), is a nuclear receptor that is expressed in virtually every cell in the vertebrate organism, from fish to man. GR belongs to the nuclear hormone receptor superfamily of ligand-activated transcription factors. Examples of other steroid receptors include the androgen receptor, the thyroid-hormone receptor, the mineralocorticoid receptor, and the estrogen receptor.

Structurally, steroid receptors are composed of a DNA binding domain containing 60-70 amino acids, which is delimited by a ligand-binding domain (LBD) on the carboxyl terminal and a non-homologous amino-terminal domain.

Upon ligand binding, each receptor undergoes a conformational change resulting in either direct interaction or interference with specific proteins, and the modulation of downstream molecular events.

Human GR transcript levels are highest in lung, spleen, brain, and liver. Alternate splicing of GR transcripts and the use of multiple promoters (1A, 1B and 1C) results in a variety of GR isoforms, which differ in the 5'-untranslated region. Promoter usage may regulate such diversity by determining membrane and intracellular isoform expression and localization (Chen et al., 1999a and 1999b) and at least one isoform serves to regulate GR mRNA translation (Diba et al., 2001). Exon 1A alone contains three alternative splicing sequences; exon 9 splicing gives rise to the alpha and beta isoforms.

GR-alpha is the recognized classical GR. It is composed of a single polypeptide chain of 777 amino acids in length, and in the absence of ligand it resides in the cytoplasm; the transcriptionally inactive and ligand averse beta form is reduced in the c-terminus by 35 amino acids (Hollenberg et al., 1985), yet both forms are identical up to amino acid 727. The variety of receptor isoforms can in part help to explain the paradox of how one receptor is able to elicit such diverse cellular- and tissue-specific responses. It is also important to note that, despite the highly conserved structural organization of the GR gene between human and mouse, alternative splicing into the beta form does not seem to occur in the latter, though GR-beta mRNA has been found in rat skeletal muscle (Korn

et al., 1998). Yudt and Cidlowski speculate that the absence of a beta form in mouse could be in part responsible for the differences in glucocorticoid response between the two species (Yudt and Cidlowski, 2002). To date, the role of GR-beta as a dominant negative regulator of GR-alpha remains to be elucidated, though sufficient evidence suggests that GR-beta might play an important pathological role in chronic inflammation and glucocorticoid resistance (Webster et al., 2001). Finally, two isoforms termed GR-P and GR-gamma has been found to influence GR-alpha mediated gene expression and might be protagonists in certain types of hematological malignancies and corticotroph adenomas (Krett et al., 2001; de Lange et al., 2001; Rivers et al., 1999).

GR-alpha acts primarily as a transcription factor to regulate the expression of genes involved in development, metabolism, neurobiology, apoptosis, as well as anti-inflammatory and immunosuppressive responses. Canonically, upon ligand binding, GR-alpha dimerizes and travels to the nucleus, where it binds to glucocorticoid response elements (GREs) in the promoter regions of downstream genes, therefore being able to modulate their expression (Nordeen et al., 1990). The typical GRE palindrome is represented by the pentadecameric imperfect palindrome GGTACAnnnTGTTCT.

Though GR is present in most tissues, the panel of genes regulated by GR can fluctuate significantly between different cell types, just as GR can act via direct and indirect transcriptional molecular mechanisms (reviewed by Labeur

and Holsboer, 2010). In fact, GREs are part of larger glucocorticoid response units (GRUs) composed of diverse binding sites for other transcription factors (Onard et al., 2004). Aside from the more common positive effect of GRs on gene activation, the complexity of such interactions between GR and different transcription factors and co-activators can exert a negative effect on gene expression; GRs are also known to influence transcriptional activity through an indirect way, which does not requite binding to GRE or even nuclear translocation (Jalonen et al., 2005). GR monomers can also interact with other transcription factors activated by other signaling pathways leading to transrepression (Labeur and Holsboer, 2010 and references within).

Glucocorticoids

Glucocorticoids are steroid receptor ligands that are produced in the adrenal cortex. Increased glucocorticoid levels are associated with metabolic responses to energy demands involving gluconeogenesis, lipolysis and proteolysis. Glucocorticoids are perhaps better well known for their role as anti-inflammatory agents, being among the most widely prescribed class of drugs in the world. Aside from the naturally occurring cortisol (or hydrocortisone), cortisone and corticosterone, synthetic ligands have been generated (e.g. dexamethasone, prednisolone) that vary in their ability to induce GRE-dependent gene expression and *trans*-repress the transcription factor nuclear factor kappa-B (NF-kB) involved in pro-inflammatory responses (McKay et al., 1999). Other interactions can involve c-jun (Yangyen et al., 1990), TFIID complex (Ford et al.,

1997) and STAT5 (Stocklin et al., 1996). The GR is also able to interact with several cytosolic proteins such as chaperones, nuclear trafficking proteins, kinases and phosphatases. Moreover, the physiological response and sensitivity to glucocorticoids is species-, individual-, tissue-, cell type-, and cell-cycle-dependent. The cellular epigenetic state can also have an influence on the response to glucocorticoids. This illustrates the ability of different natural and synthetic glucocorticoids to elicit numerous and diverse physiological responses that go far beyond a simple on/off genetic switch.

Among steroid hormones, cross-specificity can at once be of physiological importance and experimentally confounding (Yudt and Cidlowski, 2002). Dexamethasone (Dex), a 40-fold more potent GR activator than hydrocortisone, is able to down regulate GR transcript levels. The mineralocorticoid receptor (MR) agonist Aldosterone (Ald) has significant affinity for the GR at higher doses; conversely, the MR can bind endogenous glucocorticoids, despite not only having an entirely different physiological role, but also being a completely distinct gene product. The GR synthetic antagonist RU486 is highly efficacious on both the GR and the progesterone receptor (ER).

Like other nuclear receptors, GRs are phosphoproteins (Singh and Moudgil, 1985) and therefore might be controlled in their ability to interact with other signal transduction pathways by their phosphorylation state upon ligand binding. Several N-terminal serine and threonine sites can often be

simultaneously phosphorylated in the protein (Bodwell et al., 1991), as well as elicit both a constitutive and a ligand-induced phosphorylation state of the receptor. However, phosphorylation alone is insufficient to elicit transcriptional activity of GR (Weigel and Zhang, 1998). Phosphorylation primarily controls GR protein stability, half-life and promoter-specific transcriptional activity (Webster and Cidlowski, 1997), aside from its subcellular localization (De Franco et al., 1991).

The complexity of GR biology contrasts with a single gene coding for all isoforms known to date. Hence, the variety of receptors and not that of ligands is likely to be responsible for GR signaling diversity. In fact, it is far from unreasonable to envision that the variation between isoforms, at least eight phosphorylation sites, several sumoylation sites, and the ability to combine different isoforms, are able to elicit unique biological responses to the same hormone (Yudt and Cidlowski, 2002).

HNF4-alpha

The GR agonist Dex has been shown to induce an important transcription factor involved in endoderm induction and differentiation: Hnf4a. Hnf4a plays a determinant role in the developing embryo and in liver homeostasis.

Hnf4-alpha (Hnf4a) was the first HNF4-transcription family member to be identified as a regulatory factor of promoter elements, which mediate critical liver

specific transcription (Sladek et al., 1990). Hnf4a is an orphan member of the steroid/thyroid hormone receptor superfamily and is mainly known for its role as a positive transcriptional activator of many genes expressed in both embryonic and adult liver (Sladek et al. 1990, Chen et al., 1994). For the following decade, many different research groups, from academia to industry, searched for a ligand other than DNA that would allow HNF4a to fall into the classical model of steroid/thyroid hormone receptors. In 2002, Wiseley et al. reported on the finding that fatty acids filled the ligand-binding pocket of a receptor considered *orphan*. However, the high-resolution X-ray crystallographic structure of the ligand-binding domain of human HNF4a showed that these fatty acids seemed to be locked into the receptor and not accessible for exchange. This represented a paradigm that helped explain how, in the absence of an exogenously added ligand, HNF4a seemed to activate transcription in a constitutive manner (Wisely et al., 2002). Two years later, solving the structure of human HNF4a bound to both fatty acid ligand and an SRC-1 derived co-activator sequence would suggest that co-activator rather than ligand binding locks the receptor in its active conformation (Duda et al., 2004).

Hnf4a is present in visceral endoderm of mice as early as embryonic day 4.5 (E4.5), during implantation and well before gastrulation, with expression in the mouse embryo detectable in anterior primitive streak of E6.5 embryos, followed by expression in the node, notochord, and floor-plate of the neural tube and endoderm (Lai et al., 1991; Kaestner et al., 1993; Monaghan et al., 1993;

Sasaki et al., 1993; Duncan et al., 1994; Kaestner et al., 1994, Sasaki et al., 1994).

Homozygous loss of function studies involving Hnf4a result in embryonic lethality before day 10.5 due to severely impaired gastrulation (Chen et al., 1994). In fact, at day 6.5, coinciding with pre-streak or early primitive streak stages, Hnf4a null mouse embryos exhibit incomplete gastrulation phenotypes due to a dysfunctional visceral endoderm together with increased ectodermal cells (Chen et al., 1994), with a characteristic delay in the expression of T/Brachyury (Duncan et al., 1994). Notably, different Hnf4a splice variants originated from the same promoter are expressed in undifferentiated mouse embryonic stem cells, visceral endoderm, and prestreak ectoderm; later in the metanephric tubules, the developing pancreas, stomach, intestine; at even later times again in the metanephric tubules; and in the adult, from a different promoter, in endoderm-derived tissues like the liver. This important distinction between isoforms was technically not made in early studies of HNF4-family role in early mouse development. Hnf4a-1 was the first isoform to be characterized (for a review, see Sladek and Seidel, 2001) and Hnf4a-7 was originally cloned from a murine cell line (Nakhei et al., 1998).

The HNF4a gene has two known promoters and encodes at least nine isoforms through differential splicing. The use of its two promoters is responsible for developmental and tissue-specific expression of different Hnf4a isoforms

(Totrres-Padilla and Weiss, 2003). In mouse liver, promoter 2 (P2) is the first to become active and is more prominent than P1 during fetal life, a balance that switches to P1 after birth. An enhancer upstream of P1 seems to mediate both Hnf4a transactivation and glucocorticoid induction; Hnf4a-1, originated from P1, is able to repress P2 activity by recruiting at least GR to the P2 promoter (Bailly et al., 2009).

Glucocorticoids such as Dex have been shown to induce the HNF4a gene in hepatic cells (Nakei et al., 1998; Oyadomari et al., 2000; Michalopoulos et al., 2003). Mary Weiss' group has recently shown that functional glucocorticoid response element (GRE) and half-GRE consensus sites are present within sites for the LETF HNF3, HNF4a and C/EBP, and that both GR and Hnf4a are able to bind and induce Hnf4a expression in mouse embryonic liver (Bailly et al., 2009). Glucocorticoid hormone also seems to alter the balance between the Hnf4a-1 and Hnf4a-7 splice variants (Nakhei et al., 1998) the latter being present in mouse embryonic stem cells, and the former playing an important role in gut formation (Zhong et al., 1993). Hnf4a-7 was initially discovered in an immortalized mouse embryonic hepatocyte line that stopped differentiating when grown in the presence of serum; removal of serum from growth media or treatment with Dex induced Hnf4a-1 transcription, an isoform with significantly higher transactivation potential (Nakhei et al., 1998). In rat, Dex treatment also causes suppression of HNF4a P2 promoter while enhancing hepatic expression derived from P1 (Dean et al., 2010). Aside from composition, Hnf4 binding sites

can differ both in localization and in their neighboring sequences. Hnf4a isoform expression and activity can therefore be subject to the influence of environmental factors such as high glucocorticoid levels, with developmental and pathophysiological repercussions.

As discussed above, GR and Smad-3 physically interact to regulate transcription via Smad-3 Mad homology 2 (MH2) domain (Song et al. 1999), an interaction that is not dependent on Ser211 phosphorylation of GR, which is associated with its nuclear translocation, as well as ligand-binding independent (Li et al., 2003; Li et al., 2006). Smad-3 and Smad-4, but not Smad-2, have been shown to physically interact with Hnf4a via their MH1 domains both *in vivo* and *in vitro*, an interaction involving a region of Hnf4a that includes activation function 1 and DNA binding domain (Kardassis et al., 2000 and Chou et al., 2003), resulting in downstream transcription activation. However, the presence of an Hnf4a binding site on a promoter was demonstrated to be insufficient to confer responsiveness of that promoter to the TGFb-signaling pathway (Chou et al., 2003). The opposite can also be true: the ectopic insertion of an unresponsive Smad-3/Smad-4 and Hnf4a binding sites within an otherwise unresponsive heterologous promoter can result in transcriptional activation, and the absence of canonical full length Smad-3/4 and Hnf4a binding sites in responsive promoters is not sufficient to preclude the activation of downstream transcriptional events by those factors. Furthermore, Smads can recognize non-canonical DNA-binding elements present in the vicinity of hormone response elements even with low-

affinity. Thus, the importance of promoter context, intra- and extra-cellular responsiveness, cell type and developmental stage, seems to be the reflection of the formation of specific multiprotein complexes, which modulate the favorable or unfavorable Smad, Hnf4a and GR recruitment to promoters and enhancer regions. It is also important to state that in order to interact with Hnf4a, Smad-3 needs to be phosphorylated by the receptor and translocate to the nucleus as a heterodimer with Smad-4. Therefore, TGFb receptor involvement is crucial in order to promote Smad-3 phosphorylation and the resulting interaction with Hnf4a (Chou et al., 2003). Also, Smad-3/4 complexes are able to activate the Hnf4a promoter irrespective of Hnf4a binding. The fact that GR can bind to Smad-3 and together modulate downstream events suggests that GR/Smad-3/Smad-4 complexes might be involved in Hnf4 gene expression and transcriptional activities. Many of the transcription factors important in the maintenance of homeostasis in the adult also play a significant role in development. Paradoxically, despite being expressed in virtually every organ, GR involvement in the activation of the gluconeogenic program occurs predominantly in the liver and to a minor extent in the kidney (Hanson and Reshef, 1997). The regulatory regions of several genes involved in gluconeogenesis present both GRE and cAMP response elements in close proximity to binding sites for an important embryonic endoderm determinant, Foxa2. During early embryonic hepatic development from foregut endoderm, Foxa proteins bind to promoters and enhancers of liver-specific genes in anticipation of their transcription (McPherson et al., 1993). This important chromatin-remodeling role of Foxa

proteins allows the binding of other hepatic transcription factors to *cis*-regulatory elements (Gualdi et al., 1996). In the adult, expression of the transcription factor Foxa2 is required for the activation of the hepatic gluconeogenic transcriptional program, and allows the binding of transcription factors cAmp Response Element Binding Protein (CREB) and GR to CRE or GRE sites during fasting. Thus, the combination of a tissue-specific transcription factor with two ubiquitously expressed yet hormone-regulated transcriptional activators seems to function together in controlling a liver-specific transcriptional response to metabolic demands. Likewise, in the developing embryo, the cellular response to glucocorticoids might be determined by and dependent upon the co-expression of GR along with other transcription factors, such as Foxa2, that recruit liganded GR access to its target sequences. The regulation of signaling between cells is a function of the availability of ligand, receptor, and the different components of the signaling pathway. However, in order for a cell to respond to an instructive signal, it must first become competent to respond, i.e. it must express the right machinery. Competence precedes the activation of signaling in the sense that it is not a rate limiting step, but rather a determining factor in regulating the response: a cell that is not competent, cannot respond accordingly even if it receives the signal (reviewed in Freedman and Gordon, 2002). Zaret and others have shown that Foxa proteins are able to interact with histones H3 and H4, unravel chromatin, and thus facilitate the access of transcription factors like C/EBP and NF-1 (Cirillo et al., 1998; Cirillo et al., 1999; Zaret, 1999; Chaya et al., 2001; reviewed by Kaestner et al., 2006). Relevant for the findings here reported,

it is possible that Foxa2 expression in a population of anterior definitive endoderm renders these cells responsive to GRE- or SBE-mediated Hnf4a induction, resulting in the up regulation of the transcription factor Sox17, which acts in endoderm to direct the production of proteins that induce heart formation in mesoderm.

SOX17

Sox17 is a SRY-related high-mobility group (HMG) box transcription factor that is required for endoderm formation and differentiation in several species (Clements and Woodland, 2000;, Kanai-Azuma et al., 2002; Yasunaga et al., 2005). In mouse, Sox17 is first expressed in visceral endoderm at E6.0 near the ectoplacental cone, its domain of expression quickly spreading to the entire extraembryonic visceral endoderm. Sox17 is also a maintenance factor for definitive endoderm, being directly regulated by TGFbetas (i.e. Vg1 or Nodal), and by VegT and Sox17 itself, though through a different promoter (Howard et al., 2007).

During early organogenesis, the definitive endoderm forms the lining of the primitive gut. It is from this endoderm-derived epithelium that the digestive tract, liver, pancreas and associated visceral organs develop (Wells and Melton, 1999). Little is known about the genetic determinants regulating the specification and differentiation of the definitive endoderm (Kanai-Azuma et al., 2002). Gata4, present in both visceral endoderm and foregut endoderm, is required for gut

closure as well as heart morphogenesis (Molkentin et al., 1997; Narita et al., 1997). Mutations in important Nodal signaling players, such as Smad-2 (Tremblay et al., 2000) and the upstream modulator of Foxa2, Foxh1 (Hoodless et al., 2001), result in deficient definitive endoderm. Loss of Foxa2 activity also results in loss of foregut and midgut endoderm as well as the notochord (Kanai-Azuma et al., 2002 and references within). The functional requirements of these genetic determinants for the endodermal lineages are however obscured by severe defects observed in other embryonic tissues. That is not quite the case with Sox17, however, which is expressed specifically both during gastrulation in definitive endoderm, along with the mesendoderm marker Goosecoid (Gsc), and earlier in visceral endoderm (*Sox17+*, *Gsc-*), playing a key role in endoderm formation.

Sox17 has also been shown to be indispensable for cardiomyogenesis *in vitro*, as Sox17 short-hairpin RNA (shRNA), despite having no effect on mesoderm formation, suppresses the induction of cardiogenic mesoderm marked by MesP-1 and MesP-2, transcription factors which, as we will see later, are crucial to cardiac specification (Liu et al., 2007). In the embryo, however, loss of Sox17 seems to be compensated by redundancy between factors involved in early cardiac development, as Sox17-null mice have no evident abnormal cardiac phenotype (Kanai-Azuma et al., 2002). Nonetheless, ESC differentiation towards a cardiac fate seems to be highly vulnerable to proper and timely Sox17 expression and function.

Notwithstanding its essential role in cardiac muscle cell formation, few direct targets of Sox17 have been unveiled to date. Among those are *Lama1, Fn1, Foxj1, Sftpc, and Fgf3,* in the mouse, and *Hnf1-beta, Foxa1, Foxa2, Edd, and Sox17-beta* in *X. laevis* (Patterson et al., 2008 and references within). Recently, two Sox17 binding sites were found within the first intron of the zinc finger protein 202 gene (*Zfp202*), a gene whose transcript is upregulated during F9-derived endoderm differentiation (Patterson et al., 2008). Importantly, Zfp202 colocalized with Sox17 in anterior definitive endoderm on E7.75 mouse embryos, and was further shown to repress Hnf4a transcription in both human and mouse (Wagner et al., 2000; Patterson et al., 2008). Hnf4a expression lags behind Sox17 in the anterior definitive endoderm (Duncan et al., 1994; Taraviras et al., 1994, Kanai et al., 2002). It is possible that Sox17 upregulation of Zfp202 is one mechanism by which to regulate the timing of Hnf4a expression in order to control endodermal differentiation towards liver (Parviz et al., 2003). A secondary result of glucocorticoid induced Sox17 up regulation might be the induction of its target, Cerberus-1 (Cer1), a soluble and direct Nodal inhibitor that is also downstream of Nodal and BMP2.

CER1, a Nodal-signaling Inhibitor and Inducer of Cardiogenesis

Cer1 is a potent early cardiac-marker inducer expressed in the dorso-anterior endoderm. In *Xenopus* sp., Cerberus is both a BMP and Wnt antagonist that necessarily becomes down-regulated prior to the cardiogenic requirement for

BMP signaling (Bouwmeester et al., 1996; Shi et al., 2000; in Foley and Mercola, 2004). In mouse, Cer1 seems to be upregulated by Nodal as a positive feedback mechanism that aims at regulating Nodal signaling itself. Briefly, Nodal signals by binding to ActRIIB and ActRIB (i.e. Alk4), and their interaction with EGF-CFC co-receptors (e.g. Cripto), although other receptors might also be involved in mediating Nodal signaling. Nodal binding to, and the association between these receptors, results in phosphorylation of Smad-2, which fellows with Smad-4 and other transcription factors (Foxh1, Mixer) to activate transcription of downstream events, including Nodal, Nodal inhibitor Lefty (in mouse, *lefty1* and *lefty2*), and Pitx2. In mouse, Cer1 binds to and block Nodals, as well as BMPs, but not Wnt (Piccolo et al., 1999; Belo et al., 2000; Bell et al., 2003). Cer1 expression, unlike that of Leftys, does not closely reflect Nodal-signaling activity. It is predominant in anterior endoderm and plays an important role to inhibit Nodal signaling both in the overlying ectoderm and the cardiogenic mesoderm, thus allowing these tissues to develop and differentiate. Prior to this, however, the absence of Nodal-signaling abolishes the formation of all endoderm and head and trunk mesoderm, as well as their derivatives, including notochord, heart, kidney, blood, liver, pancreas and gut (Feldman et al., 1998, Gritsman et al., 1999). The requirement for Nodal signaling for the induction of all mesendoderm cell-types, with the exception of a few posterior somites, is evident: without Nodal signaling, the primitive streak does not form (Zhou et al., 1993; Conlon et al., 1994). Similarly, unchecked Nodal signaling both in zebrafish and mouse embryos results in an expansion of mesendoderm (Meno et al., 1999; Chen and Scheir, 2002; Feldman

et al., 2002).

In This Dissertation

The work here presented reports on the development of a fluorescence-based reporter system to screen chemical libraries for cardiomyocyte-inducing molecules. This was screened against a library of known drugs and small molecule pathway modulators. Active compounds included hydrocortisone and its analog Dexamethasone (Dex), and Dihydropyridine (DHP), which seem to work in different ways to modulate the signals between definitive endoderm and cardiogenic mesoderm in determining cell-fate. We propose that Dex activated GR regulates a genetic cascade in foregut endoderm that is involved indirectly in heart formation and later in lung maturation and liver homeostasis. DHP, on the other hand, seems to inhibit TGFb-signaling downstream of the receptors to promote the expansion of cardiac progenitor cells.

Our results should contribute to a better understanding of the important cross talk between endoderm and mesoderm progenitors in the establishment and expansion of a cardiac progenitor population.

INTRODUCTION TO CHAPTERS 1 AND 2

Embryonic stem cells ability to self-renew, being able to be propagated in culture virtually indefinitely, and differentiate into derivatives of all three embryonic germ layers, makes them ideal candidates for cell replacement therapies and chemical genetics. However, the yield of cardiomyocytes that can be obtained by differentiating ESCs is still unsatisfactorily low and insufficient to satiate current therapeutic demand; current therapies fail to replace loss of myocardium or to stimulate resident progenitors to repopulate the affected area after ischemic injury. The fact that the molecular signals observed during cardiogenesis within differentiating mESC cultures seem to resemble and follow a spatio-temporal pattern observed in the embryo suggests that the mESC model might serve as a valid surrogate for the study of cardiac development and the very basic biology of ESCs. Simultaneously, ESCs allow for the discovery and validation of small molecules and natural drugs that might either increase the yield of cardiomyocytes and endothelial cells available for cell-based therapies, or re-activate specific developmental programs in cells with cardiomyogenic potential *in vivo*, opening the door for true myocardial regeneration.

Small molecules and drug-like compounds are an atractive alternative to the use of growth factors for obtaining cardiomyocytes from ESCs. Many of the small molecules assembled today in large chemical libraries, and readily availabe for screening, are already well characterized in terms of activity, targets,

secondary effects, effective dose, half life, solubility and stability. They also offer the possibility to redesign a structure of interest in order to improve upon selectivity, potency and *in vivo* administration, when applicable and desirable. Simultaneously, small molecule screening allows for not only the study of know pathways, but also the discovery of new targets, new partners and new pathways involved in cardiovascular development.

When combined with the utilization of fluorescence-based reporter lines that relate specific steps in the differentiation process, cell-based assays facilitate both a quick readout to identify molecules of interest, and a more sensitive detection and detailed scrutiny by use of high-content image acquisition and analysis. This allows for live imaging, precise quantification of signal, spacial, temporal, and cellular resolution, plus subcellular compartment localization of response. It also places the molecule in a more complex and dynamic biological context, which a biochemical assay is unable to offer.

In order to identify small-molecule modulators of pathways known to regulate ESC cardiogenesis, we performed high throughput automated screens (HTS) of libraries containing natural products, known drugs, and synthetic compounds. Among several of potential interest, two molecules have shown to promote eGFP expression regulated by the aMhc promoter in murine ESCs: hydrocortisone and hydrocortisone 21-hemisuccinate. Hits were confirmed in primary screens followed by dose-response over a series of replicates. We

hypothesized that other steroid hormone receptors might also be involved in cardiomyogenesis. Secondary assays confirmed that endogenous gene activity accompanies observed reporter gene expression as well as shed light into the mechanism by which such molecules affect the cardiogenic pathways. We propose that cortocosteroids have a paracrine effect on cardiogenesis by stimulating endothelial cells to secrete ligands such as Cer-1, promoting a cross-talk between uncommitted and committed cardiac progenitors and definitive endoderm resulting in increased cardiogenesis without affecting the balance between mesoderm and endoderm progenitors.

CHAPTER 1

This chapter is being prepared for submission as a manuscript entitled:

Glucocorticoid Receptor Directs Cardiogenic Mesoderm Specification in Murine Embryonic Stem Cells by Induction of Cardiogenic Factors in Endoderm

Joaquim Cabral-Teixeira and Mark Mercola

Abstract

Cardiovascular disease accounts for the majority of deaths in the United States and Europe. Improvements in the treatment of ischemic heart disease have increased significantly both the rate of survival of patients after myocardial injury and the number of patients with heart failure secondary to ischemic episodes. Embryonic Stem Cells have the ability to be differentiated in vitro into cardiomyocytes and would be beneficial to generate and expand cardiomyocyte progenitor cultures for research and clinical applications, while also serving as probes to dissect the mechanisms that control differentiation. In order to identify novel signaling proteins and pathways involved in cardiogenesis, we developed a fluorescence-based reporter system to screen chemical libraries for

cardiomyocyte-inducing molecules. This was screened against a library of known drugs and small molecule pathway modulators. Active compounds included hydrocortisone and the artificial glucocorticoid Dexamethasone (Dex), which induced 10-fold increase in cardiomyocytes, confirmed by an upregulation of cardiac markers Tbx5, cTnt, alphaMhc, but not the endothelial cell marker CD31. Mechanistically, RNA interference to alphaGR blocks this effect showing dependence on GR. Marker analysis revealed that GR acts predominantly on definitive endodermal cells. In particular, we observe a striking induction in endodermal genes that are associated with production of Cerberus-1, a secreted inhibitor of BMP and Nodal that parallel studies in the lab have shown induces cardiomyocytes from uncommitted multipotent Flk1+, MesP1+ progenitors. We further show that GR selectively activates the induction of heart-inducing gene expression in endoderm via the upregulation of Hnf4a, without altering the number of endodermal cells or cardiac progenitor cells in the cultures. Glucocorticoids are well known to regulate the expression of genes involved in hepato-development, lung adaptation perinatally, and live homeostasis, a process involving some of the same genes regulated by Dex/GR in early endoderm in our cultures (e.g. Hnf4a, Sox17, Foxa2). Thus, we propose that GR regulates a genetic cascade in foregut endoderm that is involved indirectly in heart formation and later in lung maturation and liver homeostasis. Our results should contribute to a better understanding of the important cross-talk between endoderm and mesoderm progenitors in the establishment and expansion of a cardiac progenitor population.

Results

HTS for Small-Molecule Inducers of Cardiomyogenesis

I first adapted and optimized mESCs to growth and differentiate in adherent conditions utilizing a multi-well format suitable for High-Throughput Screening. In brief, CGR8 mESCs harboring an eGFP reporter driven by the alpha Myosin Heavy Chain (aMhc) promoter (Takahashi et al., 2003) were weaned off mouse embryonic fibroblasts (MEFs), platted at low density on gelatin coated 96 well plates in 10% differentiation media (DM) without LIF, and allowed to differentiate for 8 days, with media changes performed every other day (**Figure 1.1**, DM Assay). Platted at this density, each cell grew clonally and behaved as an embryoid body (EB) would in suspension, yet remained attached as it differentiated and eventually contacted other colonies, further promoting differentiation. A low background of spontaneous differentiation into contracting eGFP-harboring cardiomyocytes was observable between days 7 and 8, validating the competence of mESCs under these conditions to normally differentiate into fully functional immature cardiomyocytes.

Hydrocortisone Induces Cardiomyocyte Differentiation in mESCs

I performed a pilot screen of 2000 natural products, known drugs, and synthetic compounds (Chembridge MS-Discovery DIVERset Library) for their ability to induce the expression of a muscle specific myosin promoter driven reporter fluorescent protein (aMhc-eGFP).

I have identified two glucocorticoid receptor agonists, hydrocortisone (H2) and hydrocortisone 21-hemisuccinate (H4), as potential inducers of cardiomyocyte differentiation (**Figure 1.2, [A-C]**). I verified the presence of both GR transcript (GR+) and GR protein (GR) in EB cultures. Both GR+ (**Figure 1.2.D**) and GR (**Figure 1.2.D'**) were detectable in mESCs; GR seemed to follow a downregulation at the time of treatment (day 2; GR not quantified). Also present in the library, but not initially identified as an inducer, was hydrocortisone 21-acetate (H3). A subsequent assay proved H3 to be a false negative in the pilot screen, and together with H2 and H4, it induced aMhc-eGFP in a dose responsive manner (**Figure 1.2.E**).

We hypothesized that other steroid hormone receptors might also be involved in cardiomyogenesis, or that other steroid hormones might induce cardiogenesis through the same receptor. For this purpose, we tested a panel of steroid hormones across a dose range. This selection included, besides the supra mentioned glucocorticoid hormones, the mineralocorticoid receptor agonist Aldosterone (Ald), the glucocorticoid receptor agonists Dexamethasone (Dex) and Prednisone (not shown), and the progesterone receptor agonist Progesterone (not shown). We used a 384-well plate format to test these compounds and measured αMhc promoter-driven eGFP expression in CGR8 mESCs by high throughput microscopy. Of those, Ald seemed to be the most potent cardiomyocyte inducer, followed by Dex and H2 (**Figure 1.2.F**). Interestingly, Prednisone had no inductive effect even though it is known for its

agonistic effect on the glucocorticoid receptor. Ald, on the other hand, has considerable affinity for GR at the concentration used, so we focused on this receptor in subsequent studies and its activation by Dex.

We hypothesized 3 ways in which the above mentioned compounds might have an inductive effect on the numbers of aMhc-eGFP expressing cardiomyocytes, namely by (1) increasing the total number cells in culture, (2) by increasing specifically the number of cardiomyocytes, or (3) by reducing overall cell death in these highly apoptotic cultures. In order to elucidate this, I performed FACS analysis and looked at the percent eGFP positive population (not shown) as well as total eGFP cells present (**Figure 1.2.G**). Both Ald (not shown) and H2 showed dose response at concentrations ranging from 0.5 to 5uM (**Figure 1.2.G**). Furthermore, I have observed a statistically significant difference in the percentage of induced eGFP levels (demonstrated in **Figure 1.2.E,F**; FACS not shown) as well as total number of eGFP cells present in culture between vehicle (DMSO) treated cells and H2 treated samples, at 0.5uM and above (**Figure 1.2.G**).

The effect of serum in ESC cultures is not well understood. Generally, serum varies from batch to batch and often induces stem cell differentiation. Likewise, factors present in the serum can alter or predispose differentiation to adopt an undesirable fate. For instance, while bone morphogenic protein (BMP) is important for the derivation, maintenance, and proliferation of ESCs, only a

minute amount of BMP in serum is able to differentiation upon leukemia inhibitory factor (LIF) withdrawal (Ying et al., 2003). In yet another example, neural stem cells can undergo differentiation following the addition of serum to the medium (Reynolds, et al., 1992). In order to reduce the presence of confounding factors in the media, we opted for low serum to no serum media formulations in our assays, when suitable, and as stated (**see Figure 1.1**).

Dexamethasone Acts Downstream of Smad-2, -3 Phosphorylation

Nodal, BMP, and Wnt each have the ability to inhibit cardiogenesis after specification of anterior streak mesoderm. Others in our lab have shown an upregulation of Nodal between days 1 and 6 of EB differentiation, with a delayed increase of the Nodal/BMP, inhibitor Cerberus-1 (Cer1) to allow for mesoderm differentiation (Cai et al., 2010). We set out to investigate whether glucocorticoids induce cardiogenesis by inhibiting Nodal/Activin/TGFb-signaling. We employed a reporter vector consisting of a Smad-4 Response Element driving Luciferase (SBE4-Luc) to verify whether Dex could block TGFb-signaling activation. As differentiated ESCs are difficult to efficiently transfect, we employed a surrogate immortalized embryonic hepato-carcinoma cell line (293T) for our next studies. Using SBE4-Luc or a Glucocorticoid Response Element drive luciferase reporter (GRE-Lux), I reverse transfected 293T cells in media containing low serum (293T-M). Both Activin A (not shown) and TGFb-2 (**Figure 1.3.A,B**) induced both SBE4-Luc and the phosphorylation of two TGFb-signaling mediators, Smad-2, -3. Treatment with an increasing concentration of Dex both induced GRE-Lux and

inhibited SBE4-Luc (**Figure 1.3.A**). In this assay, the calculated IC50 for SBE4-Luc inhibition is 6.621uM, whereas the EC50 for GRE-Luc induction is 4.75nM, suggesting that the doses at which Dex inhibits TGFb-signaling, close to the dose used in out cardiogenic assay, might not elicit a GRE-dependent effect. Dex also did not block Smad-2, -3 phosphorylation induced by TGFb-2, both in 293T cells and on mESCs, similar to another compound studied in our lab (149) that we know does not inhibit TGFb-signaling, and unlike the bona-fide Alk-4, -5, -7 inhibitor SB-431542 (SB) that proved effective at blocking Smad-2, -3 phosphorylation in both cell types (**Figure 1.3.[B,C]**).

Dexamethasone Induces Cardiomyogenesis Through GR

We set out to determine if the nuclear hormone receptors might be the targets of the corticosteroids by testing various inhibitors (not shown). On mESC, Spironolactone, a Mineralocorticoid Receptor antagonist, was unable to inhibit Aldosterone effect on cardiogenesis, whereas Mifepristone, a Glucocorticoid Receptor antagonist, blocked the effect of Dexamethasone and Hyrocortisone but also of Aldosterone, which is known to have affinity for the GR at high doses. This suggests that GR might be activated to induce cardiomyogenesis. An unexpected outcome of testing the inhibitors was that we have discovered a synergistic effect between the estrogen receptor (ER) antagonist, Tamoxifen (Tam), and both Ald and H2 at concentrations ranging from 0.5-1uM for the former, and 2-4uM for the latter (not shown). Together with the fact that in our DM cardiogenesis assay, b-Estradiol, an ER Agonist, was unable to induce

cardiogenesis on repeated occasions, this strengthened our idea that inhibiting the ER might be important to promote cardiogenesis.

To further imply GR on Dex cardiogenic effect, I tested whether siRNA against GR could inhibit cardiogenesis in a serum free assay (**Figure 1.1**: siRNA Assay). Dex induced cardiogenesis when added to cells at day 3 (**Figure 1.4.A**) and 3.5 (**Figure 1.4.B**) of differentiation, with a downward shift in the dose curve for siGR treated mESCs, an effect more pronounced if Dex was added to the cultures 12 hours after transfection (day 3.5), likely to coincide with the degradation of GR by siGR, hence eliminating Dex target.

Dexamethasone Upregulates Early Endodermal Markers

To test whether Dex induced cardiogenesis by blocking TGFg-signaling downstream of Nodal induced Smad-2, -3 phosphorylation (**Figure 1.5**), I performed a gene profiling of transcripts relevant to meso-endoderm lineage specification in the SFM assay, at different time points. Dex was added to the cultures between days 3 and 5 of differentiation and compared to Activin only treated samples. As expected, Dex induced GR+ at day 4 and promoted an increase in meso-endoderm markers T/Brachyury (T/Bra) and meso-endoderm/endoderm progenitor marker Goosecoid (Gsc), without affecting endoderm progenitors marked by Foxa2+ (**Figure 1.6.A**). At day 5, Dex effect was quite prominent on definitive endoderm markers Sox17 and Cer1 but not on Foxa2; BMP4 was unaffected, and so were mesoderm progenitor markers

PDGFR-beta, E-cadherin and Cadherin-11 (**Figure 1.6.B**). At day 8, I did not observe a statistically significant increase on cardiac committed mesoderm markers (Nkx2.5, Mef2c) or early expression of cardiomyocyte markers (aMhc, cTnT) and endothelial cells (CD31) (**Figure 1.6.C**). However, at day 10, a dramatic increase in cardiomyocyte markers (Tbx-5, aMhc, cTnT) was observed (**Figure 1.6.E**), corroborating our observations of aMhc-eGFP induction in previous assays; the endothelial cell marker CD31 seemed unaffected, suggesting that Dex might specifically promote cardiac progenitor differentiation specifically toward cardiomyocytes.

Dexamethasone Does Not Affect Mesoderm:Endoderm Lineage Choice

To confirm or dismiss the possibility that Dex cardiac induction might be done by upregulating cardiogenic progenitors (Flk1+, MesP1+), I treated day 3 mESCs with Dex and analyzed transcript levels at day 5 and day 6 (**Figure 1.7.A**). Neither Flk1, nor MesP1, were upregulated by Dex when compared to Activin alone, suggesting a model in which Dex-liganded GR upregulates Sox17 -> Cer1 without altering the ratio between endoderm progenitors (Foxa2+) and cardiogenic progenitors (Flk1+, MesP1+) (**Figure 1.7.C**).

Dexamethasone Upregulates Endoderm Secreted Cardiac Inducing Factors

Cer1 is a secreted Nodal and BMP inhibitor whose timing of expression must coincide with the need to locally titrate Nodal signaling in anterior mesoderm in order to promote cardiac mesoderm formation (Cai et al., 2010). To determine the ideal time window in which Dex cardiogenic effect is maximized, I treated mESCs for 2 day periods from day 0 to day 8 and analyzed cardiac markers (aMhc, cTnT, Tbx5) at day 10 (**Figure 1.8.A**). Dex proved most effective when added to the cultures between days 4-6, with an illustrative 45-fold increase in cTnT over Activin A alone. In order to profile the effect of Dex at different times in differentiation, I analyzed gene transcription in CGR8 mESCs treated from days [0-2] and probed at day 4; treated from days [3-5] and probed at day 5; treated from days [4-6] and probed at day 6; and treated from days [5-7] and probed at day 7 (**Figure 1.8.[B-L]**). Dex induced an upregulation exclusively of endodermal markers (Sox17, Cer1) as well as the hepato-developmental and liver homeostasis marker Hnf4a (Figure 8J), without upregulating definitive endoderm (Foxa2) or affecting meso-endoderm specification (Flk-1, MesP-1 versus Foxa2, CXCR4).

Dexamethasone Does Not Alter Flk-1/Foxa2 Ratio and Proliferation in Culture

To further prove or dismiss the possibility that Dex might act by modulating the lineage choice between Foxa2+ and Flk1+ progenitors, I

differentiated R1::Flk1-eGFP cells as depicted (**Figure 1.1:** Flk1/Foxa2 Assay) for 3 days, platted single cells onto gelatin coated 384-well plates, and added an increasing concentration of Dex to the cultures. On day 6, cells were fixed, and areas of eGFP (Green) and Foxa2 (Red) expression were quantified (**Figure 1.9.A**; **Figure 1.9.A'** shows a representative image). Dex did not significantly alter the total area of Flk1+, Foxa2+ expression, both in terms of maximum total area (**Figure 1.9.B**) or the mean total area (**Figure 1.9.D**). Dex also did not alter the relative ratio between Flk1+ and Foxa2+ cells in terms of maximum area of each population (**Figure 1.9.C**) or mean expression area **(Figure 1.9.E**).

Hnf4a Transcriptional Activation Induces Sox17 and Cer1 in mESCs

Upon transcriptional activation, Hnf4a has been shown to be the target of zinc finger protein 202 (Zfp202), which is transcriptionally activated by an upregulation of Sox17 (Patterson et al., 2008). To test whether Hnf4a could induce Sox17 -> Cer1 (**Figure 1.10.A**), I tested a transcriptional activator (7005) and an inhibitor (BIM) of Hnf4a (kind gift from M. Dawson) for their ability to modulate Sox17 -> Cer1 in our cultures. I first verified that Cer1 was required for Dex cardiogenic effect, using an R1 mESC line constitutively expressing small-hairpin RNA against Cer1 (shCer1; generated by W. Cai). In shCer1 cells, Dex treatment between days [4-6] failed to induce cardiac markers (aMhc, cTnT, Tbx5) by day 10 of differentiation when compared to Dex treated wt ESC (**Figure 1.10.B**). To test whether Hnf4a acts upstream of Sox17 -> Cer1, I added a concentration of Hnf4a transcriptional activator 7005 to day 4 cultures and

probed for Sox17 and Cer1 transcript on day 6. At 2 and 5uM, 7005 induced significant levels of both Sox17 and Cer1 over Act alone (**Figure 1.10.[C,D]**). I subsequently tested whether Hnf4a inhibitor BIM could block Dex mediated induction of Sox17 and Cer1 and verified that a 10:1 ratio of BIM:Dex (5uM:0.5uM) was able to block Dex effect on those endodermal markers (**Figure 1.10.[C,D]**).

These data place Dex/GR upstream of Hnf4a to induce Sox17 -> Cer1. We hypothesize that GR activation of Hnf4a induces a negative feedback response resulting in the upregulation of Sox17 -> Cer1 which promotes cardiogenesis; we have not yet tested whether Sox17 induces Zfp202 to counter Hnf4a upregulation in these cultures (**Figure 1.10.E**).

CHAPTER 2

This chapter is being prepared for submission as a manuscript entitled:

Novel Activity of Dihydropyridines

in Mouse and Human Embryonic Stem Cell Differentiation

Erik Willems, Joaquim Cabral-Teixeira, Marion Lanier,

Wenqing Cai, Paul J Bushway, Zebin Xia, Marcia Dawson,

John Cashman and Mark Mercola

Abstract

Heart failure is one of the major causes of death in North America and Europe. Cardiac muscle damage is irreversible for the most part and often results in a significant decline in contractility and ejection fraction. In order to effectively replace lost cardiomyocytes, novel therapies are urgent if we are to veer away from whole heart transplantation and the scarcity of donors.

ESCs and iPSCs offer the exciting possibility of studying the biology of endogenous progenitors that have recently been found to regenerate the heart to a very minor extent. ESCs and iPSCs also provide a viable source of cell

replacements for numerous degenerative disorders. ECS propagate relatively well in culture and could potentially become an infinite source of exogenous cardiomyocytes for transplantation. Moreover, they serve as useful tools to elucidate the biology of resident progenitor cells in the adult heart and to better understand the nature of and the timing during which signaling molecules are needed to enhance the limited regenerative capability of the adult human heart.

In our studies, we found DHPs to be a class of drugs that alters the balance between mesoderm and neurectoderm. In mESCs, when added early to the cultures in a time when mesoendoderm specification occurs, they inhibit cardiogenesis. Later, however, once the mesoendoderm has been established, DHP promotes cardiogenesis by inhibiting Nodal/Activin signaling at the receptor level by a mechanism yet to be elucidated - recent data suggests this might be receptor type specific. DHP appears to mimic the effects of the natural cardiac inducer Cer1 by blocking both Nodal/Activin/TGFb-signaling and BMP-signaling in uncommitted cardiac progenitor cells, resulting in an augmentation of the cardiac restricted *Flk1+, MesP1+* progenitor population and ultimately of cardiomyocytes. Our results should contribute to a better understanding of the important cross-talk between endoderm and mesoderm progenitors in the establishment and expansion of a cardiac progenitor population.

Results

We performed a screen of roughly 20,000 small molecules for their ability to induce aMhc-eGFP expression in differentiating mECSs. We identified dihydropiridines (DHPs) as potent cardiac inducers of mESCs. DHPs are known Calcium and Potassium channel blockers/openers; a structural-activity relationship (SAR) analysis of cardiogenesis and calcium channels, however, did not overlap. This, together with data showing that *bona fide* calcium channel blockers do not induce cardiogenesis under similar conditions, suggested that a different mechanism of action was in place.

Dihydropyridines Have a Biphasic Role During Mesoderm Differentiation.

Others in the lab showed that DHPs inhibit expression of aMhc-eGFP when mESCs are treated during mesoderm induction (day 1-3) whereas they promote eGFP expression when added during mesoderm patterning (day 3-5).

Further analysis of markers by RT-qPCR revealed that DHPs repress all markers for early mesoderm (*T/Bra, Flk1, MesP1*), endoderm (*Sox17*) at day 5, as well as late markers for more mature mesoderm derivatives such as cardiomyocytes (*aMhc*), endothelium (*VE-Cad*), smooth muscle (*Sm*a) and blood (*CD34*) at day 10. In contrast, DHPs promote induction of early (*Sox1*) and late neural markers (*Pax6*). When added later in the cultures, DHP upregulated the

earliest cardiac markers *Flk1* and *MesP1*, with a corresponding day 10 increase of cardiac markers (*Nkx2.5, Mef2c*, and *aMhc*) upon DHP treatment, without affecting other mesoderm.

Flow cytometry of Brachyury-eGFP cells analyzed on day 4 of differentiation (**Figure 2.1**) revealed that the number of mesoderm cells was downregulated by DHP (Red, DMSO; Blue, 1uM; Green, 3uM) and that this could be mimicked by the Nodal/Activin/TGFb inhibitor SB-431542 (SB) (Red, DMSO; Blue, 1uM; Green, 5uM) and an inhibitor of Wnt production (WPI) (Red, DMSO; Blue, 1uM; Green, 10uM), but not by the BMP receptor inhibitor Dorsomorphin (DM) (Red, DMSO; Blue, 1uM; Green, 5uM). RT-qPCR analysis confirmed that DHP was able to block TGFb-2 induced *T/Bra* when gastrulation impaired Cripto-/- cells were treated between days 1-3, but not when Cripto-/- cells were stimulated with Wnt3a or BMP4 (**Figure 2.2.[A-C]**).

DHP Blocks SBE4-Luc Induction By TGFb-2, Activin A Upstream of Smad Phosphorylation

Based on the foregoing, we hypothesized that DHP inhibition of mesendoderm in early mESC cultures might correspond with the necessity for high Nodal signaling to induce primitive streak derivatives, and therefore that TGFb-signaling was inhibited by DHP. To test this, I transfected 293T cells with SBE4-Luc and, upon stimulation with Activin, saw a marked increase in luciferase, whereas simultaneous treatment with SB resulted in TGFb-signaling

inhibition; the same was valid for TGFb-2 (**Figure 2.3.A,C]. I** then generated dose response curves with increasing concentration of DHP and analyzed the percent inhibition of Activin A (**Figure 2.3.B**) and TGFb-2 (**Figure 2.3.D**) on the SBE4-Luc. Maximal inhibition was reached at about 70% of DMSO control for Activin A and 95% for TGFb-2.

The ability of DHP analogues to inhibit mesoderm early in the cultures correlated with inhibition of TGFb-2 induced SBE4-luc **(Figure 2.4**), suggesting that mesoderm inhibition occurred by blocking TGFb-2**.** Antigen detection by western blot revealed reduced phosphorylation of Smad-2, -3 proteins by TGFb-2 but not by Activin A, upon both a 3 hour (not shown) and an 18 hour treatment with DHP, while total Smad protein compartment was unaffected (**Figure 2.5**).

These data suggest that DHP acts to block TGFb-signaling upstream of Smad2, -3 phosphorylation, resulting in impaired gastrulation and meso-endoderm specification.

DHP Inhibits TGFb-Signaling Upstream of Smad-2 Phosphorylation

To test whether DHP inhibits TGFb-signaling downstream of the Alk receptors, I employed constitutively active Alk4, 5, 7 (Alk4ca, Alk5ca, Alk7ca) receptors to activate the SBE4-Luc. SB compound effectively inhibited SBE4-luc induced by transfection with the Alkcas (**Figure 2.6**). Similar results were

achieved with DHP for Alk4ca (**Figure 2.7.A**), Alk5ca (**Figure 2.7.B**), and Alk7ca **(Figure 2.7.C**).

DHP Blocks Lefty-1 Induced by Activin A Though Not by TGFb-2

Consistent with a blockade of TGFb receptor signaling downstream of the kinase, some aspects of signaling were intact in cells treated with DHP. Lefty1 is a member of the TGFb-family, which antagonizes Nodal signaling. Nodals can act both locally and as morphogens at a distance and in a concentration-dependent manner (Chen and Schier, 2001; Gurdon and Bourillot, 2001; Meno et al., 2001; Green, 2002). Nodal signaling can, both spatially and temporally, be blocked by the feedback inhibitor Lefty and its members Lefty1 and Lefty2 (Bisgrove et al., 1999; Meno et al., 1999, 2001; Agathon et al., 2001; Branford and Yost, 2002; Chen and Schier, 2002; Feldman et al., 2002). To verify whether DHP is able to block transcriptional activation of the TGFb-signaling downstream event *Lefty-1*, Cripto-/- ES cells were exposed to Activin A or TGFb-2. We then probed DHP for its ability to alter Lefty1 transcript levels elicited by those ligands (E. Willems). **Figure 2.8** shows that, though DHP is able to block TGFb-2 induced *Lefty1* transcript, it fails to block Activin A induced *Lefty1*,, thus suggesting a difference in DHP specificity for the different TGFb-signaling receptors, unlike a typical kinase inhibitor like SB which acts to block TGFb-signaling across the board.

DISCUSSION AND CONCLUSIONS

Our results showcase and validate the use of small molecules and drug-like compounds as an alternative to the use of growth factors for obtaining cardiomyocytes from ESCs, as well as probes to dissect pathways involved in cardiac differentiation with regenerative potential in adult heart progenitor cells upon ischemic injury.

Corticosteroids Induce Cardiogenesis in mESCs

I have identified several molecules that promote mESC cardiogenesis in a dose-dependent manner (H2, H3, H4, Ald, Dex). Validation of these results by microscopy, RT-qPCR and FACS, showed that they work to promote not only an increase in the expression of markers of cardiomyocytes (*aMhc-eGFP, aMhc, cTnT, Tbx5)*, but also an increase in the yield of myocytes obtained in culture, both in relative (%) and absolute numbers (eGFP+ cells per same unit of volume) (**Figure 1.2**). Among GR agonists surfaced a MR agonist (Ald), which raised the possibility that the MR might be involved in cardiac induction in mECS.

Ald binds to and acts primarily via the mineralocorticoid receptor (MR). Among the nuclear hormone receptors, the MR is most closely related to the GR (Rogerson et al., 2007 and references within) and cortisol, corticosterone and Dex have all been shown to bind to the MR with considerable affinity, although Dex does not activate the MR with equal potency as it does the GR (Arriza et al.,

1991). This raised the possibility that Dex might also work through MR to promote cardiogenesis. Notwithstanding, in the mouse the MR is largely unaffected by glucocorticoids by its co-localization with an enzyme, 11beta-hydroxysteroid dehydrogenase type 2 (11b-HSD2), that converts cortisol to inactive cortisone (Funder, 1995). Furthermore, in our studies, Spironolactone, a MR antagonist, was unable to inhibit Ald effect on cardiogenesis, whereas Mifepristone, a GR antagonist, blocked the effect of H2, Dex, as well as Ald (not shown). Together with the known affinity of Ald for the GR at the concentrations used in our assay, we hypothesized that GR was the main receptor involved in mediating the cardiogenic effect of corticosteroids in mESC cultures.

Dexamethasone Acts Downstream of Smad-2, -3 Phosphorylation

We further characterized Dex, a synthetic glucocorticoid that is 20 to 30 times more potent that the natural hormone cortisol. We tested conditions that would allow us to identify the possible mechanism and target population of such steroid hormones as they induce cardiomyogenesis. Several extracellular signaling molecules show bimodal effects on heart induction in embryos and ESC cultures. Wnts and TGFb-signaling activation by Nodal induce endoderm and mesoderm early in mESC differentiation, playing an important role in promoting cardiogenic differentiation. Nodal has been used to initiate cardiogenesis in ESC cultures (Xu et al. 2006; Yang et al. 2008) and high levels of Nodal in a precise temporal window seem to favor definitive endoderm and

anterior mesoderm at the expense of lateral and posterior derivatives. Nodal, Wnts and BMPs have been shown to induce cardiogenesis early, but inhibit a cardiac fate if added past anterior streak mesoderm specification.

We hypothesized that Dex might work to attenuate Nodal signaling and promote a cardiac fate. Here, I showed that Dex is able to activate luciferase driven by a Glucocorticoid Response Element in a dose dependent manner, while inhibiting TGFb-signaling activation of a Smad-4 response element by TGFb-2 in 293T cells (**Figure 1.3.A**). We further showed that Dex does not affect phosphorylation levels of the R-Smads Smad-2 and Smad-3, suggesting that Dex might modulate TGF-beta signaling downstream of Smad phosphorylation both in 293T (TGFb-2) and in day 3 mESCs (Activin A) (**Figure 1.3.[B,C]).** Dex IC50 for TGFb inhibition, however, is much lower than the EC50 for GRE-activation. This raised the possibility that Dex induction of cardiogenesis at a typical single-digit uM dose might elicit off target effects that do not involve GR. However, the closer proximity of Dex EC50 for cardiogenesis to EC50 for GRE-Luc activation is well within reason to suggest that GR is the target of Dex to induce cardiogenesis.

Dexamethasone Acts Via GR to Induce Cardiogenesis

Procedures for cardiomyocyte differentiation of mESCs often utilize varying amounts of serum. The unknown nature of the serum composition can confound characterization of the differentiation process. In order to eliminate

such factors from our cultures that could hamper our ability to dissect Dex mechanism of action, we switched to a serum-free differentiation assay (as previously described; Cai et al., 2010) and probed whether GR was necessary for Dex effect on cardiogenesis (**Figure 1.4**). When treated with siGR, aMhc-eGFP mESCs were no longer able to as efficiently differentiate into cardiomyocytes, an effect that was more pronounced when Dex addition was performed at a time in which siGR might become significantly effective in knocking down GR. Simultaneously, Dex EC50 for cardiac induction at day 3.5 is lower than that at day 3, suggesting that Dex might be most effective at a stage when meso-endoderm is already established. **Figure 1.5** summarizes the findings discussed so far.

Dexamethasone Does Not Affect Meso-Endodermal Lineage Choice

If Dex was to modulate TGFb-signaling in meso-endoderm, we should expect to see markers such as *Smad-2* and its downstream targets *Lefty1* and *Lefty2* downregulated, together with a reduction in markers of the endodermal lineage or expressed by endodermal cells (*Foxa2*, *Gsc*, *Sox17, Cer1*). Our observations pointed to the opposite: Dex induced meso-endoderm markers (*Bra, Gsc*) as well as Nodal downstream genes (*Smad-2, Lefty1, Lefty2*) marginally, while quite significantly upregulating cardiogenic factors expressed in endoderm (*Sox17 and Cer1)*, but not the endodermal lineage marker *Foxa2* (**Figure 1.6.[A,B]**). This later resulted in an upregulation of cardiac markers (*aMhC, Tbx5, cTnT*) that contradicts the positive effect on Nodal targets seen

earlier, along with data showing that Dex inhibits TGFb induced SBE4-Luc. We therefore concluded that, even though Dex is able to inhibit TGFb-signaling at higher doses, at the working doses in this SFM assay that is not likely to be its the mechanism of action. I confirmed this by showing that Dex does not affect markers of cardiogenic progenitors (*Flk1, MesP1*) in the same way it did not affect endoderm progenitors marked by *Foxa2*.

Dexamethasone Upregulates Endoderm Specific Cardiac Inducing Factors

Figures 1.7.B and 1.9.[B-E] dismiss the possibility that Dex changes the ratio between endoderm and cardiogenic mesoderm in mESCs. Both by RT-qPCR analysis, and by ICC, I have shown that *Flk1*/*Foxa2* populations remain unaffected by Dex, and that Dex does not promote or hamper an expansion of either or both in culture. We therefore constructed a model in which Dex-liganded GR upregulates Sox17 in endoderm progenitor cells that signals through Cer1 to inhibit Nodal signaling locally uncommitted cardiogenic progenitor cells and promote a cardiac fate. In order to identify potential candidates that might mediate this interaction as well as cell-types as potential targets of Dex as mESCs progress through differentiation, I tested Dex during different time windows and verified a maximum effect of Dex when added to mESCs between days 4 an 6, as per cardiac marker analysis (*aMhc, cTnT, Tbx5*). Further examination (**Figure 1.8.[B-L]**] revealed that, while early addition of Dex to the cultures does not have a pronounced effect in regulating mesendoderm

induction, when present between days [4-6], it significantly upregulated endodermal cardiogenic factors (*Sox17, Cer1*) but not endoderm markers (*Hex, Foxa2, CXCR4*) or cardiogenic mesoderm markers (*Flk1, MesP1*). Particularly, the hepato-differentiation marker *Hnf4a*, a transcription factor shown to interact with GR both directly and at the transcription level (Nakei et al., 1998; Oyadomari et al., 2000; Michalopoulos et al., 2003), was also upregulated by Dex during the same time window.

In the adult, expression of the transcription factor Foxa2 is required for the activation of the hepatic gluconeogenic transcriptional program, and allows the binding of transcription factors cAMP Response Element Binding Protein (CREB) and GR to CRE or GRE sites during fasting. This suggested that GR and Hnf4a might interact in Foxa2 endodermal progenitors to induce a cascade of events resulting in an upregulation of Sox17 -> Cer1 to promote cardiogenesis. Of interest, two Sox17 binding sites were recently found within the regulatory region of the zinc finger protein 202 gene (*Zfp202*), a gene whose transcript is upregulated during F9-derived endoderm differentiation (Patterson et al., 2008). Importantly, Zfp202 colocalizes with Sox17 in anterior definitive endoderm on E7.75 mouse embryos, and was further shown to repress Hnf4a transcription, in both human and mouse (Wagner et al., 2000; Patterson et al., 2008). Hnf4a expression lags behind Sox17 in the anterior definitive endoderm (Duncan et al., 1994; Taraviras et al., 1994, Kanai et al., 2002). It is possible that Sox17 upregulation of Zfp202 is one mechanism by which to control the timing of Hnf4a

expression in order to direct endodermal differentiation towards liver (Parviz et al., 2003).

Hnf4a Acts Downstream of GR to Induce Sox17 and Cer1 on mESCs

A secondary result of GR -> Hnf4a induced Sox17 up regulation might be the induction of Sox17 target, Cerberus-1 (Cer1), a soluble and direct Nodal inhibitor that is also downstream of Nodal and BMP2. **Figure 1.10.A** positions Cer1 as a factor upon which Dex is dependent to elicit a cardiogenic response. To probe whether Hnf4a transcriptional activation alone could induce both *Sox17,* and *Cer1*, I tested a transcriptional activator of Hnf4a for its ability to bypass the need for Dex in the cultures (molecule 7005; kind gift from M. Dawson). **Figure 1.11.[C,D]** shows a concentration dependent induction of *Sox17* and, more effusively, *Cer1* by 7005. Furthermore, Hnf4a inhibitor BIM (kind gift from M. Dawson) was able to block Dex effect on cardiogenesis, reinforcing the model in which Dex/GR upregulate and/or transcriptional activate Hnf4a, which in turn upregulates Sox17 and as a consequence Cer1 to promote cardiogenesis. We have yet to elucidate the mechanism by which Hnf4a upregulates Sox17. However, this seems to suggest a scenario in which Sox17 indirectly represses Hnf4a expression through Zfp202 to prevent hepatocyte differentiation until the end of gastrulation. In day 4-6 mESC, a secondary but rather interesting result of that outcome seems to be cardiogenesis, as Sox17 also ends up inducing the cardiogenic factor Cer1 while responding to Hnf4a upregulation.

DHP TGFb-Signaling Acts at Distinct Times in Differentiation With Contrasting Results

DHP shows a biphasic effect on cardiac induction typical of secreted factors like BMP, Wnt and Nodal. Early in mESC differentiation, high Nodal is required to induce endoderm and mesoderm, and for this reason is important in laying the foundation for cardiogenesis to occur. Wnts and Nodal have been extensively used in ESC cultures to initiate cardiogenesis. (Xu et al., 2006; Yang et al., 2008). However, as gastrulation ensues, Nodal can have an inhibitory influence in cardiac specification. High Nodal induces definitive endoderm and anterior mesoderm, whereas lower Nodal levels pattern lateral and posterior derivatives. A narrow dose and window of exposure to Nodal is required for the right kind of mesoderm to be induced and progress down the cardiac path of differentiation.

DHP, we have shown, is able to mimic TGFb-signaling inhibitor SB in reducing the number of *T*/*Bra* expressing cells when added early to ESC cultures **(Figure 2.1).** Other markers of early mesoderm are also reduced, and so is endoderm. This suggests a suppression of much required high Nodal signaling in primitive-streak stage mESCs by DHP to impair gastrulation and favor neural tissue. We show that DHP has the ability to block TGFb-signaling both at the receptor level and upstream of R-Smad phosphorylation, an activity that correlates with mesoderm inhibition by SAR (**Figure 2.4).**

As Nodal is required to taper down after gastrulation and allow cardiomyogenesis, so increases the efficiency of DHP in inducing committed multipotent progenitors to assume a cardiac fate. Cer1, we have seen, blocks both BMP and Nodal, and induces cardiac mesoderm by allowing *Flk1+*/*MesP1+* progenitors to overcome the inhibitory effect of Nodal and BMP (Cai et al., 2010 and others). DHPs, we have shown, when added between days 3-5, suppress Nodal signaling to upregulate early cardiac lineage markers (*Flk1*, *MesP1*) and late cardiac markers (*Nkx2.5, Mef2c*, and *aMhc*). This alters the differentiation balance to confer a mesoderm bias at the expense of endoderm, just as caused by the Nodal inhibitor Cer1, or knockdown of TGFb-signaling effector Smad-2 by siRNA (**Suplemental Figure 1**). Dex/GR does not seem to mimic the Smad-2 knockdown effect on the lineage choice between *Flk1*+ and *Foxa2*+ progenitor cells, suggesting that Dex/GR mechanism of action does not involve Smad-2 and is dissimilar to that DHP, despite sharing a cardiogenic effect.

DHP and Dexamethasone Differently Modulate Nodal-Signaling to Induce Cardiogenesis

Figure 2.9 best summarizes our findings on the different effects of Dexamethasone and DHP in mECS cardiac differentiation *in vitro*. By inhibiting Nodal-signaling at the receptor level, DHP has a bimodal effect on cardiogenesis. Early, it inhibits meso-endoderm formation and favors an ectodermal fate. Later, by inhibiting the Nodal pathway, in prevents *Flk1+*/*MesP1+* progenitors from responding to Nodal signaling and allows them to assume a cardiogenic fate,

ultimately differentiating into cardiomyocytes. During early embryonic hepatic development from foregut endoderm, Foxa proteins bind to promoters and enhancers of liver-specific genes in anticipation of their transcription (McPherson et al., 1993). In the adult, expression of the transcription factor Foxa2 is required for the activation of the hepatic gluconeogenic transcriptional program, and allows the binding of transcription factors cAmp Response Element Binding Protein (CREB) and GR to CRE or GRE sites during fasting. In our cultures, Dexamethasone might therefore require the co-expression of Foxa2 to grant GR access to chromatin regions where it can unregulate *Hnf4a*, or alternatively for *Hnf4a* to be present, allowing GR/Hnf4a interaction to occur. This could result in an up regulation of a negative feedback reaction, by which we think Sox17 attempts to prevent precocious differentiation of hepatic endoderm otherwise induced by Hnf4a. Our results unequivocally show that Hnf4a transcriptional activation upregulates Sox17. As a result of the elevation of Sox17 in endoderm, Cerberus-1 is also expressed, locally and temporally inhibiting Nodal directly to allow uncommitted progenitors to become committed to the cardiac lineage. We further wish to dissect out this proposed mechanism so as to better elucidate this sequence of events.

FINAL REMARKS

"Dissertations are not finished; they are abandoned."

Fred Brooks

Much work still sits at the bench-top waiting to be done. For instance, we recognize the need to show that Dex acts specifically in endoderm and that there is no other effect on cardiac progenitors. Rescue experiments that first separate, treat with relevant ligands and factors, and then combine the endodermal and mesodermal populations, together with siRNA knockdown of GR and Hnf4a, should allow us to address that question. We also have yet to completely characterize the effects of Hnf4a transcriptional activator 7005 and Dex on other lineage markers, as well as profiling gene expression when BIM, or siHnf4a in its stead, inhibits Dex cardiogenic effect, to rule out an unspecific inhibition of cardiogenesis. Furthermore, embryological relevance needs to be addressed for the role of GR in cardiac development. The analysis of the relevant developmental stages of the GR-/- mouse should allow us to clarify this important aspect. It is our intention to answer these open questions in the near future, in preparation of our manuscripts for submission.

Through this work, we hope to have contributed, if modestly, to a better understanding of the timing and nature of the signaling molecules that specify

lineage differentiation in mESCs, the signals required to increase the yield of cell progeny of interest *in vitro*, and to have furthered our understanding of how to best modulate such signals in order to promote endogenous regeneration of the myocardium by endogenous progenitor cells.

MATERIALS AND METHODS

Cell Culture and ES Differentiation

CGR8 and R1 mESCs were cultured in growth media (GM) as previously described (Bushway et al., 2006) and in gelatin coated plates. For serum-based differentiation (DM), mESCs were cultured in identical formulation of GM, with the exception of leukemia inhibitory factor (LIF). For serum-free differentiation assays, ESCs were cultured in Iscove's Modified Dulbecco Media (IMDM) supplemented with 25% DMEM/Ham's F-12, 2mM L-glutamine, 4.5×10^{-4} M monothioglycerol, 0.5mM ascorbic acid, B27 supplement without Vitamin A (Gibco), N2 supplement (Gibco) and differentiated as EBs. Recombinant TGFb-2 and Activin A were purchased from R&D Systems; SB-431542 was purchased from Sigma.

HEK239T (293T) cells were grown in DMEM High Glucose with sodium pyruvate (Hyclone), supplemented with 10% (v/v) FBS and 1mM L-glutamine (293T-M). Cells were passaged when 80% confluent. For transfection and western blot analysis, cells were weaned off serum for 24 hours in 1% (v/v) FBS containing 293T-M before start of assay.

Automated Quantitative Microscopy for Fluorescence

mESCs harboring an eGFP reporter driven by either the aMhc promoter (Takahashi et al., 2003) or Flk-1 promoter were dissociated at day 3 and plated

onto 384-well plates. Flk-1-eGFP was counterstained with mouse anti-GFP antibody (Abcam) detected by Alexa488-conjugated rabbit anti-mouse polyclonal antibody (Molecular Probes). aMhc-eGFP was imaged at differentiation day 10 (InCell 1000, GE/Amersham) and quantified by CyteSeer (Bushway et al. 2006).

Plasmids, Lentivral Infection and siRNA Transfection

SBE4-Luc was a kind gift from Dr. Michael Kuehn. Cignal GRE Reporter (GRE-lux) was purchased from SABiosciences (Qiagen).

shRNA lentivral vectors (Cer1 shRNA-1, -2, -3 and scrambled luciferase shRNA) were provided by Michael Schneider (Imperial College, London). shCer1 and shControl R1 mESCs were generated by, and kindly obtained from, W. Cai.

Pre-designed siRNAs against murine GR, Smad-2 and Smad-3 (Ambion) and validated negative siRNA (Ambion) were transfected into R1 or CGR8 cells using Lipofectamine 2000 as per manufacturer's instructions.

RNA Extraction and RT-qPCR

Total RNA was extracted with TRIzol (Invitrogen) and reverse transcribed to cDNA with QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturers' instructions. cDNA samples synthesized from 1ug of total RNA

were subjected to RT-qPCR with LightCycler 480 SYBR Green I Master kit (Roche) performed with LightCycler 480 Real-Time PCR System (Roche). Values were normalized to *GAPDH* transcript. Primer sequences are listed in **Supplementary Table 1**.

FACS and Flow Cytometry

mESCs were harvested and gently dissociated by trypsinization. Dissociated single cells were resuspended in 1% FBS in PBS containing 50uL/mL of fluorescent polystyrene microspheres (LinearFlow™ Carmine Flow Cytometry Intensity Calibration Kit, for 488 nm excitation/620 nm). Propidium iodide was used to detect dead cells and cell debris. Single cells were analyzed with FACSCalibur (BD Biosciences).

Statistics

Each experiment was repeated at least two times using a minimum of three biological replicates per condition. Unless otherwise stated, statistical analyses were performed using unpaired Student's T-test and reported as average ± s.d. Significance was considered at p-value < 0.05.

ACKNOWLEDGEMENTS

This research was supported by the Science and Technology Foundation of the Portuguese Ministry of Science, Technology and Higher Education, and by the Howard Hughes Med-into-Grad Program.

APPENDIX

Figures

Summary of Different Experimental Designs

day	0	2	3	4	5	6	8	10
Assay	naïve mESC begin differentiation No LIF		Gene Profile					
DM	10% FBS Adherent	Add compounds		Add compounds		Withdraw compounds		Analyze
SFM	SFM Suspension EBs	+Activin	+Act +Dex	Dex, 7005, BMI later assays		Withdraw treatment		Analyze
siRNA	SFM Suspension EBs	+Activin	siRNA Dex			Withdraw treatment		Analyze
Flk1/Foxa2 Assay	SFM Suspension EBs	+Activin	Dex			ICC Analyze		

Figure 1.1. High-Throughput Screening for Small-Molecule Inducers of Cardiomyogenesis: Experimental Design.

DM Assay. CGR8 aMhc-eGFP mESCs were weaned off MEFs for 2 days in GM. On day 0, LIF was withdrawn and cells were dispersed onto gelatin-coated 96-well cell culture plates at a density of 1000 cells per 100uL of DM per well. Cells were allowed to differentiate for 2 days. On day 2, media was changed and the Chembridge DIVERset MS-Discovery collection was added at 4uM per well. Media was changed at day 4 and library re-added. On day 6, media was changed. On day 8, cells were fixed in 4% PFA, nuclei counterstained with DAPI, and imaged using a EIDAQ™ 100 (Q3DM) High Throughput Microscopy System. Images were analyzed using Q3DM metrics and integrated fluorescent intensity quantified. Confirmatory analyses were performed to dismiss false positives.

SFM Assay. mESCs were grown in suspension in serum free media (SFM). On day 2, cells were dispersed and treated with Activin A (15ng/mL). on Day 3, cells were dispersed and platted onto cell culture dishes coated with gelatin. Treatment with Dex was performed at day 3, 3.5 for initial assays, and at day 4 (Dex, 7005, BMI) on later assays.

siRNA Assay. Differentiation was done as described (**SFM Assay**). On day 3, cells were reverse transfected with siRNA and Dex treatment performed (day 3, 3.5).

Flk1/Foxa2 Assay. Similar to **SFM Assay**, cells were treated with Dex between days 4-6, fixed and analyzed for Flk1-eGFP and Foxa2 expression.

A lineage gene profile of differentiation was obtained for the different assays.

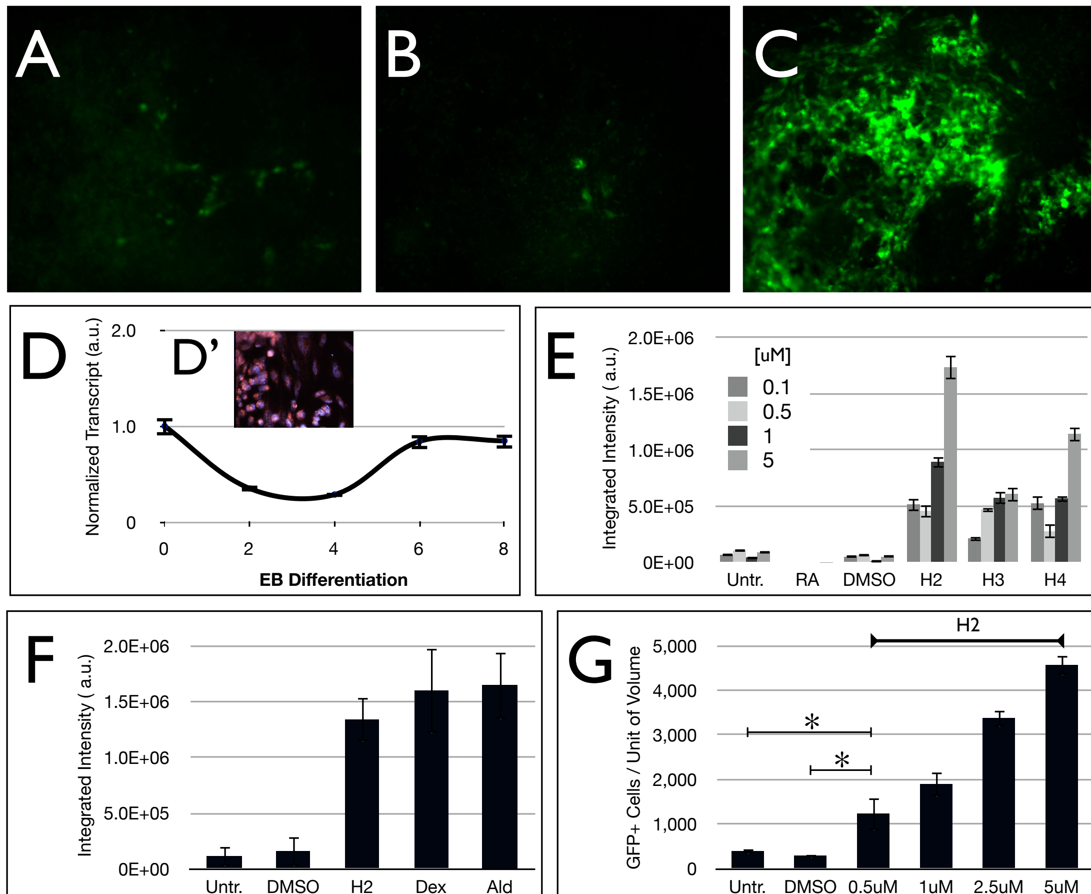


Figure 1.2. GR-Ligands Induce mESCs Differentiation into Cardyomyocytes.

[A-C]. Representative panel of cardiomyocyte induction on aMhc-eGFP mESCs. Hydrocortisone **[C]**, added to aMhc-eGFP mESCs at day 2 and day 4 of differentiation, induces significant day 8 eGFP expression when compared to media alone **[A]**, or 0.1% DMSO **[B]**. Images were acquired by inverted microscopy.

[D-D']. Glucocorticoid receptor (GR) transcript **[D]** is detectable by RT-QPCR analysis in naïve mESCs (day 0) and throughout EB differentiation (days 2-8). GR protein **[D']** is detectable by immunocytochemistry at the time of Hydrocortisone treatment (day 2). Red, GR; Blue, DAPI. Images were acquired by inverted microscopy.

E. Hydrocortisone (H2) and Hydrocortisone 21-hemisuccinate (H4) were both identified in the initial blind-screen for inducers of cardiomyogenesis. Also present in the set was the false-negative Hydrocortisone acetate (H3). H2, H3, and H4 were confirmed for their ability to induce cardiomyogenesis in a dose dependent manner [0.1, 0.5, 1, 5; uM]; untreated (Untr.) and vehicle-only (DMSO) controls were negative in comparison. Retinoic acid (RA) was used as a negative control. Images were acquired through High Throughput Microscopy and integrated fluorescent intensity quantified.

F. At 5uM, bona-fide GR-ligands, Dexamethasone (Dex) and Aldosterone (Ald), mimicked H2 induction of cardiomyogenesis.

G. Flow cytometry quantification of H2 induced cardiomyocytes. aMhc-eGFP mESC were treated from day 2-6. On day 8, cells were gently dissociated and resuspended in PBS containing fluorescent polystyrene microspheres. Samples were analyzed by FACS. GFP+ cells were counted and data normalized against number of red fluorescent polystyrene microspheres. Data represents number of GFP+ cells per arbitrary unit of volume, as inferred by number of fluorescent beads. Treatment with H2 [0.5uM; 1uM; 2.5uM; 5uM] significantly induces a higher number of total cadiomyocytes (eGFP+) in the culture, when compared to media alone (Untr.) or vehicle only (DMSO).

Error bars indicate s.d. For **D**, n=3; **E**, n=8; **F**, n=8; **G**, n=3. Data are representative of two independent experiments. Asterisk (*) indicates $p < 0.05$ (Student's t-test).

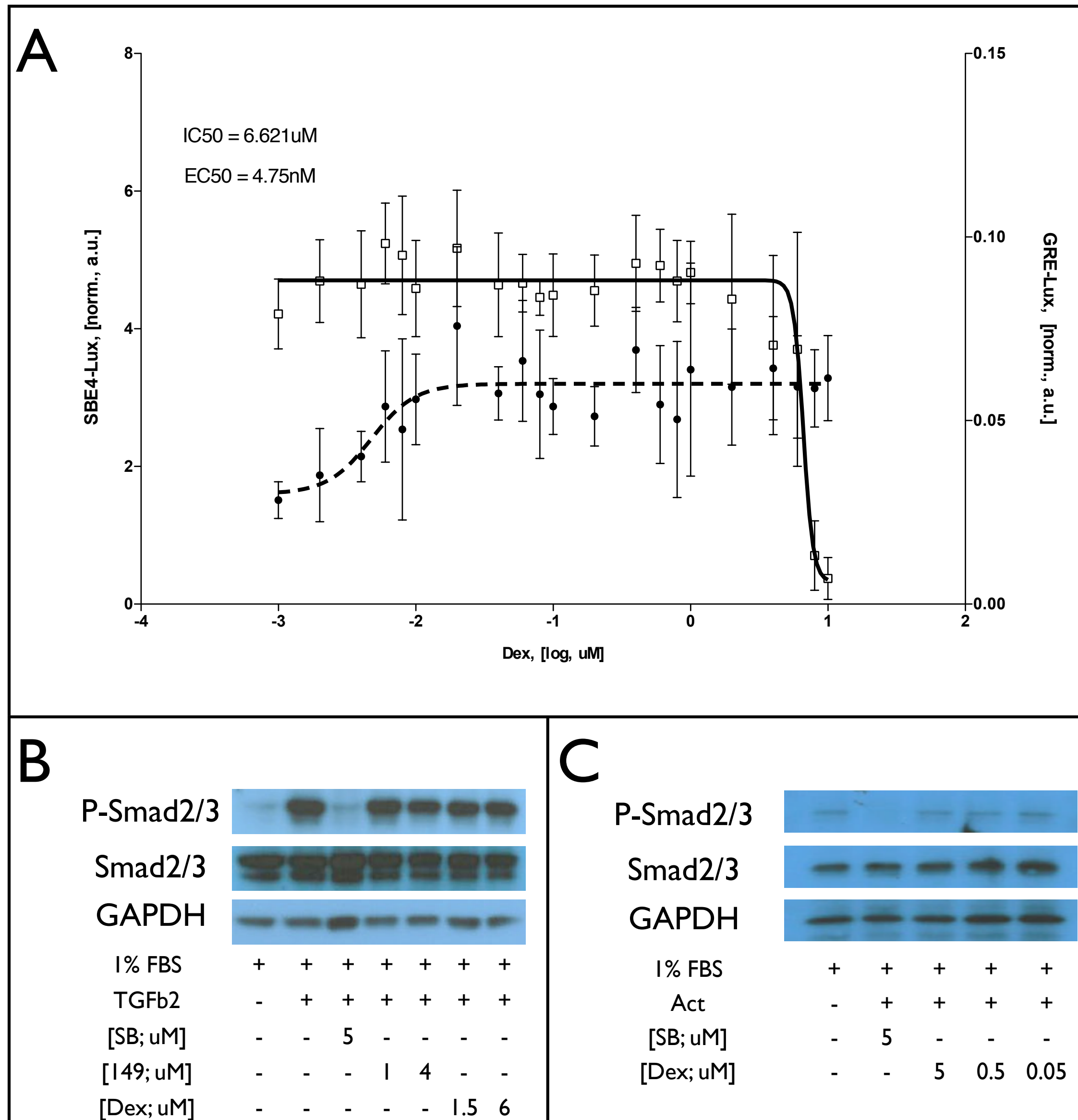


Figure 1.3. Dexamethasone Acts Downstream of Smad-2, -3 Phosphorylation in 293T and in CGR8 mESC.

A. 293T cells were reverse transfected onto cell-culture plates in 1% 293T-M with either 10:2 ng/well [SBE4-Luc:Ren-FF] or 100:2.5 ng/well [GRE-Luc:Ren-FF]. TGFb-2 induces luciferase driven by a Smad-4 response element (SBE4-Luc) and can be inhibited by Dex. Dose response curve generated with increasing Dex concentration; Dex inhibitory effect on TGFb-2 signaling induced luciferase is shown (SBE4-Luc; left Y-axis). IC50 value is indicated. Dex induces luciferase driven by a GR Response Element (GRE-Luc; right Y-axis). EC50 value is indicated. Data normalized to renilla luciferase luminescence driven by CMV promoter. n=8.

[B-C]. Dex does not inhibit Smad-2, -3 phosphorylation on 293T cells **[B]** or differentiated mESCs **[C]**. **B.** Cells were grown on 6-well plates in 1% 293T-M for 24 hours. Cells were treated as indicated by a plus sign (+) or dose (number; uM). SB, Alk-4, -5, -7 inhibitor SB-431542 (5uM); 149, verified negative control from a different screening (1, 4; uM); Dex, Dexamethasone (1.5, 6; uM). **C.** mESCs were differentiated in SFM as described (see **Methods**). On day 3, EBs were dispersed and platted onto gelatin coated 6-well plates, and treated as indicated by a plus sign (+) or dose (number; uM). **[B-C].** Total protein was harvested 24 hours after treatment and proteins of interest detected by western blot. GAPDH was used as loading control.

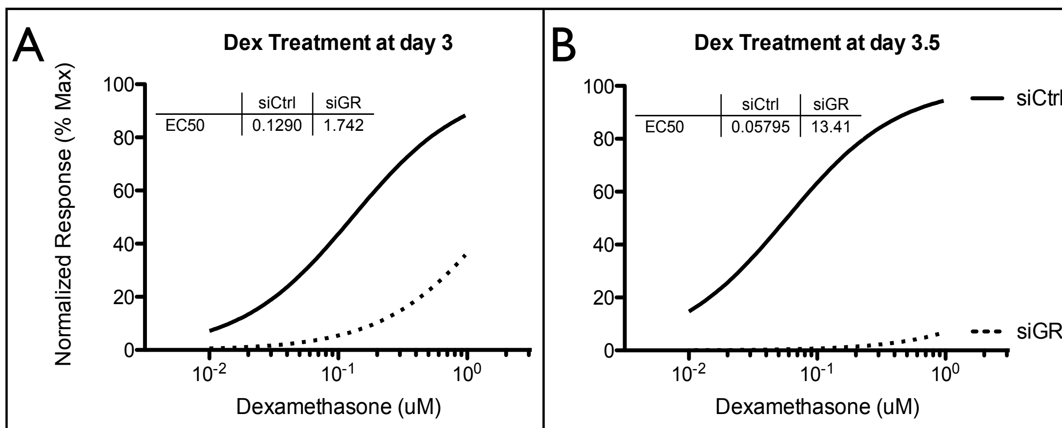


Figure 1.4. Dex Induces Cardiomyogenesis Through GR. aMhc-eGFP mESC were differentiated as EBs for 3 days, and reverse transfected with Control-siRNA (siCtrl) or siRNA against GR (siGR). Cells were allowed to adhere to 384-well plates. Treatment with a dose of Dex was performed on day 3 **[A]** or day 3.5 **[B]**; Dex withdrawn at day 5. On day 10, cells were fixed with 4% PFA, DAPI stained, and imaged by High Throughput Automated Microcopy. Dex induction of cardiomyogenesis [solid line; (— siCtrl] is significantly reduced if GR mRNA is targeted [dashed line; (- - siGR)]. Inhibitory effect is more pronounced if siGR transfection precedes Dex treatment **[B]**, as reflected by a higher EC50 for siGR and a lower EC50 for siCtrl, compared to day 3 **[A]**.
Data represents fit curves of dose response. Data points considered not diverging significantly from fit model. For A, n=8; B, n=8. Data are representative of two independent experiments.

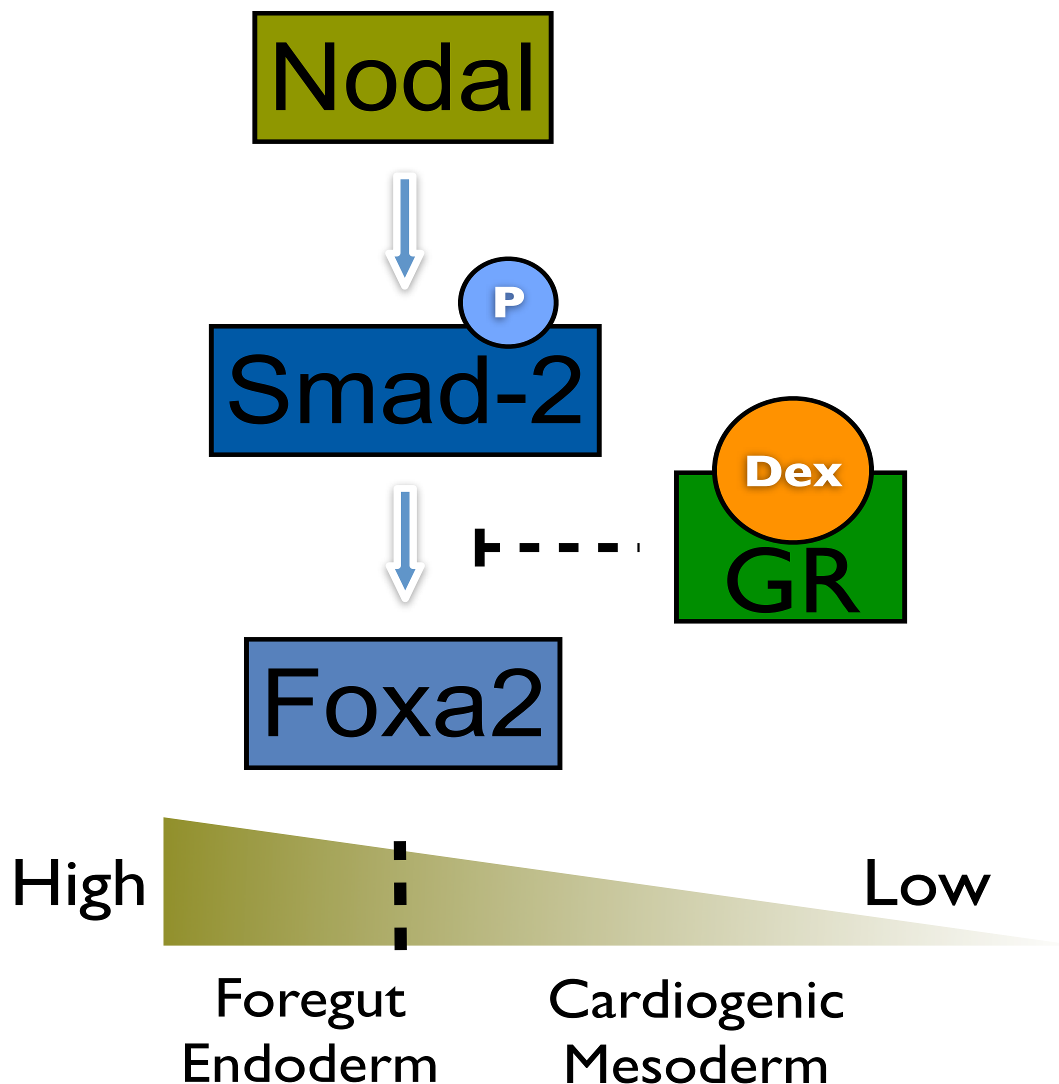


Figure 1.5. Proposed Intermediate Model in Which Dex Induced GR Acts Downstream of Smad Phosphorilation. Dex induction of cardiomyogenesis is GR dependent. Together with Dex inhibition of SBE4-luc, the data suggests that Dex may act through GR to block Nodal signaling on mesendoderm and promote a cardiac fate. Dex liganded GR fails to inhibit TGFb-signaling both on 293T cells and mESCs, suggesting it might block TGFb-signaling downstream of Smad phosphorylation. If this model is true, we should expect to see markers of mesoderm and cardiac progenitors to be upregulated (*Flk1, MesP1*) as well as markers of committed precursors (*Nkx2.5, Mef2c)*, at the expense of endoderm progenitor cell markers *(Foxa2, Hex, Sox17, Cer1*).

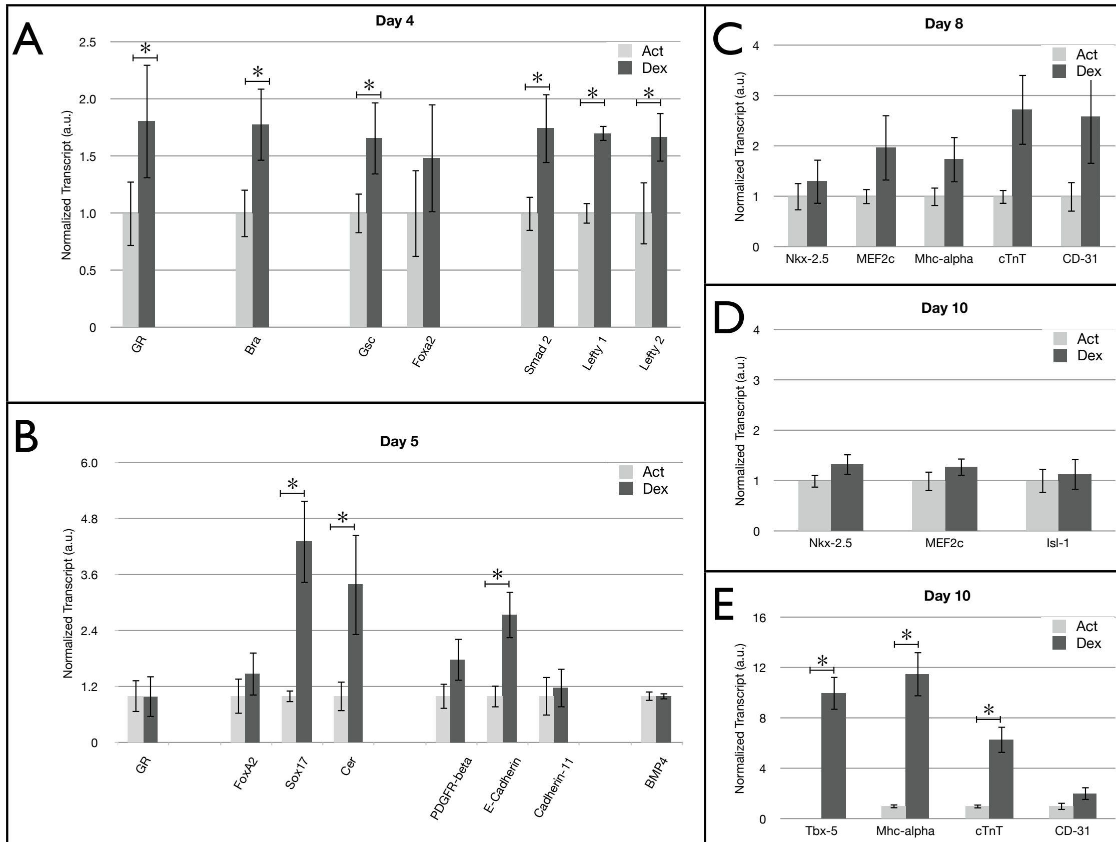


Figure 1.6. Dex Upregulation of Early Endodermal Markers Contrasts With Later Cardiogenic Effect. mESC were differentiated as described. Cells were treated with Activin between days 2-5 and Dex was added to cultures from day 3-5. Samples were collected and transcript levels analysed by RT-QPCR at day 4 [A], day 5 [B], day 8 [C] and day 10 [D, E]. Data was normalized against *GAPDH* and represented as fold over Activin treatment (Act). Gene expression analysis shows that Dex upregulates endodermal markers involved in cadiac induction (*Sox17*, *Cer1*) [B], but not by upregulating definitive endoderm (*Foxa2*) [A, B] or committed cardiac precursors (*Nkx2.5*, *Mef2c*, *Isl-1*) [C, D]; cardiomyocyte markers are upregulated without affecting endothelial cells [E]. Error bars indicate s.d.; n=3. Asterisk (*) indicates $p<0.05$ (Student's t-test). Data are representative of at least two independent experiments.

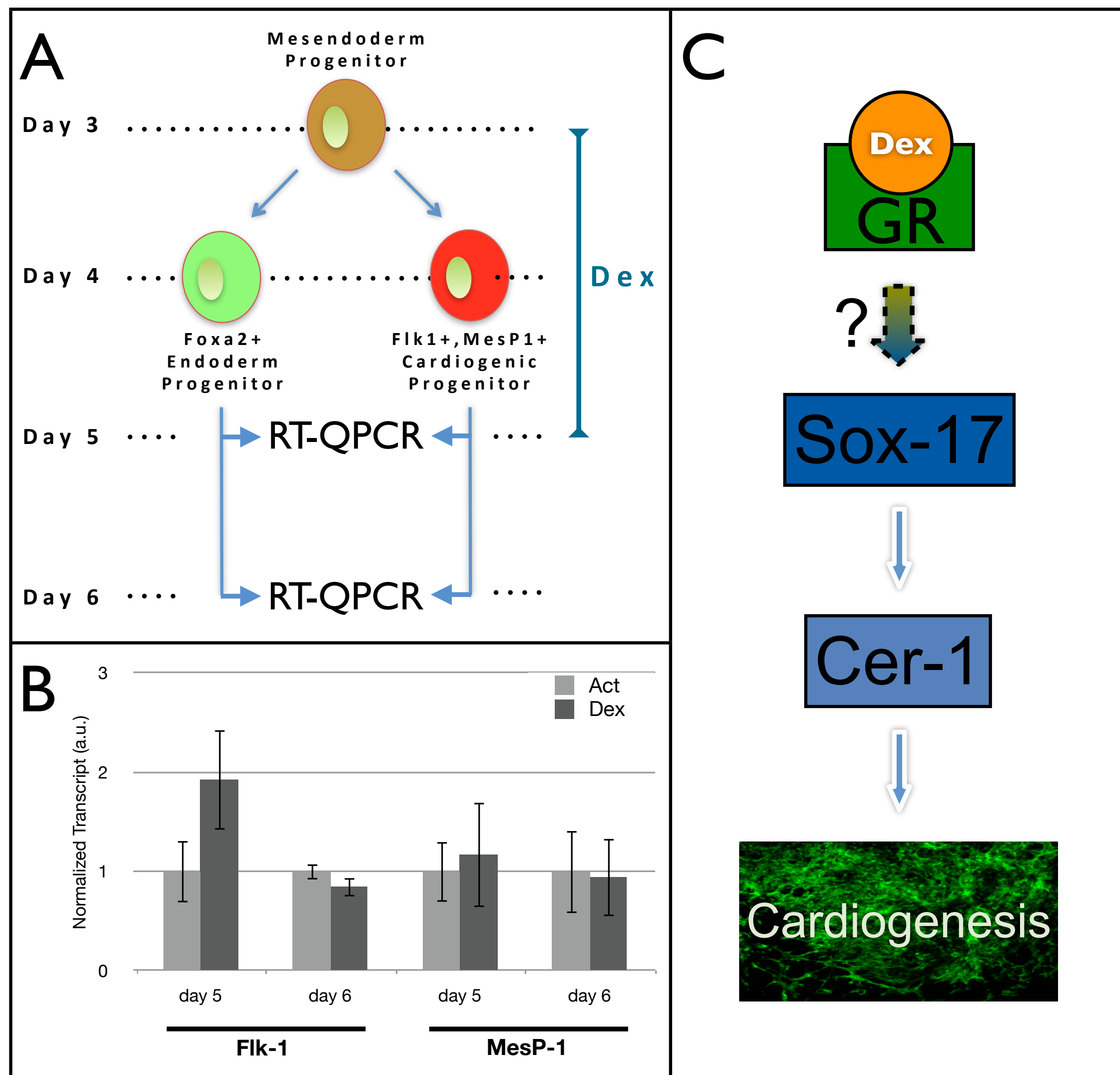


Figure 1.7. Dex Does Not Promote Cardiogenesis by Affecting Mesoderm:Endoderm Lineage Choice.

A. Experimental design**.** Day 3 Flk1-eGFP mESC were treated with Dex and allowed to differentiate for 2 days. RNA samples were collected at day 5 and 6. The model predicts that, if Dex acts by promoting endoderm progenitors at the expense of mesoderm, cardiogenic progenitor markers *Flk1* and *MesP1* should be reversely affected.

[B-C]. Day 5 and day 6 markers of cardiogenic progenitors are not significantly affected by Dex, when compared to Activin treated samples **[B]**. This suggests a model in which Dex activated GR is able to induce a cascade of signaling through Sox17 and Cer1 that promotes cardiogenesis without altering early lineage relationships.

n=3. Data are representative of at least two independent experiments.

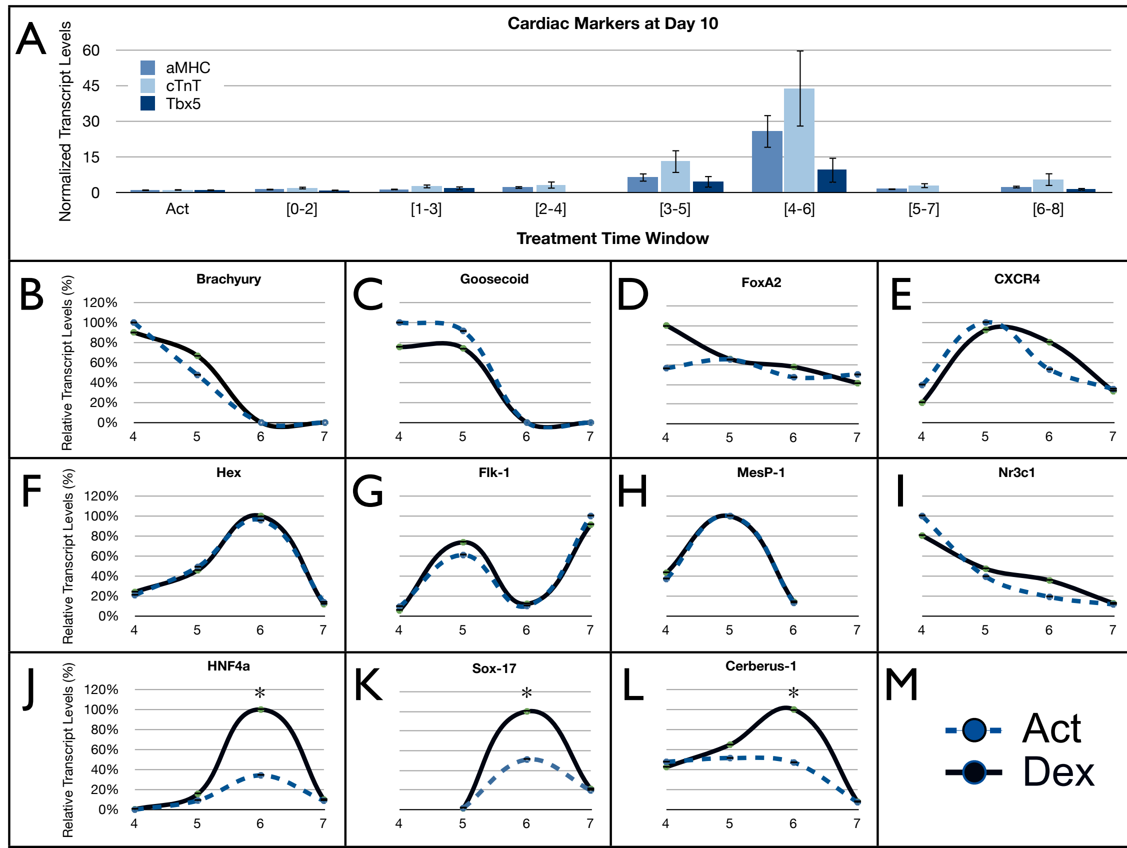


Figure 1.8. Dex is Most Cardiogenic Between Days 4-6 and Promotes Upregulation of Endoderm Specific Cascade of Cardiac Inducing Factors. R1 mESCs were differentiated from day 0-10 and treated with Dex for 2-day periods. Activin was present in the cultures from days 2-5 (Dex and Act). Samples were collected at days 4, 5, 6, 7 **[B-L]** and 10 **[A-L]**. Data was normalized against *GAPDH,* and plotted as fold over Activin treatment **[A]**; maximum induction for panels **[B-L]** was set at 100%.

A. Dex induced day 10 cardiac markers (*aMhc*, *cTnT*, *Tbx5)* when added between days 3-6, with best time of addition between days 4-6.

[B-L]. Gene expression profiling of mESCs treated with Dex from days [0-2], [3-5], [4-6], and [5-7], and analyzed at day 4, 5, 6, and 7, respectively. Data shows upregulation of a cascade of cardiogenic signaling typical of endoderm (*Sox17*, *Cer1*), without altering meso-endoderm specification (*Flk-1*, *MesP-1* vs *Foxa2*, *CXCR4*). Importantly, Dex also upregulates endodermal marker *Hnf4a* which might be indirectly involved in the upregulation of Sox17 -> Cer1.

Error bars indicate s.d. **[A]** and e.r.o.m. **[B-L]**. For **[A-L]**, n=3. Asterisk (*) indicates p<0.05 (Student's t-test). Data are representative of at least two independent experiments.

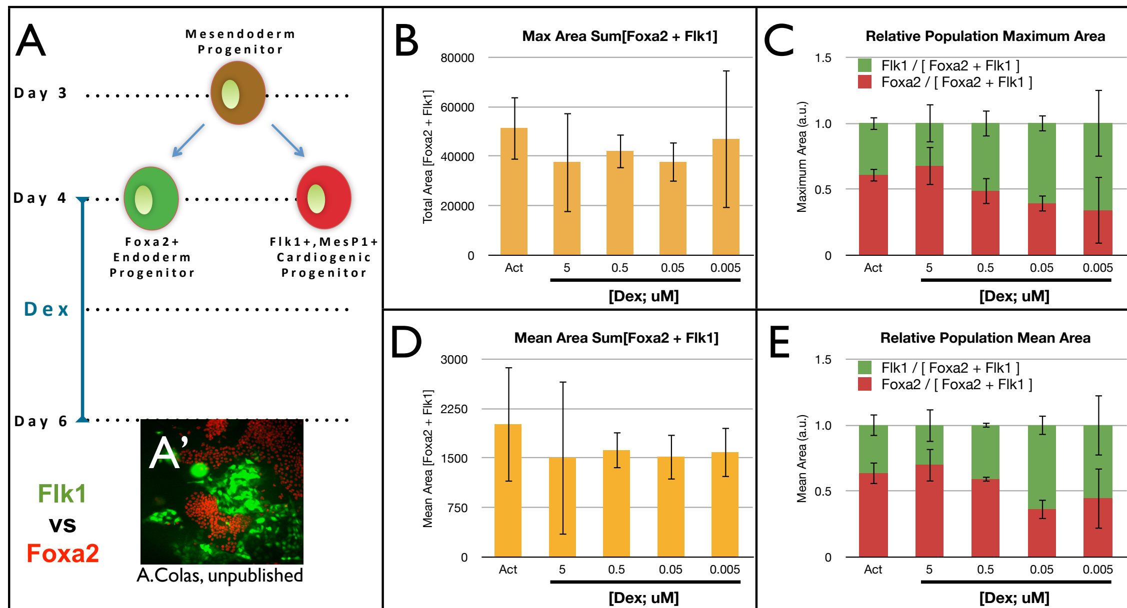


Figure 1.9. Dex Does Not Alter Flk-1/Foxa2 Ratio and Proliferation in Culture.

A. Experimental design. Flk1-eGFP mESCs were differentiated for 3 days unders standard serum free conditions. Cells were treated with Activin (15ng/mL) from days 2-6. Dex [0.005, 0.05, 0.5, 5; uM] was added to cultures from days 4-6. Plates were fixed, antigens detected by immunofluorescence (Green, eGFP; Red, Foxa2), and images (InCell100). Images were analyzed and quantified by Cyteseer software. Representative image shown **A'** (A. Colas, unpublished).

[B-E]. Dex did not significantly alter the total area of *Flk1+*, *Foxa2+* expression, both in terms of maximum total area **[B]** or mean of total area **[D]**. Dex also did not alter the relative ratio between *Flk1+* and *Foxa2+* cells in terms of maximum area of each population **[C]** or mean expression area **[E]**.

Error bars indicate s.d. **[B-E]**, n=3. Data are representative of at least two independent experiments.

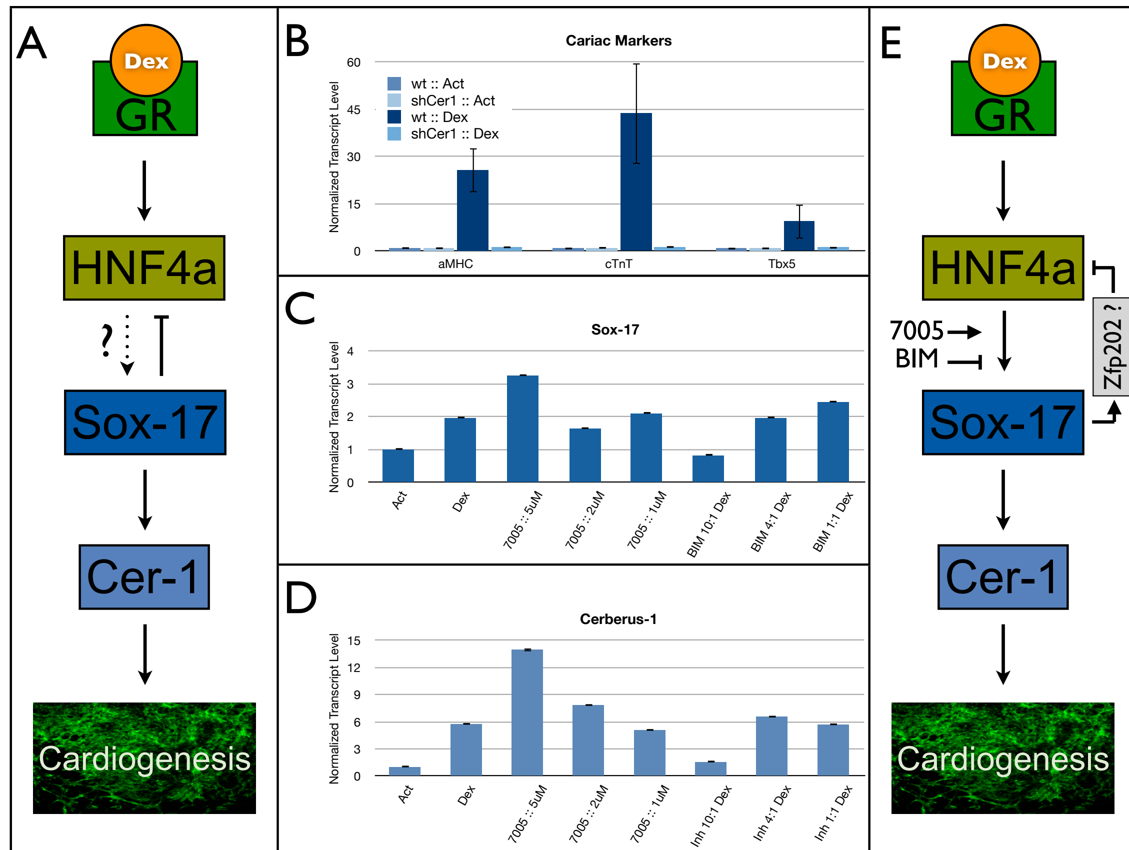


Figure 1.10. Hnf4a Transcriptional Activator Induces Sox17 and Cer1 on mESC.

A. Hypothesis: Dex activated GR upregulates *Hnf4a*, which in turn induces *Sox17 -> Cer-1 ->* Cardiogenesis.

B. Day 4-6 treatment with Dex induced cardiac markers by day 10 in wt but not in shCer1 mESC.

[C-D]. Hnf4a transcriptional activator (7005) induces *Sox17* **[C]** and *Cer1* **[D]** in a dose dependent manner. A 10:1 treatment with Hnf4a inhibitor (BIM) blocks Dex induction of *Sox17* and *Cer1*. For Dex, 0.5uM; for 7005, [5, 2, 1; uM]; for BIM, [5, 2, 0.5; uM].

E. GR Cardiogenic Mechanism of Action: Dex liganded GR acts upstream of Hnf4a to induce Sox17 and Cer1. Dex cardiogenic effect is dependent on Cer1, as Dex fails to significantly induce cardiogenic markers on shCer1 above Act. Hnf4a inhibition (BIM) also blocks Dex induction of *Sox17* and *Cer1*, an effect replicated by Hnf4a transcriptional activation (7005). Together, these results place Hnf4a downstream of GR and upstream of *Sox17* and *Cer1* to induce cardiogenesis. Sox17 downregulation of Hnf4a via upregulation of *Zfp202* has been shown by others (Patterson et al., 2008).

Error bars indicate s.d. **[B]** and e.r.o.m. **[C-D]**. For **[B-D]**, n=3. Asterisk (*) indicates $p<0.05$ (Student's t-test). Data are representative of at least two independent experiments.

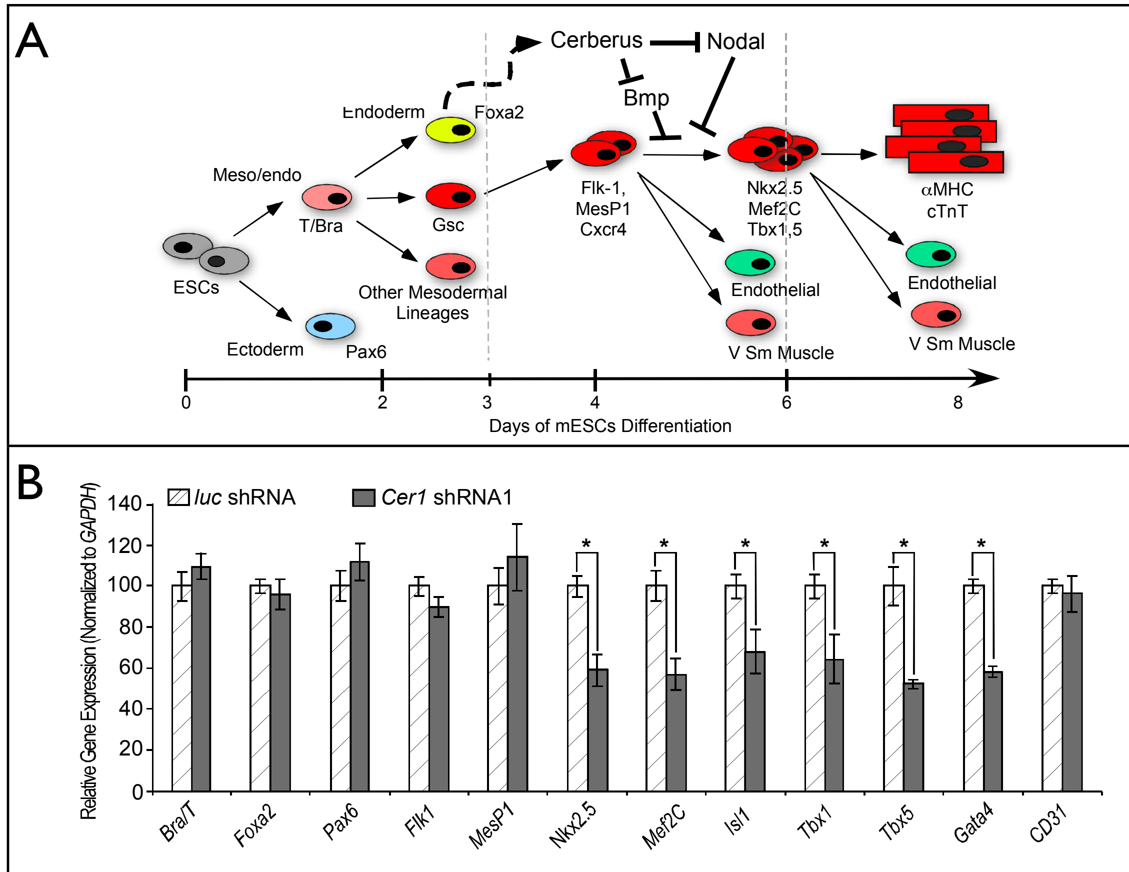


Figure 1.11. Cer1 is Selective for Cardiomyocyte-Committed Cells.

A. Cai et al. (2010) have demonstrated that Cer1 localizes to a Cxcr4+, Flk1- population enriched for anterior definitive endoderm-like cells which Morrison and others have show is able to induce cardiomyocytes when ectopically positioned near uncommitted mesodermal cells (Morrison et al., 2008; Holtzinger et al., 2010).

B. Cai et al. (2010) have shown that Cer1 is needed for cardiomyocytes but not other cardiopoietic lineages. Cer1 knockdown does not affect mesoendoderm formation (*T/Bra+*), endoderm (*Foxa2+*), ectoderm (*Pax6+*), or cardiac progenitors (*Flk1+*, *MesP1+*); it does not alter specific endothelial markers (*CD31+*) or smooth muscle (*sm-MHC+*, not shown here). Cer1 knockdown significantly reduces cardiomyocyte differentiation (*Nkx2.5*, *Mef2c*, *Isl1*, *Tbx5*; also *aMhc*, *cTnT, not shown*). These data suggest that Cer1 is needed after the *Flk1+*, *MesP1+* stage of differentiation. Our data on upregulation of Cer1 by Dex supports these findings.

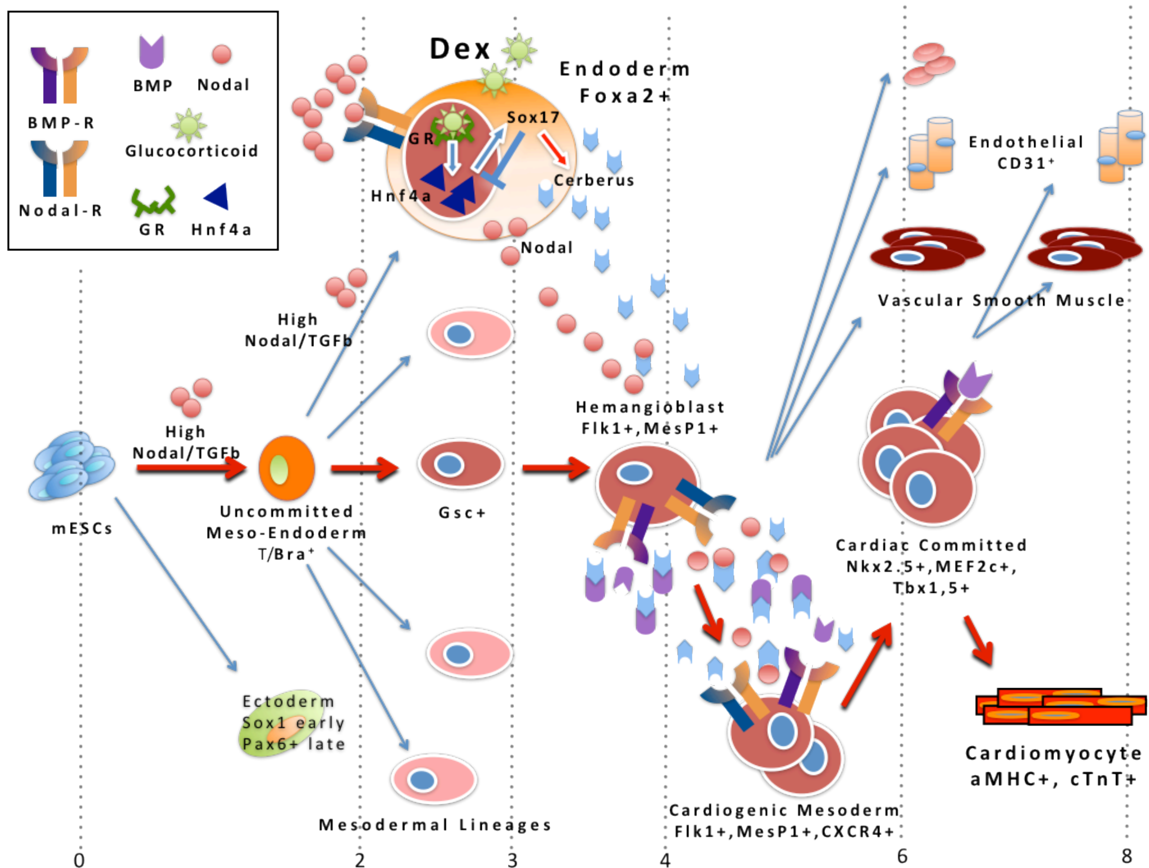


Figure 1.12. Model of Cer1 Induction by GR to Specify Heart Field.
Schematic depicting the embryonic germ layers as they progress through mESC differentiation *in vitro*. Cer1 has been shown to be required for differentiation of Flk1, MesP1 progenitors. Definitive endoderm (depicted in light orange) and mesoderm (shown in red) share a common progenitor in the primitive streak. Red arrows follow the lineages leading to cardiomyocytes. High Nodal promotes endoderm while low to no Nodal promoted mesoderm cell fates. Dex liganded GR promotes an upregulation of *Hnf4a* that triggers a feedback mechanism upregulating *Sox17* to prevent premature hepatic differentiation. Sox17 in turn will induce *Cer1*, and Cer1 acts to locally bind and transiently inhibit BMPs, Nodal, and Wnt (not shown) on cardiogenic mesoderm, thus establishing the heart field. We propose that this results in an increased recruitment of cardiac committed progenitors to adopt a cardiomyocyte fate, and not at the expense of endodermal or endothelial progenitors or their derivatives.

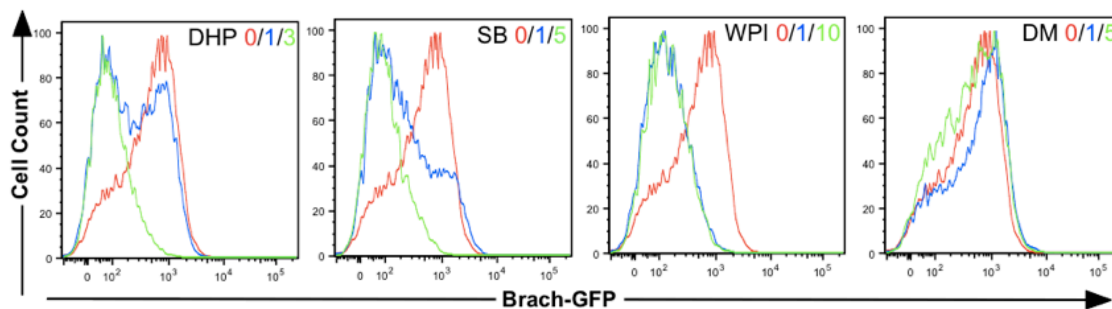


Figure 2.1. DHPs inhibit Mesoderm in ESC by Blocking the TGFb/Activin/Nodal pathway. Flow cytometry of Brachyury-eGFP analyzed on day 4 of differentiation. Compounds were added at day 1 of differentiation. The number of mesoderm cells is donwregulated by DHP (Red, DMSO; Blue, 1uM; Green, 3uM) and can be mimicked by the Nodal/Activin/TGFb inhibitor SB-431542 (SB) (Red, DMSO; Blue, 1uM; Green, 5uM) and an inhibitor of Wnt production (WPI) (Red, DMSO; Blue, 1uM; Green, 10uM), but not by the BMP receptor inhibitor Dorsomorphin (DM) (Red, DMSO; Blue, 1uM; Green, 5uM).

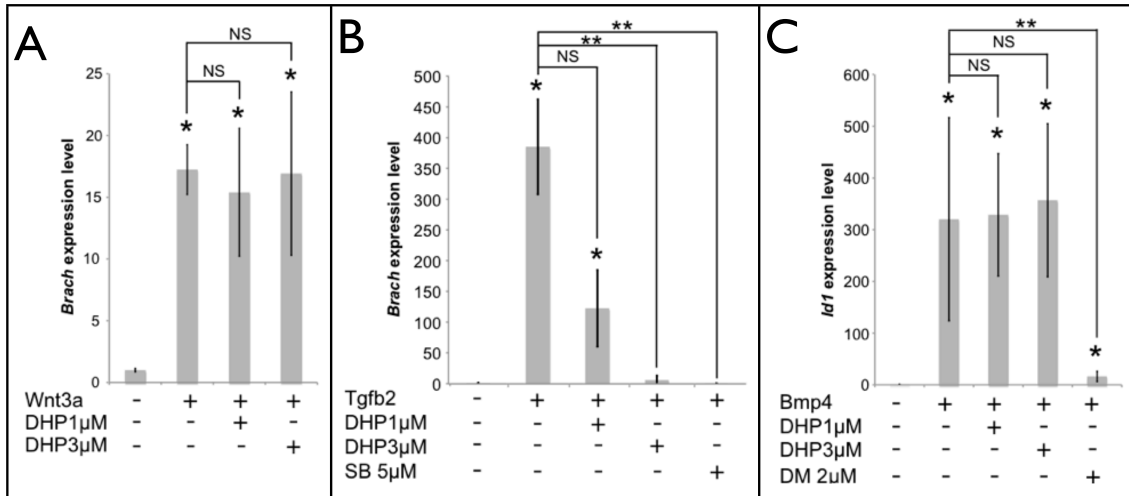


Figure 2.2. DHPs block TGFb-2 but not Wnt3a or BMP4 induced *Bra*+ expression in Cripto-/- ES cells.

A. DHP does not block Wnt3a induced *Bra*+ expression in Cripto-/- ES cells, as assessed by RT-qPCR at day 4 of differentiation.

B. DHP blocks TGFb-2 induced *Bra*+ expression in Cripto-/- ES cells in a concentration dependent manner, as assessed by RT-qPCR at day 4 of differentiation.

C. DHP does not block BMP4 induced BMP4 direct target, *Id1*, in Cripto-/- ES cells as assessed by RT-qPCR at day 3 of differentiation.

D. Error bars indicate s.d. For **[A, C]**, n=3.

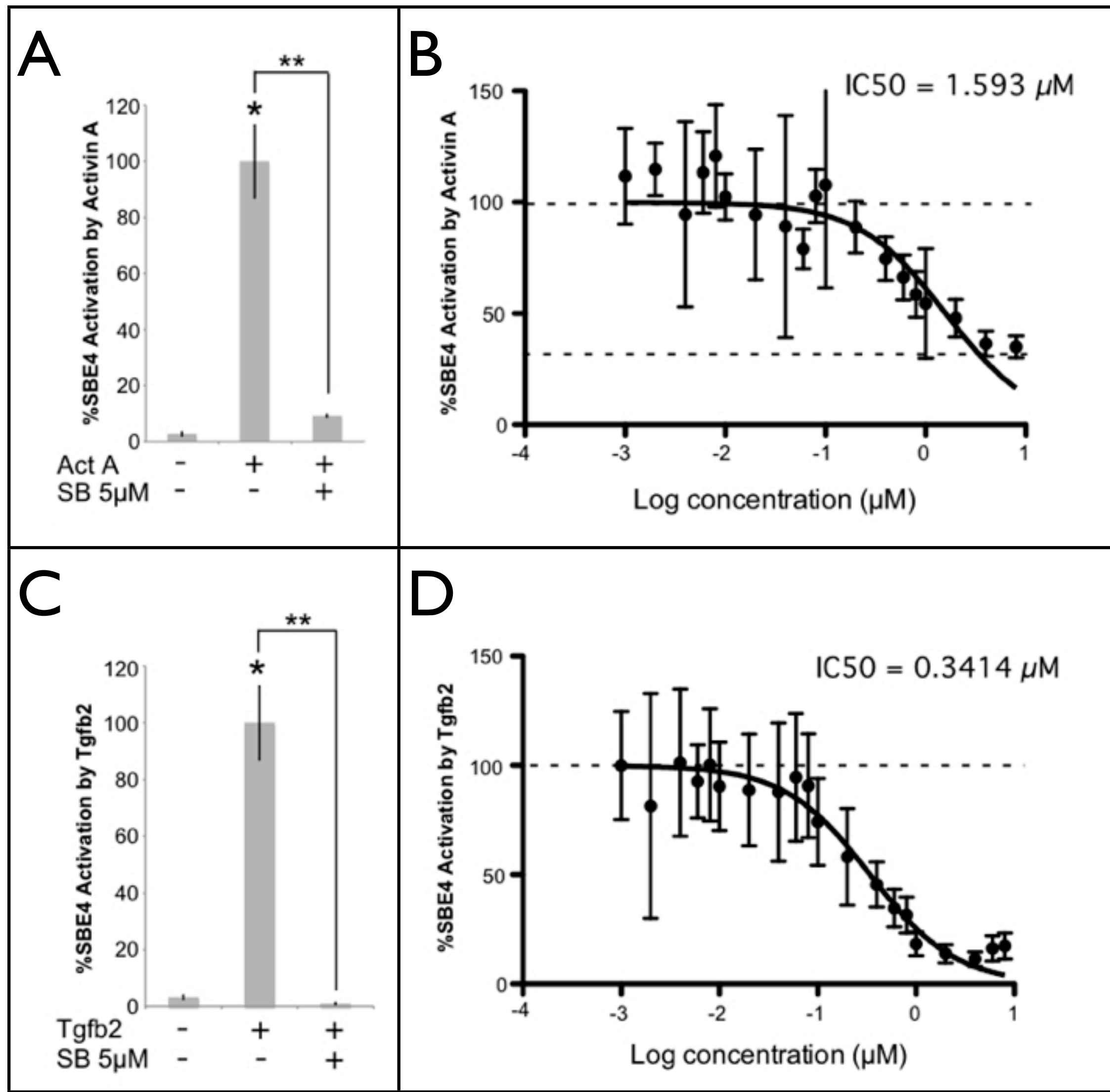


Figure 2.3. DHP blocks SBE4-Lux Induction by Activin A and more Effectively by TGFb-2.

A. Activin A induces luciferase driven by a Smad-4 response element (SBE4-Luc). and can be inhibited by the Activin/TGFb kinase receptor inhibitor SB.

B. Dose response curve generated with increasing concentration of DHP as % (percent) activation of Activin A signaling on the SBE4-Luc reporter, with maximal inhibition reached at about 70% of DMSO treated samples. IC50 value is shown.

C. TGFb-2 induces luciferase driven by SBE4-Luc and can be inhibited by the Activin/TGFb kinase receptor inhibitor SB.

D. Dose response curve generated with increasing concentration of DHP as % activation by TGFb-2 signaling on the SBE4-Luc reporter, with maximal inhibition reached at about 95% of DMSO treated samples. IC50 value is shown.

Error bars indicate s.d. For **[A, C]**, n=3; for **[B, D]**, n=8.

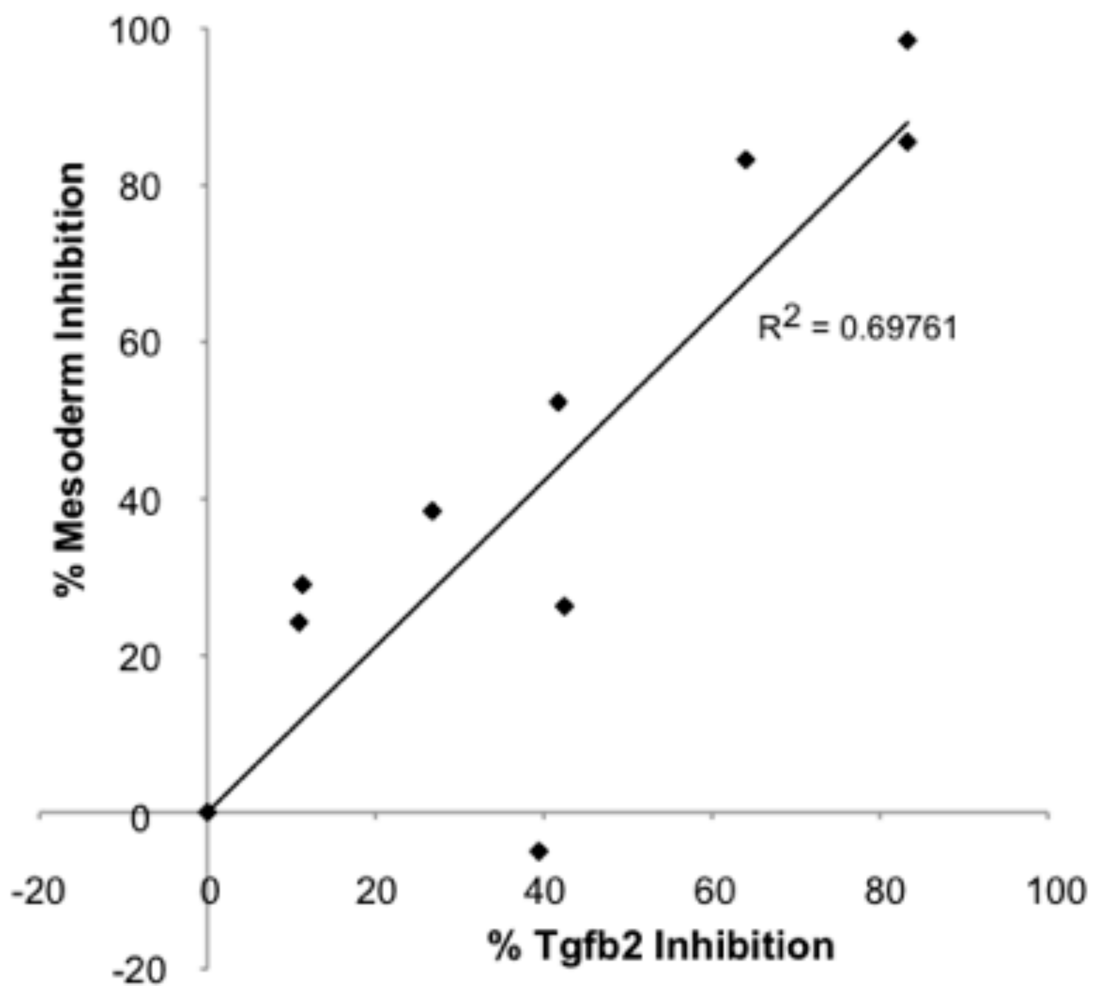


Figure 2.4. Correlation Between DHP Analogs Mesoderm Inhibition in mESCs versus SBE4-Inhibition in the SBE4-Luc assay activated by TGFb-2. Indicated trend line is predicted based on the plotted data points.

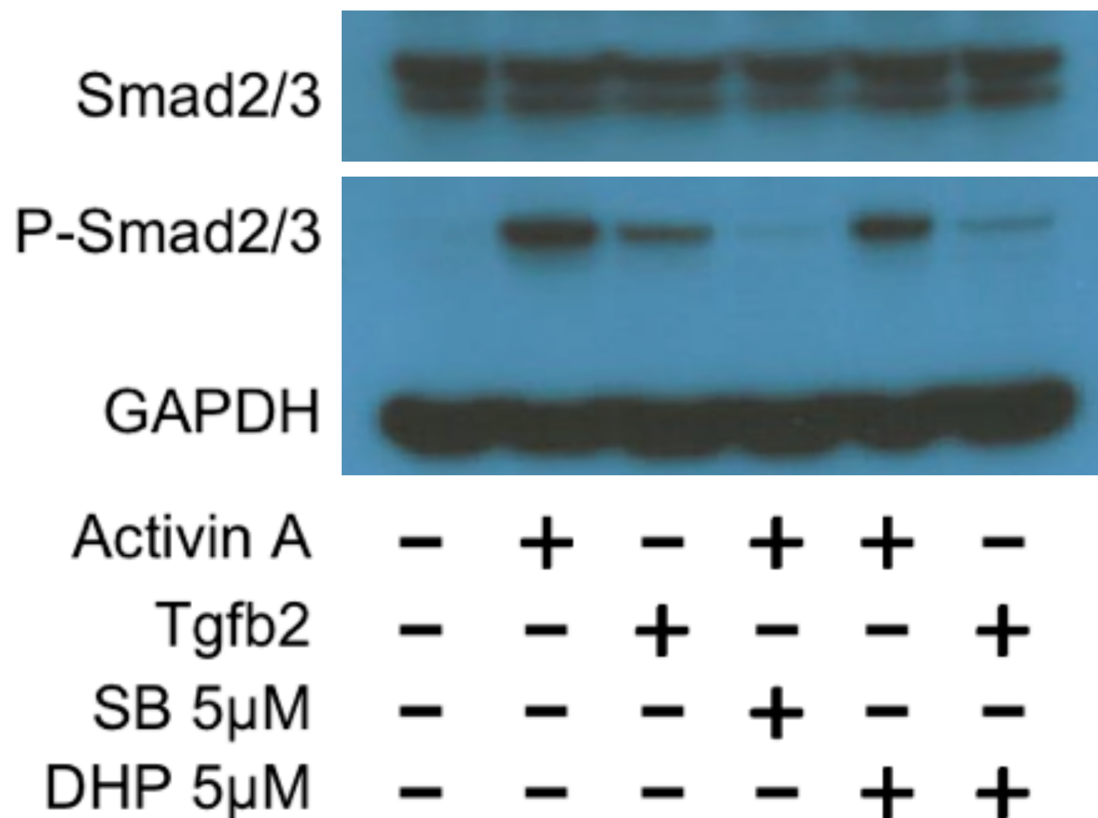


Figure 2.5. DHPs Inhibit Activin/TGFb Signaling Upstream of Smad-2 Phosphorylation. Western blot in 293T cells for phospho-Smad-2, -3 demonstrates reduced phosphorylation of Smad-2, -3 proteins by TGFb-2 but not by Activin A, upon both a 3 hour (not shown) and 18 hour DHP treatment, while total Smad-2, -3 protein compartment is unaffected. Kinase inhibitor SB-431542 illustrates inhibition of phosphorylation (SB).

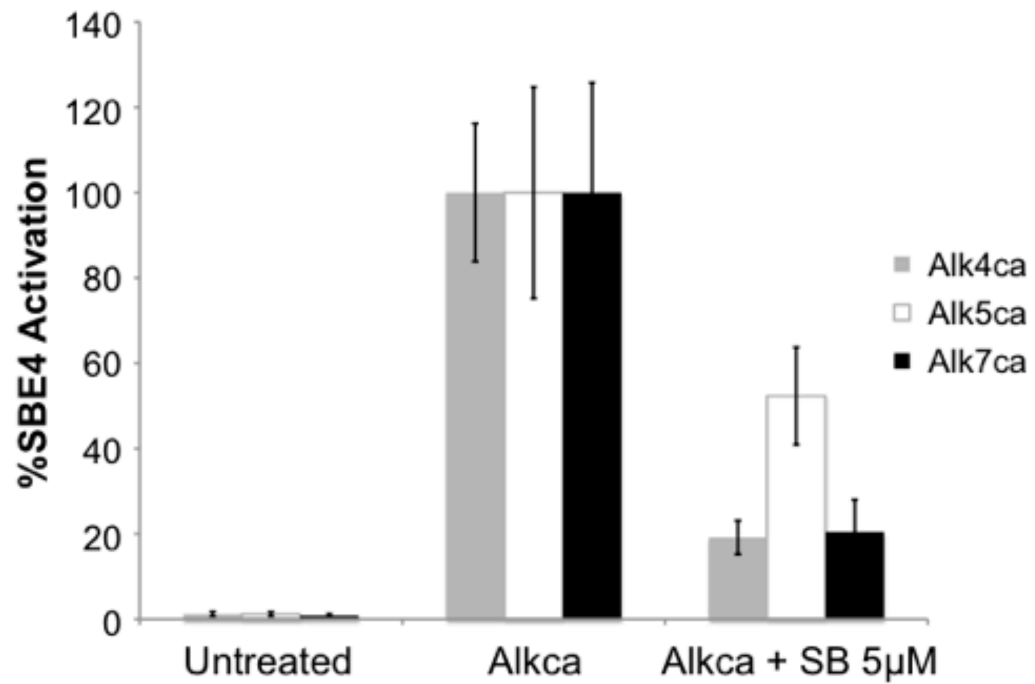


Figure 2.6. **Constitutively Activated (ca) Receptors of the Activin A/TGFb Pathway, Alk4ca, Alk5ca and Alk7ca, All Activate the SBE4-Luc Element.** This effect can be inhibited by SB.

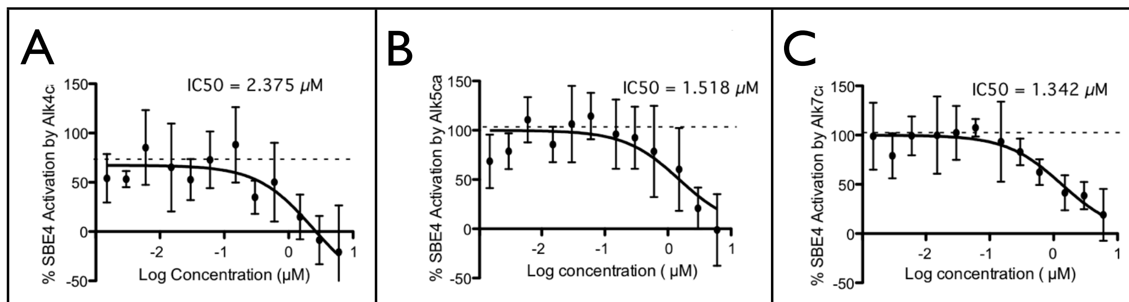


Figure 2.7. Dose Response Curves for DHP Inhibits Activation of the SBE4-Luc Reporter by Alkca. DHP inhibits the percent activation of the SBE4-Lux reporter by Alk4ca **[A]**, Alk5ca **[B]** and Alk7ca **[C]**.

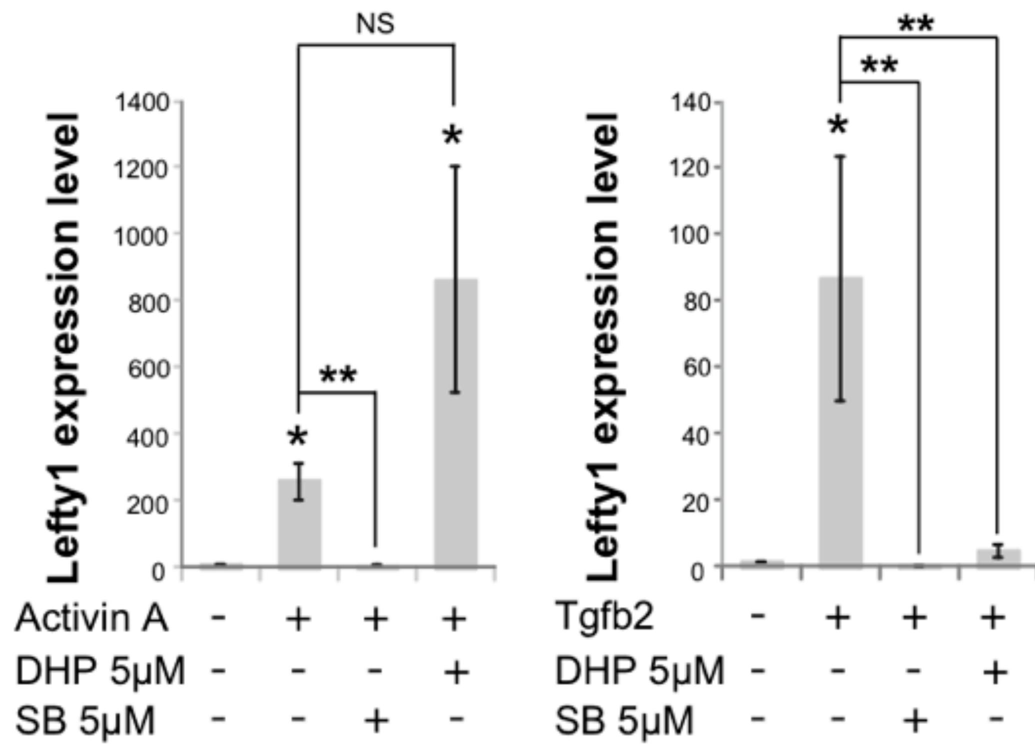


Figure 2.8. DHPs Blocks Activin A Induced *Lefty-1*, But Not Similar Induction by TGFb-2. DHP cannot block Activin A induced *Lefty1* **[A]**, whereas it can block TGFb-2 induced *Lefty1* **[B]** as determined by RT-qPCR analysis of *Lefty-1* in day 3 samples in Cripto-/- ES cells treated with Activin A or TGFb-2. This suggests a difference in DHP specificity when comparing to a typical kinase inhibitor, i.e. SB.

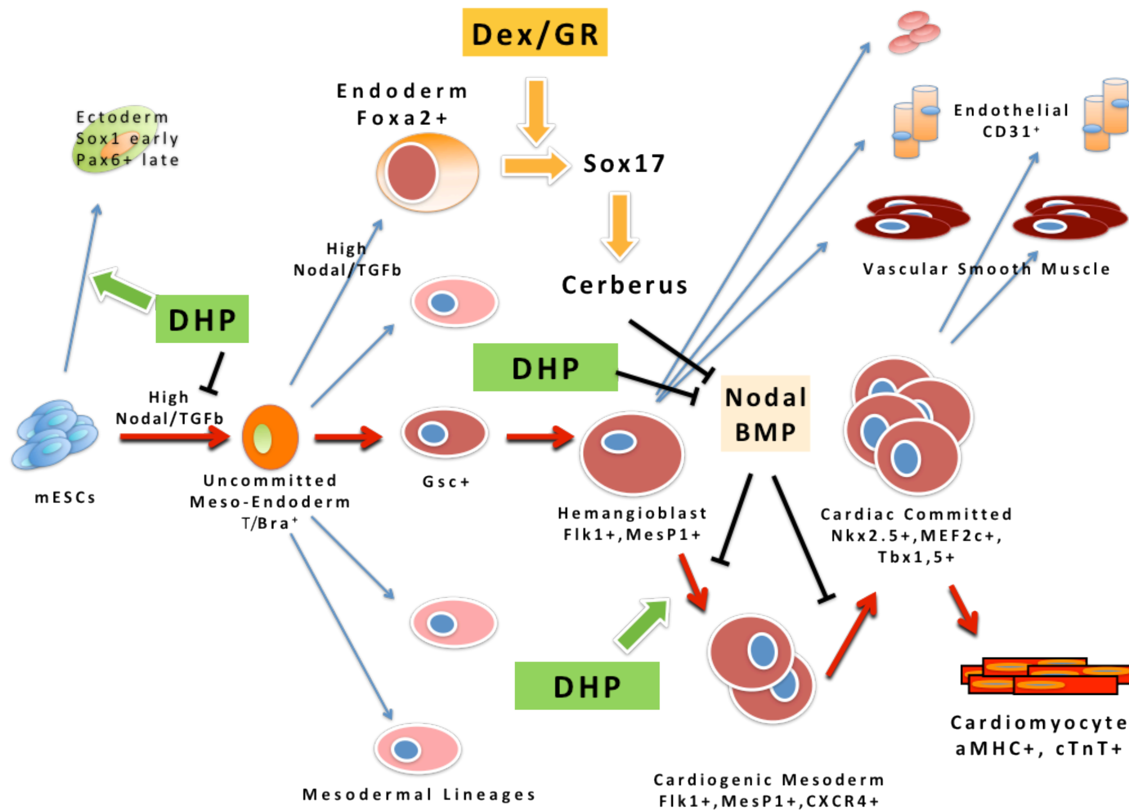
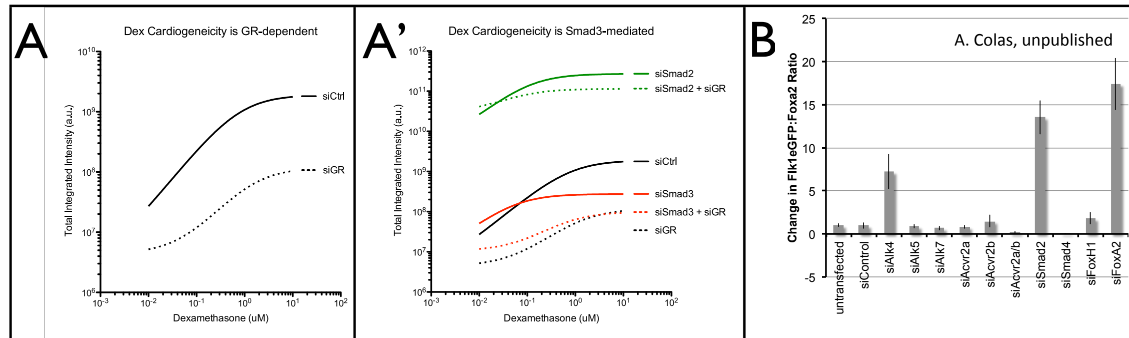


Figure 2.9. Model Combining the Proposed Mechanism of Action of GR and DHP on Cardiogenesis.

- Ligand activated GR seems to upregulate a cascade of events in *Foxa2+* definitive endoderm that begins with *Hnf4a* induction and results in Sox17 -> Cer1 upregulation. Cer1 is a known Nodal inhibitor and has been shown to promote cardiogenesis by locally blocking Nodal in committed cardiac progenitors (*Flk1+*, *MesP1+*) and allowing them to proceed with the cardiac program. Dex does so without affecting meso-endoderm lineage choice.
- DHP seems to alter the balance between mesoderm and neurectoderm when added early to the cultures, in a time when mesoendoderm specification occurs. Later, once the mesoendoderm has been established, it promotes cardiogenesis by inhibiting Nodal/Activin signaling at the receptor level by a mechanism yet to be elucidated - recent data suggests this might be receptor type specific. It appears to mimic the effects of the natural cardiac inducer Cer-1 by blocking Nodal/Activin/TGFb- and BMP-signaling both in cardiac progenitors, resulting in an augmentation of the a cardiac restricted *Flk1+, MesP1+* progenitor population and ultimately of cardiomyocytes.

Supplementary Data



Supplementary Figure 1. Dex Cardiomyogeneicity Through GR Might Involve the Cooperation of Smad3. aMhc-eGFP mESC were differentiated as EBs for 3 days, and reverse transfected with Control-siRNA (siCtrl) or siRNA against GR (siGR). Cells were allowed to adhere to 384-well plates. Treatment with a dose of Dex was performed on day 3.5**;** Dex withdrawn at day 5. On day 10, cells were fixed with 4% PFA, DAPI stained, and imaged by High Throughput Automated Microcopy.

A. Dex induction of cardiomyogenesis [solid black line; (— siCtrl] is significantly reduced if GR mRNA is targeted [dashed black line; (- - siGR)].

A'. siSmad3 [solid red line; (— siSmad3)] attenuates and synergizes with siGR [dashed green line; (- - siSmad3 + siGR)] to attenuate Dex induction of cardiogenesis. siSmad3 contrasts with the known effect of siSmad2 on promoting cardiogenesis by attenuating the Alk4/Smad2/Foxa2 Pathway **[B, C]**, suggesting a different mechanism for Dex/GR and a potential synergy with Smad3 in inducing Hnf4a/Sox17/Cer1.

B. Colas et al., unpublished: knockdown of Smad2, Alk4, and Foxa2 results in an increase in the ratio between Flk1/Foxa2 cells in mESC cultures. This suggests that Dex/GR cardiogenic mechanism of action does not involve Smad2 inhibition.

Data represents fit curves of dose response. Data points considered not diverging significantly from fit model. For A, n=8; B, n=8. Data are representative of two independent experiments.

Supplementary Table 1. RT-qPCR primers

Gene (ACCESSION NO.)	Forward Primer	Reverse Primer
Cerberus-like (NM_009887)	gcagacctatgtgtgga	atgagacatgatcgcttt
Hex (NM_008245.3)	ggaggctgatcttgact	gtagggactgcgtcat
Goosecoid (NM_010351)	accatcttcaccgatgagcagc	cttggctcggcggttcttaaac
FoxA2 (NM_010446)	tggtcactggggacaagggaa	gcaacaacagcaatagacaac
Nodal (NM_013611)	ccagacagaagccaact	aagcatgctcagtggct
Lefty1 (NM_010094)	ctcgatcaaccgcca	ccattccgaacactagc
Lefty2 (NM_177099)	ccaagacacatgtgagga	ccacatacaaagggtgt
Bmp4 (NM_007554)	ttcctggtaaccgaatgctga	cctgaatctcggcgacttttt
αMHC (M76601)	catgccaatgacgacct	cctacactcctgtactgcc
Brachyury /T (NM_009309)	agcttcgtgacggctgacaa	cgagtctgggtggatgtag
Pax6 (NM_013627)	gacctcctcatactcgtg	gtgcttctaaccgcca
Flk1 (NM_010612)	tgccggcatggtcttctg	aaatcaagccccacatt
MesP1 (BC012689)	aatgcaacggatgattgt	agcgtgtaccctattgg
Gata4 (NM_008092)	catcaaatcgcagcct	aagcaagctagagtcct
Mef2c (NM_025282)	agatacccacaacacaccacgcgcc	cattatccttcagagagtcgcatgcgctt
Tbx5 (NM_011537)	ccagctcggcgaagggatgttt	ccgacgccgtgtaccgagtgat
Tbx1 (NM_011532)	agacgaatgttccccac	gcaggttattggtcagtt
Nkx2.5 (NM_008700)	aagtgctctcctgctttcccag	ttgtccagctccactgccttc
Isl1 (NM_021459)	cgtctgatttccctgtgtgttgg	aagtcgttcttgctgaagcctatg
CD31 (NM_001032378.1)	tgcacccatcacttaccacc	cttcatccaccggggctatc
cTnT (NM_011619)	cagaggaggccaacgtagaag	tcgatcagagtctgtagctcatt
VE-Cad (NM_009868)	tgccctcattgtggacaagaa	tggcacagatgcgttgaatac
Sox17 (NM_011441)	gcatccacgaaaccacctat	tccacatctgctggaaggta

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