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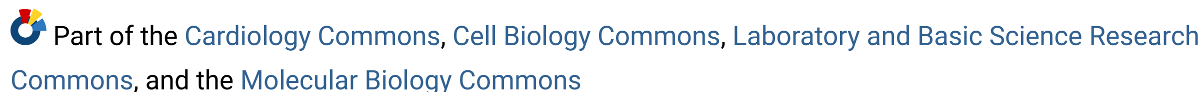
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THE ROLE OF THE ERBB SIGNALING PATHWAY IN

CARDIOVASCULAR PROGENITOR CELL-BASED REPAIR

A Thesis

Presented to the

Faculty of

California State University,

San Bernardino

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

In

Biology

---

by

Christopher Ramos

December 2021

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Christopher Ramos

December 2021

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ABSTRACT

Adult mammalian hearts lack self-renewal and proliferative capabilities necessary for cardiovascular regeneration. Current treatments using cardiac progenitor cells (CPCs) for cell-based repair do not restore cardiac function in patients who experience a myocardial infarction (MI). Our laboratory has been studying Islet-1+ neonatal CPCs as a promising candidate for cell-based repair due to their ability to significantly improve cardiac function after MI in sheep. The current study addresses the hypothesis that the ERBB pathway is linked to the Hippo-pathway to activate YAP1 by the involvement of an autocrine loop that upregulates neuregulin (NRG). In our sheep model of MI and cardiovascular progenitor cell-based repair, tissue sections from the infarct zone of three neonatal Islet-1+ CPC treated sheep and corresponding control cardiac tissue sections were used to extract RNA, prepare cDNA and perform RT-qPCR to quantify transcripts associated with the NRG-ERBB signaling pathway. Induced levels of NRG1 and NRG2 were identified in the cardiovascular repair zone of Islet-1+ CPC treated infarcted sheep. ERBB receptors 1-4 and PIK3C2B levels were induced following cell-based treatment. In addition, RT-qPCR and western blot experiments were done on human neonatal Islet-1+ CPCs treated with and without Wnt5a to investigate transcripts associated with the ERBB pathway and YAP1 protein expression. We showed an increase in the expression of NRG1,

ERBB3, PIK3CD, and YAP1 after 72 hrs of Wnt5a treatment along with overexpression of YAP1 protein at 144 hrs following Wnt5a treatment. Our studies provide new insight into the mechanism by which ERBB signaling contributes to Islet-1+ cell-based repair.

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CHAPTER ONE

INTRODUCTION

Background

Stem Cell Populations Used as Therapeutic Treatment for Myocardial Infarction

Myocardial Infarction (MI) can result in the formation of scar tissue leading to cellular injury or death. Left ventricular dilatation is a precursor of ventricular dysfunction and congestive heart failure (CHF) after MI (Yeboah, 2014). Left ventricle damage after MI undergoes progressive remodeling and the heart wall muscle becomes permanently impaired. Adult hearts are incapable of regenerating lost cardiac cells and cannot repair scar tissue. With this in mind, stem cell- based therapies are becoming important treatments in the area of heart disease. There are many stem cell candidates that are being tested in cardiac regenerative therapy. Bone marrow mononuclear cells (BMNCs) were the first hematopoietic cells to have been used in cardiac therapy (reviewed in Behfar, 2014). In some cardiac repair clinical trials, BMNCs contributed to angiogenesis, neovascularization, improved cardiac function, and increase in left ventricular ejection fraction (LVEF) (Ashara, 1999; Fujii, 2004; Liu, 2014). However, other studies reported that intracoronary infusion of BMNCs did not promote functional myocardium (de Jong, 2014; Lunde 2006). Other types of stem cells were then considered as candidates for cardiac repair.

Mesenchymal stem cells (MSCs) are derived from bone marrow stem cells that have plastic adherence characteristics and are capable of expressing CD105, CD73, and CD90 but not CD34, CD45, CD14 or CD11b, CD79α or CD19, and HLA-DR (Dominici, 2006). *In vitro*, MSCs have the potential to form adipocytes, chondrocytes, and osteoblasts (Dominici, 2006). MSCs are, however, beneficial for cardiac repair due to their release of cytoprotective molecules (reviewed in Genecchi, 2008). These molecules contain cardioprotective effects and stimulants that activate repair in the MI heart (Hatzistergos, 2010). In clinical trials, therapeutic efficiency of MSCs, when transplanted along with autologous or allogeneic cell sources showed promising signs of reduced scar mass and improved cardiac function in heart failure patients (Hare, 2012). MSCs have benefits, but they do have some disadvantages in the therapeutic setting, including the inability to differentiate into functional cardiomyocytes (CMs) (Rose, 2008).

C-kit+ cells represent a population of cells that was the focus of attention in the cardiac repair field for many years (Gude, 2018). Recently, however, the capability of some of these c-kit+ cells to differentiate into CMs *in vivo* has become controversial. The use of c-kit+ cells to repair the adult mouse heart after MI was based on an early study. Mice with infarcted hearts that were treated with cells isolated from sorted, bone marrow derived lineage Lin− and c-kit+ cells that were tagged with enhanced green fluorescent protein (eGFP) showed myocardium regeneration that consisted of CMs and vasculature, which

encompassed 68% of the infarcted region after transplantation (Orlic, 2001). However, other research labs failed to reproduce differentiation of these cells into CMs in adult mice (Jesty, 2012; Murry, 2004). In a phase 1 clinical trial called SCIPIO, c-kit+ cells were reported to promote cardiac regeneration (reviewed in Mauretti, 2017), but the study was later retracted due to issues with the data. Next, cardiospheres have also been studied in models of cardiovascular repair. Cardiospheres contain mixed cell populations; cardiac stem cells (c-kit+, etc.), endothelial precursors, and mesenchymal cells (Messina, 2004). Cardiosphere-derived cells (CDCs) have been used *in vivo* in animal models (Chimenti, 2010; Johnston, 2009) and in human clinical trials (Makkar, 2012; Makkar, 2020). In MI animal models, CDC administration reduced infarct size, improved LVEF, and improved cardiac hemodynamics *in vivo* (Chimenti, 2010; Johnston, 2009). In human studies, cardiosphere-derived autologous stem cells were shown to be safe, but exhibited no reduction of scar size in the infarct zone (Makkar, 2020).

Due to the need to identify a potentially beneficial cell type with the ability to differentiate into functional CMs, induced pluripotent stem cells (iPSCs) gained increasing attention. iPSCs can differentiate into CMs similar to embryonic stem cell (ESC) derived CMs (Pfannkuche, 2009). In MI mice, iPSC injection resulted in differentiation of CMs, improved cardiac function and increased ventricular wall thickness (Nelson, 2009). Other studies show that after iPSC transplantation, there was no evidence of increased T-cell proliferation or antigen specific secondary immune response, hence no risk of immunorejection of autologous

iPSCs (Guha, 2013). There are some risks associated with iPSCs, however. Teratoma formation can occur when the genetic material in iPSCs randomly integrates in the host genome (Li, 2011).Various sources of iPSCs can contribute to differences in genotype, phenotype, and the epigenome (reviewed in Liang, 2013). The use of iPSCs can be complex due to variable factors. Small molecules may be needed to enhance iPSC production without the use of viral vectors for gene modification (Li, 2011). Using iPSCs in cardiac therapy can lead to variable results in engraftment rate and risk of arrhythmia (reviewed in Hashimoto, 2018). Despite these issues, generation of mature iPSC- derived CMs has been improved and the cells can be produced with greater purity (Hashimoto, 2018). Hydrogels and cell sheets used as scaffolds for iPSC-derived CMs have been used in the therapeutic setting for treating diseases (Chow, 2017). Overall, these cells remain a promising approach for heart repair after injury.

Islet-1+ Cardiac Progenitor Cells

Islet-1 is a transcription factor that plays a role in regulating heart development during embryogenesis. This marker is expressed in multipotent cardiac progenitor cells (CPCs) that can differentiate into all three cardiac cell types (cardiomyocytes, smooth muscle cells, and endothelial cells) (Moretti, 2006). Islet-1+ is necessary for heart development and is the specific marker for development of the SHF, which generates cells to form the large portions of the right ventricle, outflow tract, and atria (Cai, 2003). Interestingly, Islet-1+ protein

was found to be expressed in the7.5 day old mouse throughout the anterior intra-embryonic coelomic walls and proximal head mesenchyme, regions found in the FHF and SHF (reviewed in Laugwitz, 2008; Prall, 2007). Studies have determined Islet-1+ as a marker of CPCs (Bartulos, 2016; Domian, 2009**).** In rodents, Islet-1+ CPCs administered to infarcted hearts showed improvement of left ventricular function, enhanced vascularization, reduced myocardial dysfunction, and enhanced CM proliferation (Bartulos, 2016; Barzelay, 2012). Islet-1+ CPCs are the main focus of research in our lab because they are self-renewing, clonogenic, and multipotent (Baio, 2017). We have shown that Islet-1+ neonatal CPC transplantation in MI sheep improves overall cardiac function (Hasaniya, 2018) and that Islet-1+ CPCs maintained on a hyper-cross linked polymer scaffold promote proliferation and differentiation (Baio, 2017). Other studies showed improvement of cardiac function in MI mice after Islet-1+ CPC transplantation in SIS-ECM and gelatin microsphere scaffolds (Feyen, 2016; Wang, 2017). Islet-1+ human embryonic stem cells (hESC) promote an increase in CM differentiation rather than stabilizing the cardiac precursor cell state (Quaranta, 2018). Our lab demonstrated proliferation and differentiation of Islet-1+ CPCs *in vivo* into CMs and endothelial cells at the site of repair following MI in a sheep model (Hasaniya, 2018). The mechanistic basis for efficient cardiovascular regeneration in this model is currently under investigation. Several signaling pathways appear to be activated following Islet-1+ cell transplantation for cardiac repair, including Hippo-YAP1.

Hippo- YAP1 Role in Cardiac Regeneration

YAP1 is an important regulator in promoting cell proliferation, survival, and growth during cardiac development and repair (Gumbiner, 2014). Activation of the Hippo signaling pathway stimulates MST1/2 kinases along with the binding of SAV1 and PDK1. This complex promotes activation of LATS1/2 kinases, which results in phosphorylation of YAP1 (Fig. 1). As a result, phosphorylated YAP1 is prevented from entering the nucleus, which leads to apoptosis (Gumbiner, 2014). On the flip side, inactivated Hippo pathway promotes accumulation of dephosphorylated intranuclear YAP1, which allows it to interact with other transcription factors (TEAD1-4) that regulate cell growth, survival, and proliferation (Fig. 1) (Gumbiner, 2014). Studies in mice have shown that deletion of the upstream kinases MST1/2 and LATS1/2 induces CM proliferation during mouse embryogenesis (Heallen, 2011; Heallen, 2013). In MI mice, cardiac deletion of YAP1 significantly impairs cardiac regeneration, but overexpression of a constitutively active form of YAP1 reduces scar size, increases CM proliferation and improves cardiac function (Xin, 2013). In our lab, we showed increased expression of YAP1 in a sheep model of myocardial infarction following transplantation of neonatal sheep Islet-1+ CPCs when compared to controls (Camberos, 2019). In addition, high levels of YAP1 are expressed in human neonatal Islet-1+ CPCs, but declines to insignificant levels inadultIslet-1+ CPCs (Camberos, 2019). This is a contributing factor in human neonatal Islet-1+ CPC proliferation and efficacy for repair. Overall, YAP1 plays a vital role in protecting

cells from apoptosis and is sufficient for induction of cardiac regeneration after heart injury (Xin, 2013).

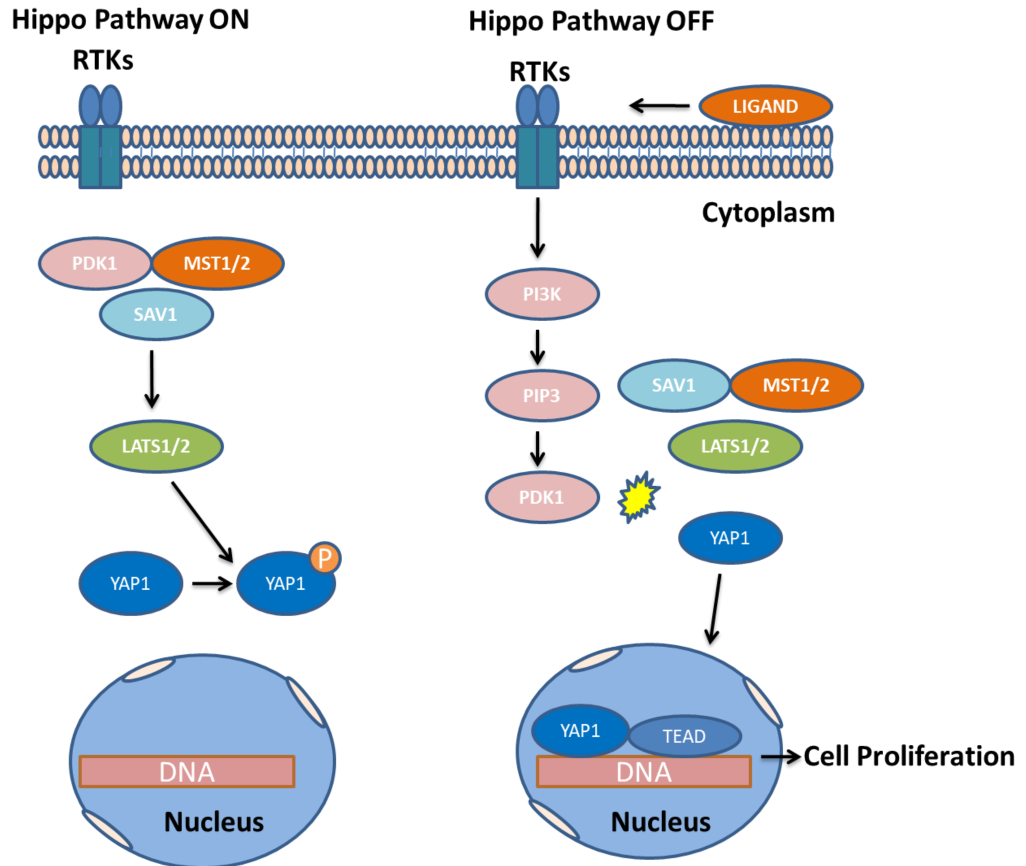

Figure 1: Hippo-YAP1 Signaling Pathway
YAP1 is phosphorylated by LATS1/2, which prevents YAP1 from entering the nucleus when the Hippo pathway becomes active (on). Ligand binding to receptor tyrosine kinase (RTK) inactivates (off) the Hippo pathway, thereby promoting dephosphorylation of YAP1 to enter the nucleus and interact with transcription factors (TEAD 1-4) to regulate signals that promote cell proliferation.

ERBB Signaling Pathway

It is now understood that signaling pathways stimulating cardiovascular repair activate proliferation and dedifferentiation (reviewed in Xin, 2013). In humans, CM regeneration also involves the signaling pathways of ERBB family proteins. This family contains four receptor tyrosine kinases (RTKs), which include ERBB1, ERBB2, ERBB3, and ERBB4. These receptors interact with neuregulins (NRGs), which are epidermal growth factor ligands. NRGs are homologous proteins that are encoded by genes NRG1, NRG2, NRG3, and NRG4. In mammalian cells, NRGs bind and activate ERBBs, which triggers a network of signaling pathways that promotes proliferation, dedifferentiation, cell migration, and survival (reviewed in Xu, 2010). More specifically, NRG1 activates ERBB3 and ERBB4 both of which form heterodimers with the ERBB2 receptor to further activate multiple signaling pathways (reviewed in Citri, 2006). ERBB2 lacks binding sites for growth factor ligands (including NRGs), so ERBB2 requires heterodimerization with other ERBB family receptors to function (Citri, 2006). ERBB3 activates the PI3K pathway by interaction with ERBB2 (Song, 2015). PI3K activity initiates signaling cascades that impact AKT (reviewed in Jean, 2014). AKT controls multiple pathways (including activation of FOXO, mTORC1, and YAP1) to regulate cell proliferation, growth, metabolism, survival, and autophagy (reviewed in Vanhaesebroeck, 2012). ERBB2 is important in reactivating proliferation in the heart and dedifferentiation in postnatal mice after

MI trauma (D’Uva, 2015) and triggers epithelial mesenchymal transition (EMT) during regeneration of cardiac tissue (Aharonov, 2020).

NRG-1 has been evaluated as a therapeutic option in treating acute/chronic heart disease in pre-clinical rodent models. Recombinant human NRG-1 (hrNRG-1) was used to pretreat rodents prior to MI by left anterior descending coronary artery ligation (LAD) and this treatment reduced ventricular arrhythmia (VA) and improved overall cardiac function in the heart (Gu, 2010; Rao, 2019). In addition, hrNRG-1 has been administered intravenously in chronic heart failure (CHF) in patients with LVEF < 40 % in a phase II, randomized, double-blind trial (Gao, 2010). In this trial, rhNRG-1 treatment improved LVEF by 31.99 % when compared to a 15.05% increase in patients treated with the placebo for three months (Gao, 2010). Short term treatment with rhNRG-1 was shown to have long term effects in reversing adverse cardiac function (Gao, 2010). Currently, there are hrNRG-1 clinical trials that are actively recruiting patients with CHF to better understand the underlying mechanisms by which damaged CMs can be repaired and function restored (reviewed in De Keulenaer, 2019). Surprisingly, there is limited literature addressing NRG2 and whether or not it can impact cardiac regeneration through interactions with ERBB receptors. NRG2 mRNA has been found in the endothelial lining of the heart in adult rats (Carraway Iii, 1997). The highest NRG2 levels were found in the atrium, the lower levels of the ventricle, and in the outflow tract (Carraway Iii, 1997). NRG2 interacts with ERBB3 and ERBB4, similar to NRG1. The mechanistic basis by

which Neuregulins, ERBB and YAP1 signaling pathway interact to facilitate cardiac repair have not been elucidated.

In summary, transplantation of Islet-1+ CPCs in MI sheep has been shown to stimulate CM proliferation and improve overall cardiac function but the signaling pathways that play a role in this process have not been fully elucidated. Understanding the molecular events that lead to cardiac repair following neonatal cardiovascular stem cell transplantation may lead to further improvements in cell-based cardiac therapy. Specifically, the Hippo-Yap1 pathway plays a key role in cardiac repair but the regulatory pathways that activate YAP1 are still under investigation. ERBB activation by NRG relays signals downstream to the PI3K pathway and this signaling may subsequently activate the Hippo-Yap1 pathway to promote cell survival, differentiation, and proliferation of CMs. The current study will address the hypothesis that the ERBB pathway activates YAP1 by the involvement of an autocrine loop that upregulates NRG (Fig. 2). This concept will be studied in a sheep model of myocardial infarction and cardiovascular progenitor cell-based repair and investigated in human neonatal Islet-1+ CPCs *in vitro*.

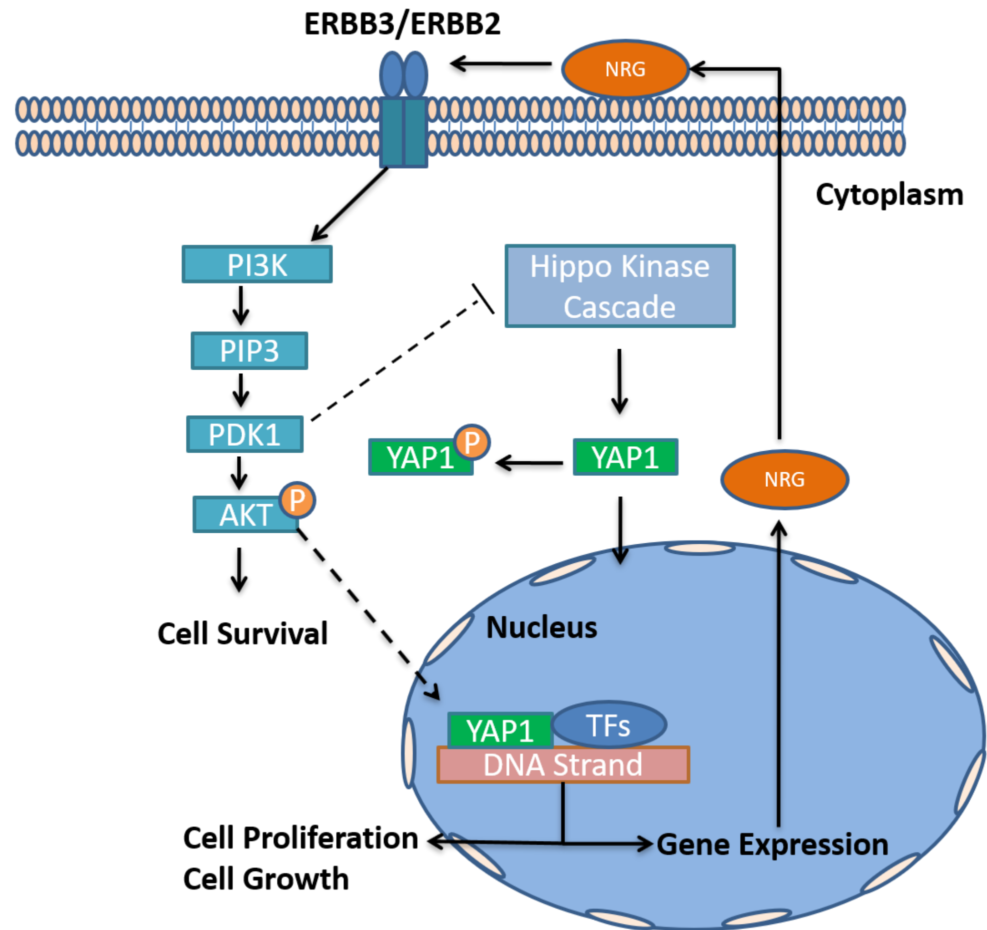

Figure 2: ERBB/ YAP1/ NRG Signaling Forms an Autocrine Loop
This diagram shows NRG binding to the ERBB3/ERBB2 receptor tyrosine kinases (RTKs), which stimulates downstream signaling to the PI3K-AKT pathway to promote cell survival. In addition, activation of PDK1 indirectly inhibits the Hippo Kinase Cascade (Fan, 2013), which activates YAP1 by dephosphorylation, allowing YAP1 to pass through the nucleus to interact with transcription factors (TFs) on the DNA strand (Gumbiner, 2014). YAP1 stimulates gene expression to induce cell growth and proliferation with indirect positive regulation from AKT (Fan, 2013). The autocrine loop interaction may occur when the expression of intranuclear YAP1 is elevated, resulting in the upregulation of NRG downstream of YAP1. NRG is transported through the cell membrane to rebind with ERBB3/ERBB2 receptors on the extracellular surface.

Definition of Terms

CHF: Congestive heart failure. MI: Myocardial infarction. CPCs: Cardiovascular progenitor cells. CSCs: Cardiac stem cells. iPSCs: Induced pluripotent stem cells. MSCs: Human mesenchymal stem cells. CDCs: Cardiosphere derived cells. BMNCs: Bone marrow mononuclear cells. HSCs: Hematopoietic stem cells. HESCs: Human embryonic stem cells. EPDCs: Epicardial-derived cells. HUVECS: Human umbilical vein endothelial cells. NRG: Neuregulin. CMs: Cardiomyocytes. LECs: Lymphatic endothelial cells. EF: Ejection fraction. LVEF: Left ventricular ejection fraction. EMT: Epithelial mesenchymal transition. ECM: Extra-cellular matrix. RTKs: Receptor tyrosine kinases. RT-qPCR: Reverse transcription quantitative PCR. GPCRs: GTP-binding protein-coupled receptors. SEM: Standard Error of the Mean.

CHAPTER TWO

MATERIALS AND METHODS

Sheep Model of Myocardial Infarction

Sheep (<1 year old) were previously infarcted via left anterior descending coronary artery ligation. After three to four weeks following infarction, sheep were injected with 10 million sheep neonatal cardiac progenitor cells. The sheep were euthanized after two months of cell injection and their hearts were collected. Sheep hearts were mapped and sections were taken according to their proximity to the infarct site. Tissue samples from neonatal sheep (n=5) were flash frozen for experimental use. The animal studies were approved by the Institutional Animal Care and Use Committee of Loma Linda University and performed within the regulations of the Animal Welfare Act. Tissue samples from normal sheep, non-infarcted sheep, infarct-only sheep and sheep which were previously infarcted and treated with Islet-1+ cells for cardiac repair were used for RNA isolation and purification.

Islet-1+ Cardiac Progenitor Cell Isolation and Clonogenic Culture

Human and Sheep cardiac progenitor cell clones have been previously isolated as described by Fuentes and Hou, respectively (Fuentes, 2013; Hou, 2012). Human cardiac progenitor cell clones were cultured and grown on 0.1% gelatin coated 6 well plate in medium 199 (Life Technologies, Carlsbad, CA)

containing 10% fetal bovine serum (Thermo Scientific, Waltham, MA), 100 µg/mL penicillin–streptomycin (Life Technologies, Carlsbad, CA), and 1% minimum essential medium non-essential amino acids (Life Technologies, Carlsbad, CA). The Institutional Review Board at Loma Linda University approved the protocol for using discarded human cardiovascular tissue obtained after cardiac surgery without the use of identifiable patient information, following a waiver of informed consent.

Treatment of Human Neonatal Islet-1+ Cardiac Progenitor Cells with Wnt5a

Wnt5a treatment has been shown to induce intranuclear YAP1 expression (Park, 2015). High levels of YAP1 in the nucleus may result in an increased expression of NRG, which binds and activates ERBB signaling pathway in an autocrine effect. In order to demonstrate this, human neonatal Islet-1+ CPC clones were grown to 60-70% confluency and were treated with 100 ng/ml of recombinant human/mouse Wnt5a (R&D Systems, Minneapolis, MN) in CPC growth media as previously described by (Baio,2018). Islet-1+ CPCs were cultured with Wnt5a for 48 hrs, 72 hrs, and 96 hrs time periods in order to induce YAP1 expression. Untreated CPCs were grown in normal CPC growth media for use as a control. At each corresponding time period of Wnt5a treatment, treated and control Islet-1+ CPCs were washed with phosphate buffered saline followed by TRIzol for RNA extraction and purification. In addition, protein was isolated

from treated and untreated Islet-1+ CPCs for western blot analysis at the 96 and 144 hour timepoints.

RNA Purification and RT-qPCR

Purification of total RNA from sheep and Wnt5a treated human neonatal Islet-1+CPC samples was performed by using an RNeasy mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The integrity of the RNA was validated using gel electrophoresis prior to preparing cDNA for each sample. 2 μg of RNA was used to prepare cDNA using Superscript III (Invitrogen, Carlsbad, CA). RT-qPCR was performed with a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). PCR plates were run under the following conditions: 94 °C for 10 min, 94 °C for 15 s, 58 °C for 1 min, 72 °C for 30 s, repeated for 40 cycles. The primers for our genes of interest were developed by NCBI Primer-BLAST (Table 1). RT-qPCR products were visualized using 1%-2% agarose gel electrophoresis and low mass DNA ladder (Invitrogen, Carlsbad, CA).

Western Blot and Protein Purification of Human Neonatal Islet-1+ Cardiac Progenitor Cells after Wnt5a Treatment

Human neonatal Islet-1+ CPCs treated with Wnt5a at 96 hr and 144 hr time periods were each washed with cold phosphate buffer saline and treated with cold trypsin. Trypsinized cells were placed on ice at room temperature until all cells rose. These two samples (96hr and 144hr) were each collected and agitated for 1 hr at 4 °C in protein lysis buffer consisting of RIPA buffer, 0.5M

EDTA, protease inhibitor cocktail, sodium orthovanadate, and sodium fluoride, before being centrifuged at 14,000× g, and aliquoted for use. Protein concentrations at each Wnt5a treated time point were quantified using the Micro BCA Protein Assay Kit (ThermoFisher, Waltham, MA, USA). Protein Simple Wes (Protein Simple, San Jose, CA, USA) was used to analyze protein levels according to the manufacturer's protocol. Antibodies used in western blot (Table 2) were provided by the manufacturer (Cell Signaling, Danvers, MA, USA).

Statistical Analysis

Data was analyzed using PRISM and was reported as mean +/- standard error. P values <.05 were deemed significant. Triplicate qPCR replicates were used for RT-qPCR experiments. GAPDH and actin were used as normalizing genes in these experiments. Data was calculated using the $\Delta\Delta$ CT method (Livak, 2001)

Table 1: Primer Pairs Used in RT-qPCR

Human Genes	Forward Sequence	Reverse Sequence
NRG1	GAA AAG AAA TCC TGT GTG TCG CT	TGG CAG GTG ATC ACT GGG ATA
NRG2	CGA GAA GAG CCA CTC GAC AG	ACA TTT CAG GAC AGG ATT CCC A
ERBB1	ACT TCT ACC GTG CCC TGA TG	TGT CTG CAA ATC TGC CAC TGT
ERBB2	CAT TGG GAC CGG AGA AAC CAG	GAC CTG CCT CAC TTG GTT GT

ERBB3	TGA GAT TGT GCT CAC GGG AC	ATC TCG GTC CCT CAC GAT GT
ERBB4	TTC AGG ATG TGG ACG TTG CC	GGG CAA ATG TCA GTG CAA GG
PIK3C2B	GGC CAT CAC TTC CCT GAG AAA	GCT AAG GCT TCC TTC AGC CA
PIK3CD	CTG CGC CGG GAC GAT	GGA AGA GCG GCT CAT GG
YAP1	TCC CAG ATG AAC GTC ACA GC	TCA TGG CAA AAC GAG GGT CA
Sheep Genes		
NRG1	TGG TGA TCG CTG CCA AAA CT	CAG CTG TGA CTG GGA GTC TG
NRG2	GGT AAC CCT CTG CCC TCC TA	CAT TGC GAA CTG CTG ACA CC
ERBB1	GAC TTT ACT GGG GCC TGA CC	ACG TGT TAC CTG GAA GGC TG
ERBB2	AGA TCC TCA AGG GAG GGG TC	GAA GGT ATA ACG CCC CTC GG
ERRB3	AGT GCC TAT CTT GCC GGA AC	CTT GTA GAT GGG GCC CTT GG
ERBB4	AGT CAC AGG CTA CGT GTT GG	CAG GTT GGA AGG CCA TGG AT
PIK3C2B	CGC AGG TGC CCA GAC A	GTA GAG TGG TTG GAC AGC CC
PIK3CD	AAC CCC AAC ACT GAG AGT GC	CTC GGA GGT TAG TTT CCG CA

Table 2: Antibodies Used for Western Blot:

Antibody	Manufacturer	Sample Used (mg/µl)	Antibody Dilution	Species	Size (kDa)	Catalog No.	Lot No.
YAP1	Cell Signaling	0.4	1:200	Rabbit	65-75	14074S	2
Phosphorylated YAP	Cell Signaling	0.4	1:200	Rabbit	65-75	13008T	5
Actin	Cell Signaling	0.4	1:50	Mouse	45	3700S	14

CHAPTER THREE

RESULTS

Islet-1+ Progenitor Cell-Treated Sheep

NRG1 and NRG2 Transcripts are Induced in Islet-1+ Progenitor Cell-Treated Sheep

In order to assess the role of ERBB signaling in cardiovascular repair, RNA was isolated from sheep tissues preserved from the cardiovascular infarct zone. cDNA was synthesized and RT-qPCR was performed to assess levels of NRGs that may be induced after myocardial injury alone and during cardiovascular regeneration after neonatal sheep Islet-1+ CPC treatment. Myocardial infarcted sheep treated with neonatal sheep Islet-1+ CPCs in the infarct zone and normal control age- matched sheep were compared. The normal control aged-matched sheep is a healthy sheep with no infarction and without cell treatment. NRG1 and NRG2 gene expression analysis by RT-qPCR demonstrated that both NRG1 and NRG2 gene levels were significantly elevated in the cardiovascular repair zone of Islet-1+ CPC treated infarct sheep when compared to normal control aged- matched sheep (Fig. 3A, 61.9 ± 3.64 fold change, **** $p<.0001$. Fig. 3B, 24.1 ± 1.9 fold change, *** $p=.0003$, respectively). NRG1 was more highly expressed in Islet-1+ cell treated sheep (Fig. 3A) when compared to NRG2 in Islet-1+ cell treated sheep (Fig. 3B). In

addition, NRG1 and NRG2 gene expression levels were both elevated in the cardiovascular repair zone of Islet-1+ cell treated sheep when compared to the non-infarct region in Islet-1+ cell treated infarcted sheep by RT-qPCR analysis (Fig. 3C, 3.7 ± 0.96-fold change, ns. P=0.1028. Fig. 3D, 5.4± 0.18-fold change, **P=0.0017). Next, NRG1 and NRG2 gene expression in the infarct region of myocardial infarcted sheep with no cell treatment was compared to normal control aged-matched sheep by RT-qPCR. NRG1 and NRG2 gene transcripts were both downregulated in infarct only sheep when compared to normal control age-matched sheep (Fig. 4A, .43 ± .01-fold change, ns. Fig. 4B, .125 ± .03-fold change, ****P<.0001, respectively).

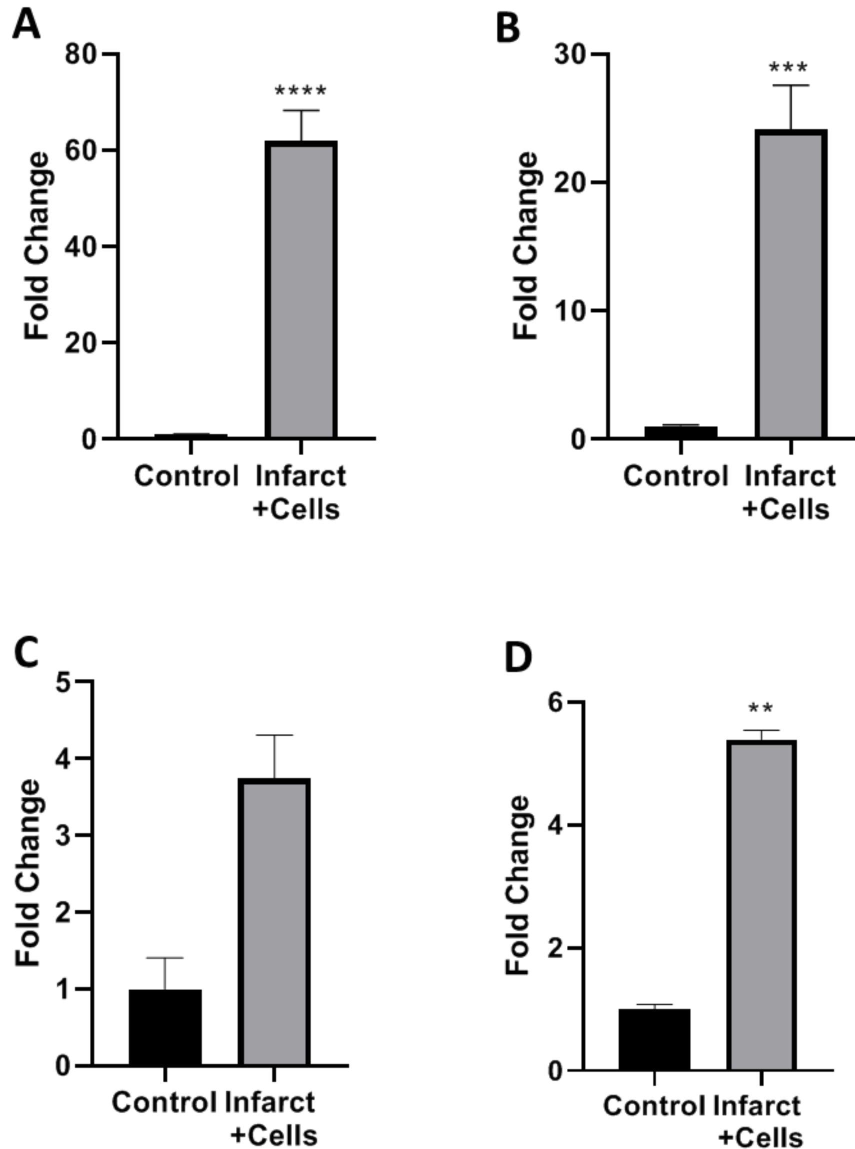

Figure 3: NRG1 and NRG2 Transcripts are Elevated in Infarcted, Islet-1+ Cell Treated Sheep
NRG1 (A) and NRG2 (B) gene transcripts were elevated in Islet-1+ cell treated infarcted sheep (Infarct + Cells) when compared to normal control age-matched sheep (Control) as shown by RT-qPCR. NRG1 (C) and NRG2 (D) gene transcripts were also elevated in Islet-1+ cell-treated infarcted sheep (Infarct + Cells) when compared to the non-infarct region in Islet-1+ cell treated infarcted sheep (Control) as shown by RT-qPCR. Fold changes are shown as the mean ±

SEM (standard error of the mean). All samples were run in triplicates and were normalized to GAPDH. Data was calculated using ΔΔ CT.

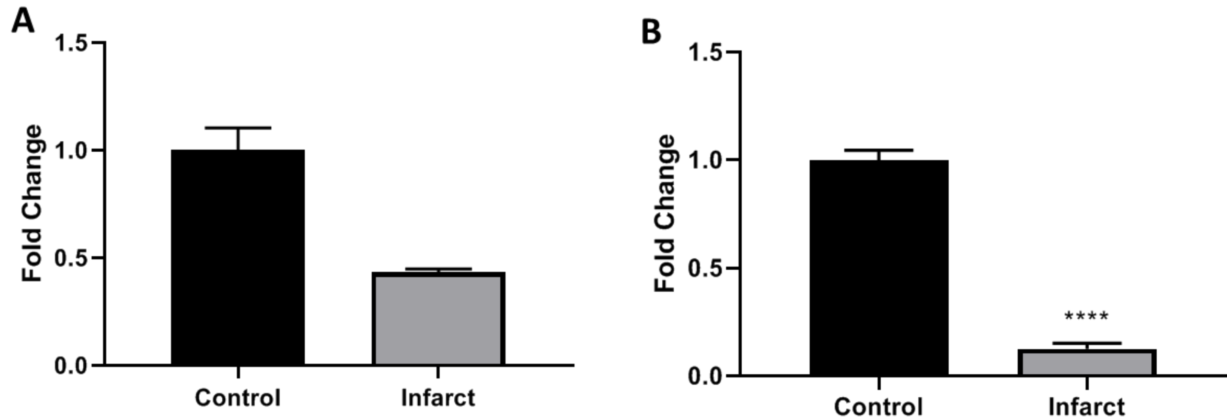

Figure 4: NRG1 and NRG2 Transcripts are Downregulated in the Absence of Cell Treatment in Infarcted Sheep
NRG1 (A) and NRG2 (B) gene transcripts were downregulated in the infarct region of sheep in the absence of cell treatment when compared to normal control age-matched sheep (control) as shown by RT-qPCR. Fold changes are shown as the mean ± SEM. All samples were run in triplicates and were normalized to GAPDH. Data was calculated using ΔΔ CT.

ERBB Receptor Expression is Elevated in the Cardiovascular Repair Zone following Islet-1+ Progenitor Cell Treatment in Infarcted Sheep

ERBB expression levels in the cardiovascular repair zone of three Islet-1+ progenitor cell-treated sheep were compared to ERBB levels in their respective non- infarct regions. Gene expression levels identified by RT-qPCR are shown as an average of three sheep per group in Figure 5. ERBB1 (3.62 ± .48 fold change, ****P<.0001), ERBB2 (2.37 ± .52 fold change, * P=.0172), ERBB3 (5.87 ± 1.74 fold change, *P=.0127), ERBB4 (2.24 ± .44 fold change, ****P=.0123), and PIK3C2B (2.72±.32 fold change, ***P<.0001) were significantly induced in all

sheep samples treated with Islet-1+ progenitor cells (Fig. 5). ERBB3 transcripts were most highly expressed in response to cell treatment in myocardial infarcted sheep. Activation of this receptor stimulates expression of PIK3C2B (class 2 PI3K), thereby triggering YAP1 expression downstream (Camberos, 2019) to promote cardiomyocyte proliferation, cell survival, and cell growth.

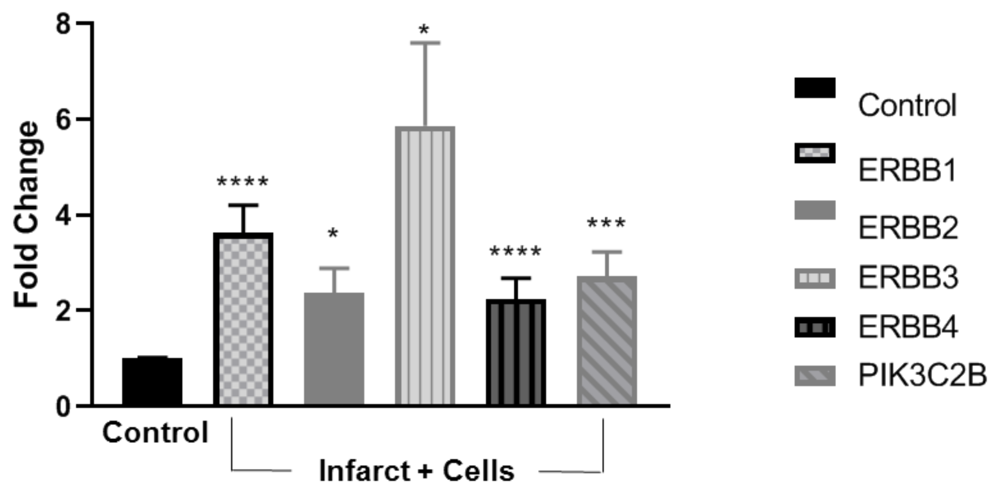

Figure 5: ERBB 1-4 Transcripts are Elevated in Infarcted, Islet-1+ Cell Treated Sheep
By using RT-qPCR, ERBB1, ERBB2, ERBB3, ERBB4, and PIK3C2B transcripts were all significantly elevated following neonatal sheep Islet-1+ CPC transplantation in sheep post-infarction (Infarct + Cells) when compared to the non- infarct region (Control). Fold changes are shown as the mean $\pm$ SEM. All samples were run in triplicate and were normalized to GAPDH. Data was calculated using $\Delta\Delta$ CT.

Islet-1+ Progenitor Cell-Treated Sheep Summary

In summary, we showed elevated transcripts encoding NRG1, NRG2, PIK3C2B, and ERBB 1-4 after neonatal sheep Islet-1+ CPC transplantation in

infarcted sheep. Cell-based treatment induces NRG1 and NRG2 which bind and activate the ERBB3 signaling pathway, resulting in downstream activation of the PIK3C2B (class 2 PI3K) pathway. Our lab has previously shown induced expression of YAP1 in Islet-1+ cell treated neonatal sheep (Camberos, 2019). With this in mind, PIK3C2B activation turns off the Hippo pathway and promotes YAP1 expression in the nucleus where it can upregulate NRG1 and NRG2 to rebind with ERBB3 receptors in an autocrine loop effect (Fig. 2). These findings led us to investigate whether the autocrine loop concept is activated within the ERBB signaling pathway in our human neonatal Islet-1+ CPCs *in vitro*.

Human Neonatal Islet-1+ Cardiac Progenitor Cells

Activation of YAP1 Induces NRG and Activates ERBB Signaling in Human Neonatal Islet-1+ CPCs *in Vitro*

We treated human neonatal Islet-1+ CPCs with Wnt5a to determine whether activation of YAP1 promotes upregulation of NRG and subsequently elevates ERBB expression as an autocrine loop. Studies have shown that cells treated with Wnt5a promotes expression of YAP1 (Park, 2015). Human neonatal Islet-1+ CPCs were therefore treated with Wnt5a in our study for 48 hrs, 72 hrs, 96 hrs, and 144 hrs. RNA was extracted from these cells at 48 hrs and 72 hrs for RT-qPCR analysis. In addition, protein was extracted from a separate sample of cells at 96 hrs and 144 hrs for Western blot analysis. The cells were treated with Wnt5a for 96 hrs followed by removal of Wnt5a from the media until the 144 hr

timepoint. The Wnt5a was removed to allow assessment of the potential for YAP1 activation in the cells to continue to be induced in the absence of Wnt5a and as a consequence of amplification via an autocrine loop. These experiments were designed to determine whether activation of YAP1 results in dephosphorylation of YAP1, translocation to the nucleus and elevated expression of NRG, which subsequently activates the ERBB signaling pathway (Fig. 2). Activation of ERBB signaling then continues to maintain YAP1 expression at high levels by signaling through PI3K-AKT pathways.

Activation of YAP1 in Human Neonatal Islet-1+ CPCs Results in Elevated Expression of NRG1 and ERBB3

Human neonatal Islet-1+ CPCs were treated with 100 ng/ml of recombinant human/mouse Wnt5a in CPC growth media for 48hrs and 72 hrs. After 48 hrs of Wnt5a treatment, NRGs, ERBBs, PI3Ks, and YAP1 gene expression levels were evaluated by RT-qPCR (Fig. 6). NRG1 gene expression in Wnt5a treated human neonatal Islet-1+ CPCs was significantly elevated when compared to NRG1 gene expression levels in the untreated control group (2.49 $\pm$. 097-fold change, ** P=.0048). In addition, ERBB3 transcript levels were significantly induced in treated CPCs when compared to the control group (2.36 $\pm$ .26-fold change, * p=.0337). There was also an increasing trend towards elevated transcripts encoding NRG2 (Fig. 6B), ERBB2 (Fig. 6G), and PIK3CD (Fig. 6D). No difference in gene expression was identified for PIK3C2B (Fig. 6C) and YAP1 (Fig. 6E). Furthermore, there was a trend

towards downregulation of gene expression for ERBB1 (Fig. 6F) and ERBB4 (Fig. 6I) in treated vs. control groups.

Next, NRGs, ERBBs, PI3Ks, and YAP1 gene transcripts after 72 hrs of Wnt5a treatment were analyzed by RT-qPCR (Fig. 7). NRG1 gene expression was significantly elevated in treated human neonatal Islet-1+ CPCs when compared to the untreated control group (3.49 $\pm$ .097-fold change, ** P=.0048). ERBB3 gene expression was also significantly upregulated in the treated group (3.5$\pm$ .37-fold change, * P=.012). PIK3CD gene expression was significantly elevated after treatment (12.5 $\pm$ .26-fold change, *** p=.0337) and YAP1 gene transcripts were significantly induced (2.12 $\pm$ .065-fold change, ** p=.0034). Next, there was a slight increase in transcripts encoding NRG2 (Fig. 7B) in treated cells. PIK3C2B (Fig. 7C), ERBB1 (Fig. 7F), ERBB2 (Fig. 7G), and ERBB4 (Fig.7I) gene expression levels were all downregulated in Islet-1+ CPCs treated with Wnt5a when compared to the control group. After 72 hrs of Wnt5a treatment, NRG1 was upregulated and may therefore have the potential to bind and activate the ERBB3 receptor tyrosine kinase, thereby inducing PIK3CD (class 1 PI3K) expression downstream. This activation of PIK3CD may further increase expression of YAP1, which then may promote upregulation of NRG1 and ERBB3 in autocrine loop fashion.

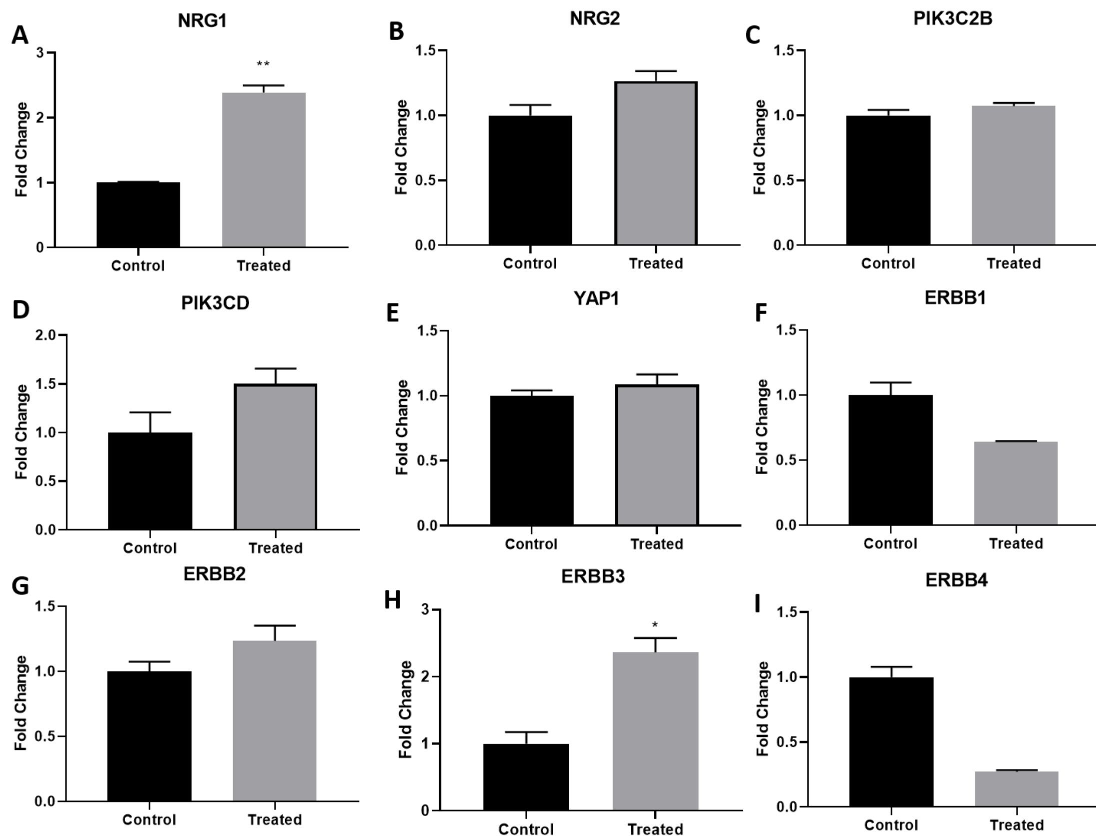

Figure 6: NRG1 and ERBB3 Transcripts are Elevated after 48 hrs of Wnt5a Treatment in Human Neonatal Islet-1+ CPCs

RT-qPCR analysis revealed that NRG1 (A) and ERBB3 (H) gene transcripts were significantly elevated in Wnt5a treated human neonatal Islet-1+ CPCs at 48 hrs (treated) when compared to the untreated control group. Fold changes are shown as the mean ± SEM. All samples were run in triplicates and were normalized to actin. Data was calculated using ΔΔ CT.

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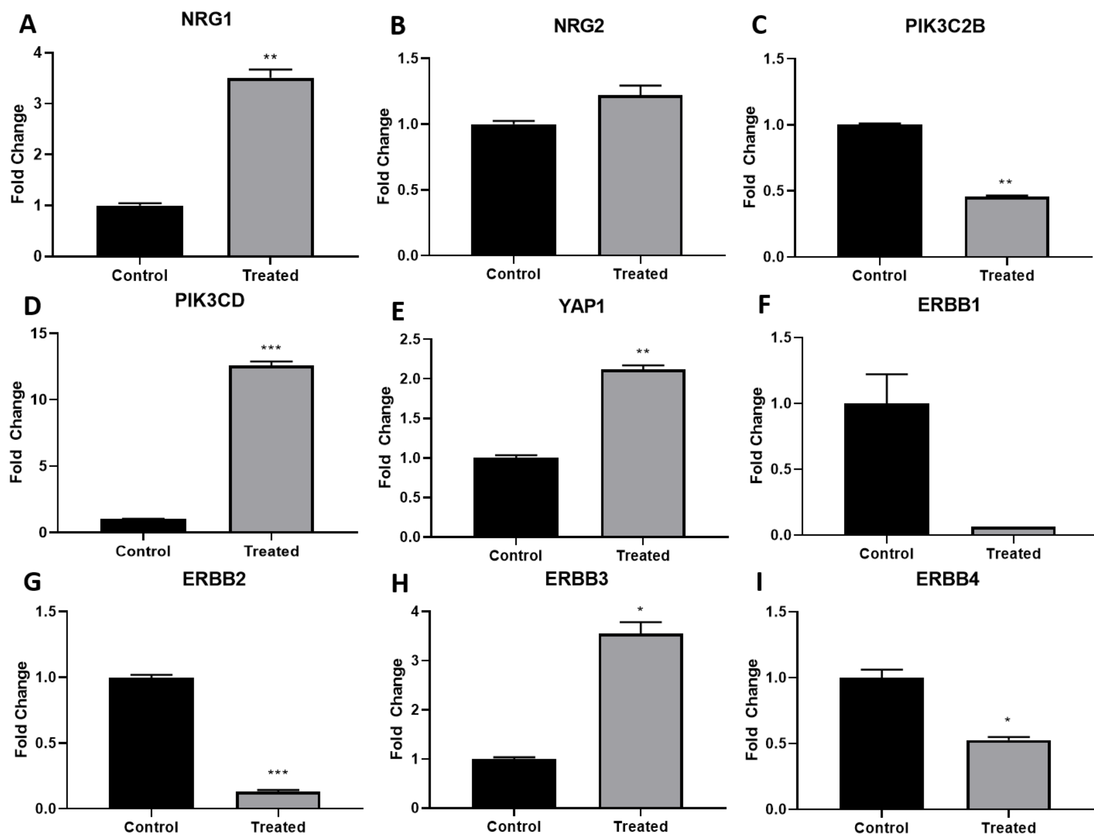


Figure 7: Activation of YAP1 after 72 hrs of Wnt5a Treatment in Human Neonatal Islet-1+ CPCs Results in Elevated Expression of NRG1 and ERBB3
RT-qPCR analysis revealed that NRG1 (A), ERBB3 (H) PIK3CD (D), and YAP1 (E) transcripts were all significantly elevated in Wnt5a treated human neonatal Islet-1+ CPCs at 72 hrs (treated) when compared to untreated control group. Fold changes are shown as the mean ± SEM. All samples were run in triplicates and were normalized to actin. Data was calculated using ΔΔ CT.

YAP1 Protein Expression is Elevated in Human Neonatal Islet-1+ Cardiac Progenitor Cells Treated with Wnt5a at 96 hrs and 144 hrs

Protein Simple Wes is an automated gel free Western blot device that is capable of performing multiple protein assays in their respective lanes as well as quantifying the amount of protein from a protein collection sample. 100ng/ml of

recombinant human/mouse Wnt5a in CPC growth media was used to activate human neonatal Islet-1+ CPCs. YAP1 protein and phosphorylation levels were assessed at 96 hrs and 144 hrs by Western blot (Fig. 8A) after removal of Wnt5a from the cell culture. These cells were grown without Wnt5a stimulant for 3 days (144 hrs total). As a result, there was a gradual increase in YAP1 expression from 96 hrs (Fig. 8B. 1.41 ± .02-fold change, ***P<.0001) to 144 hrs (Fig. 8B. 2.55 ± .14-fold change, **P<.0001) when compared to the control, respectively. In addition, YAP1 expression was significantly induced in the 144hr Wnt5a treated group (Fig. 8B. 2.55 ± .25-fold change, **P<.001) when compared to the 96 hr Wnt5a treated group. CPCs examined at 144 hrs showed an increase in YAP1 protein expression levels after removal of Wnt5a when compared to earlier timepoints (Fig. 8B) demonstrating continuing activation of YAP1 in this model. Protein expression of YAP1 was compared in the control and treated groups (Fig. 9A). The control and 144 hr Wnt5a treated human neonatal Islet-1+ CPCs were analyzed to determine P-YAP protein levels by taking the ratio of P-YAP/YAP1 protein from each group (Fig. 9B, 9C). At 144 hrs, in the Wnt5a treated group, the P-YAP/YAP1 protein levels declined (Fig. 9D). Reduced P-YAP expression indicates that YAP1 translocates to the nucleus promoting NRG1 expression which is downstream of YAP1 (Fig. 10). NRG1 can then rebind to the ERBB3 receptor there by inducing an autocrine loop-mediated activation of this pathway.

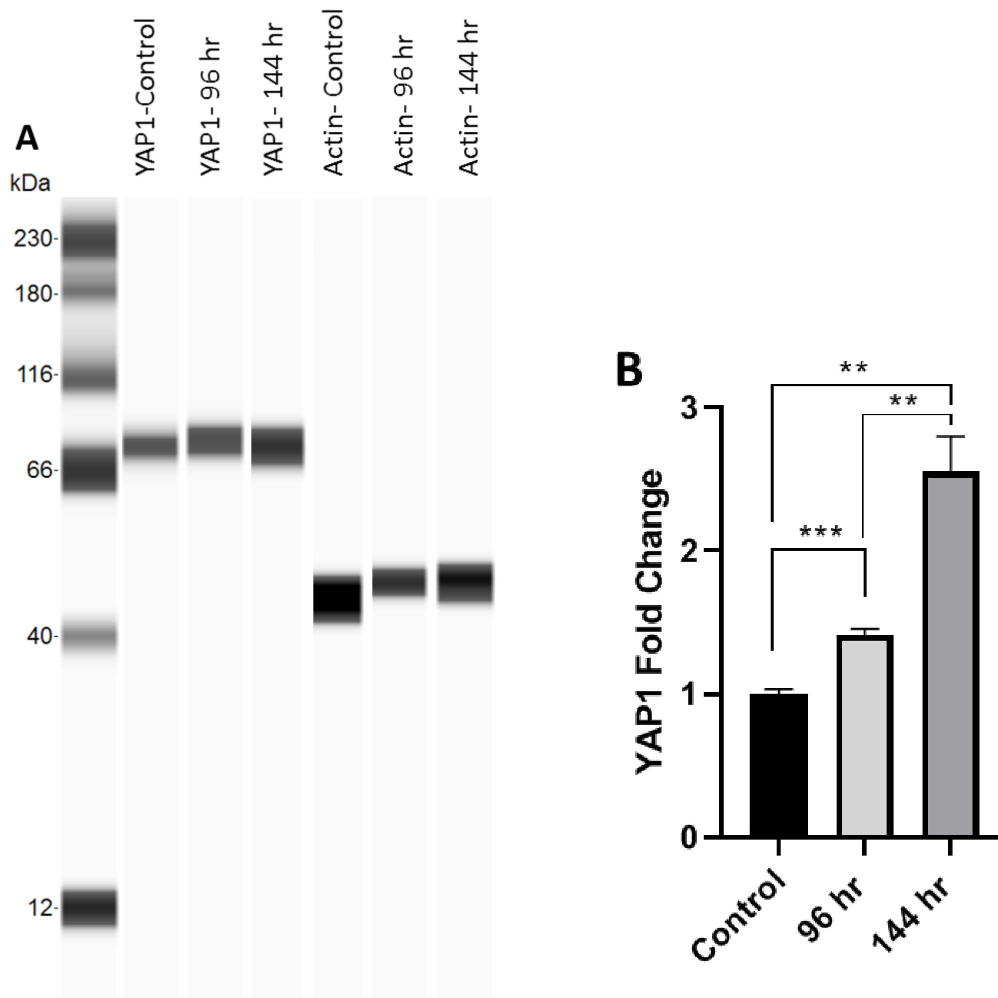
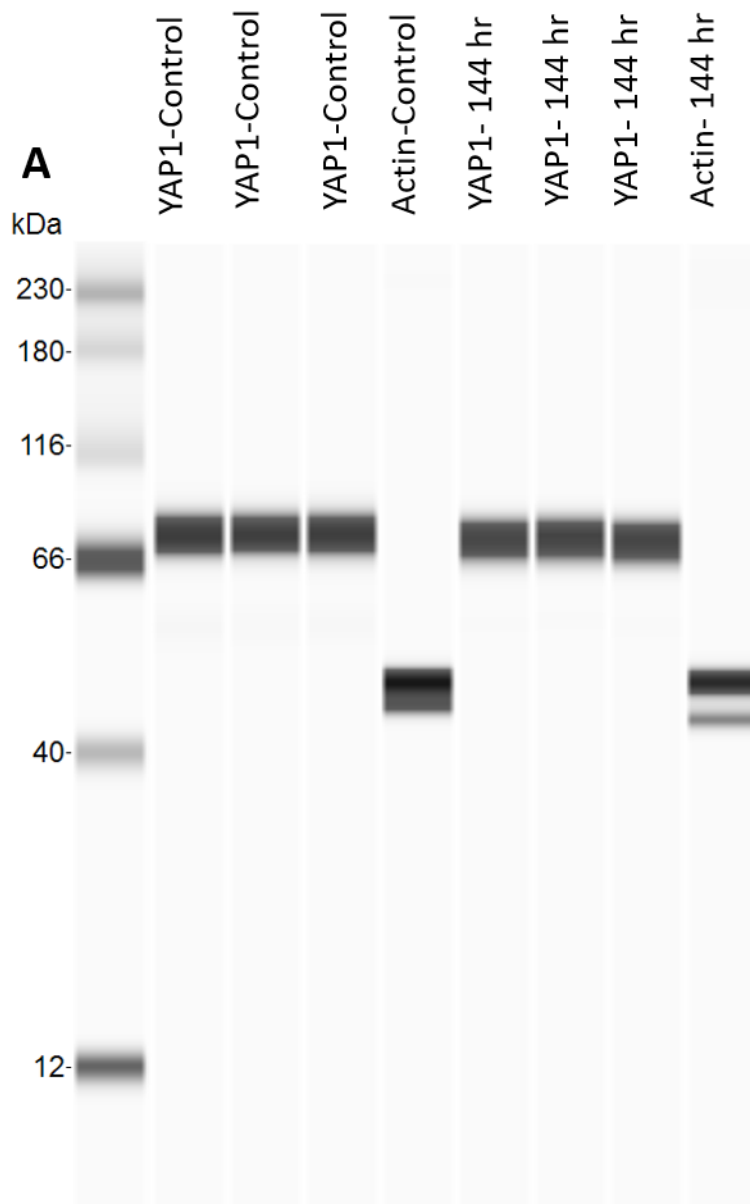


Figure 8: YAP1 Protein Expression is Elevated in Human Neonatal Islet-1+ Cardiac Progenitor Cells at 96 hrs and 144 hrs after Treatment with Wnt5a
Western blot showing protein expression of YAP1 and actin in Wnt5a treated human neonatal Islet-1+ CPCs at 96 hrs and 144 hrs when compared with controls (A). YAP1 protein expression at 144 hrs following Wnt5a treatment was significantly induced when compared to the control and 96 hr treated group (B). Fold changes are shown as the mean + SEM. All samples were run in triplicates and bar graphs were quantified and normalized to actin as a control.


A
kDa
YAP1-Control
YAP1-Control
YAP1-Control
Actin-Control
YAP1- 144 hr
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Actin- 144 hr
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180-
116-
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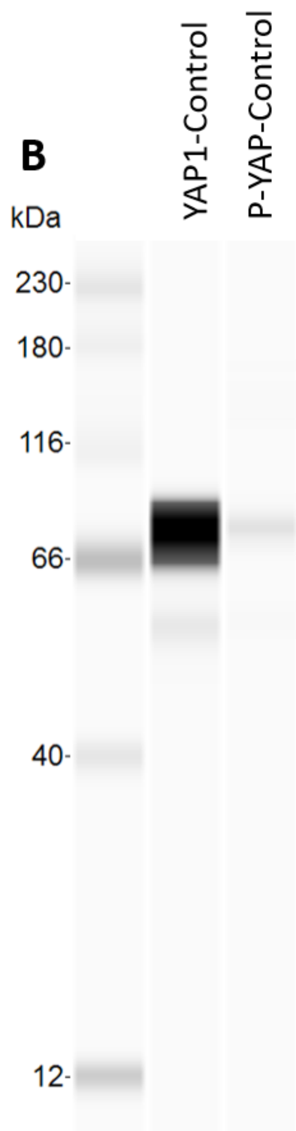
40-
12-

B



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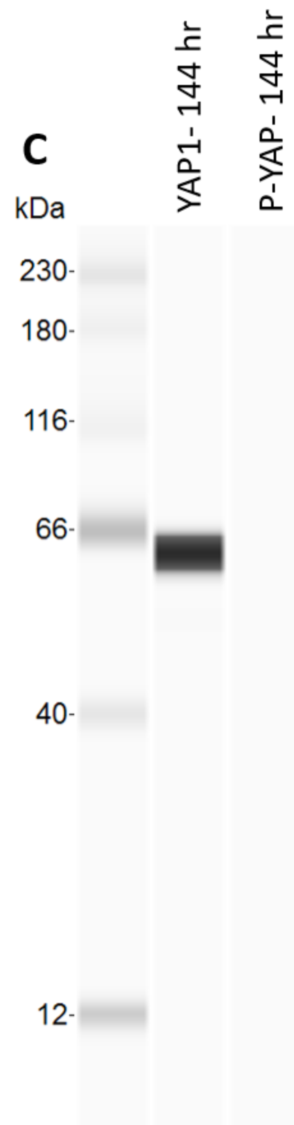

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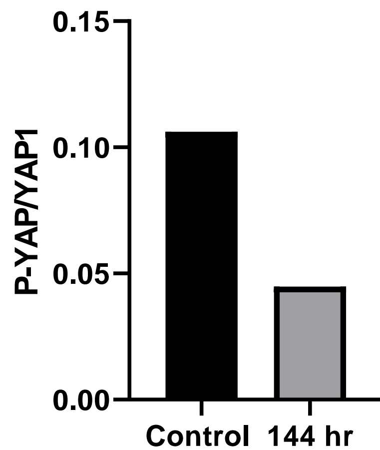

Figure 9: P-YAP Protein Expression is Reduced in Wnt5a Treated Human Neonatal Islet-1+ CPCs at 144 hrs
Western blot showing YAP1 protein levels in Wnt5a treated human neonatal Islet-1+ CPCs at 144 hrs when compared with controls. (A). P-YAP protein expression was compared in the control (B) and Wnt5a treated (C) Islet-1+ CPCs at 144 hrs. The relative levels of P-YAP/YAP1 in the 144 hr Wnt5a treated and control group were quantified (D). Protein samples in Figure 9A were run in triplicate.

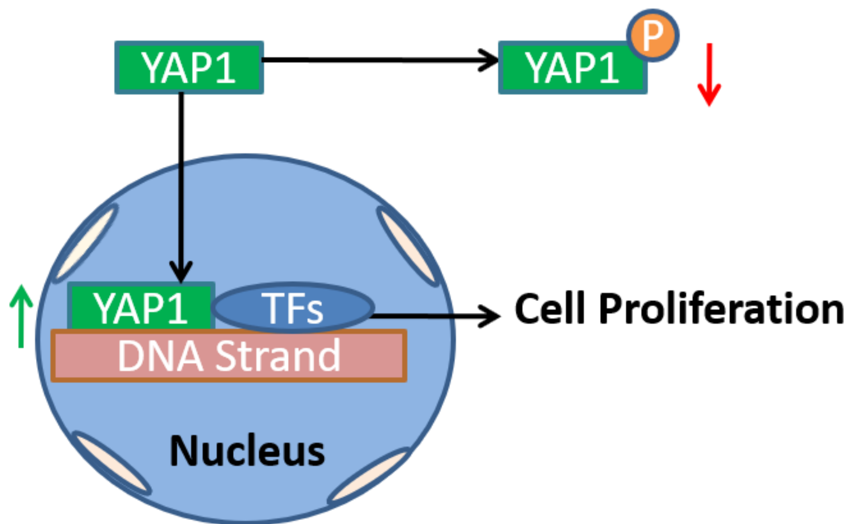

Figure 10: Decreased P-YAP Expression Promotes Translocation of YAP1 to the Nucleus
Diagram showing subsequent increase in intranuclear YAP1 expression (green arrow) when P-YAP expression is decreased (red arrow) from 96 hrs to 144 hrs of Wnt5a treatment in human neonatal Islet-1+ CPCs.

CHAPTER FOUR

DISCUSSION

In this study, the ERBB pathway was explored to determine whether ERBB signaling participates in cell-based repair following transplantation of neonatal sheep Islet-1+ CPCs in a sheep model of myocardial infarction. Using sheep as a cell-based repair model can provide new treatment options that can be applied to humans in restoring cardiac function and structure after MI. With this model, we tested our hypothesis that ERBB activation by NRG stimulates YAP1 expression, resulting in upregulation of NRG in an autocrine loop effect. In support of this, we showed induction of NRG1, NRG2, ERBB 1-4, and PIK3C2B gene expression after neonatal sheep Islet-1+ CPC transplantation in MI sheep. We also investigated the hypothesis in human neonatal Islet-1+ CPCs as a clonal population and demonstrated that this autocrine loop can be reproduced *in vitro* where NRG1, ERBB3, and PIK3CD gene transcripts were elevated following intranuclear translocation of YAP1. The data supports the concept that a feedback autocrine loop occurs and is sufficient to maintain YAP1 levels that are required for cardiovascular regeneration in this model. NRG1 gene expression was not induced solely by infarction, but required the introduction of Islet-1+ cell-based treatment. Similarly, NRG1 gene expression in infarcted fetal tissue was reported to be unchanged when compared to sham tissue after heart injury (Lock, 2019). NRG1 activation can stimulate CM proliferation at the site of injury through the ERBB pathway. In support of this, we showed increased expression

of ERBB1, ERBB2, ERBB3, ERBB4, and PIK3C2B in Islet-1+ cell treated neonatal sheep when compared to control regions. ERBB gene transcripts signal downstream to the PIK3C2B pathway and promote activation of YAP1 expression by inactivating the Hippo-pathway. Our lab has previously reported that YAP1 is induced in Islet-1+ cell treated neonatal sheep (Camberos, 2019). Activated YAP1 promotes cardiovascular regeneration. We demonstrate here that upregulation of NRG-1 subsequently induces elevated ERBB receptor expression which further activates and maintains high levels of YAP1.

The ERBB pathway has previously been investigated in mice undergoing cardiac repair after heart injury. Injection of NRG1 in the cardiovascular zone of mice after MI stimulated CMs to transition from a differentiated state to proliferative state through the ERBB4/ERBB2 receptors and PI3K pathway (Bersell, 2009). It was shown that activation of this signaling pathway leads to reduction in infarct size, reduction of CM hypertrophy, and improvement of myocardial function in mice after MI (Bersell, 2009). These positive effects were also observed when overexpressed ERBB2 was transiently induced in neonatal mice after the first week of birth (D’ Uva, 2015). In addition, overexpression of ERBB4 in MSCs that were transplanted in MI mice upregulated NRG1, which formed a NRG1-ERBB4-NRG1 autocrine loop, promoting CM proliferation and dedifferentiation (Liang, 2015). These studies highlight the importance of the NRG1-ERBB pathway during cardiac regeneration in response to heart injury.

The Hippo-YAP1 signaling pathway has previously been investigated in cardiac studies of neonatal and adult mice.YAP1 expression was reported to be robustly detected in neonatal mice, but this expression declines with age (Gise, 2012). High levels of YAP1 enhance CM proliferation, which is important during neonatal cardiac development (Gise, 2012). Conditional inactivation of YAP1 resulted in the death of neonatal mice due to a decrease in CM proliferation (Gise, 2012; Xin 2013). In addition, Yap1 induction post-MI in adult mice stimulated CM proliferation, which led to reduced infarct size and improved cardiac function (Lin, 2014). Next, the Hippo-Yap1 signaling pathway has previously been investigated in porcine models of cardiac repair using RNA technology. A study has shown that knockout of the Sav gene by treatment with AAV9-Sav–short hairpin RNA (shRNA) post-MI in pigs improved ejection fraction, promoted CM division, and reduced scar tissue size in the infarct zone (Liu, 2021). Furthermore, another study using MicroRNA-199a was shown to stimulate cardiac repair in pig hearts following MI by interfering with the Hippo-Yap1 signaling pathway (Gabsonia, 2019). This interference promoted CMs to return to a cell cycle state of proliferation (Gabsonia, 2019). These studies demonstrate the importance of YAP1 and its contribution to CM proliferation for cardiac repair.

In ovarian cancer, ERBB3/ERBB2 activation by NRG1 signals downstream to the Hippo-Yap pathway through PI3K in form of an autocrine loop (He, 2015; Sheng, 2010). In this model, ERBB3 and NRG1 gene transcripts were upregulated after overexpression of YAP1, triggering an autocrine effect (He,

2015). These findings show that YAP1 can regulate cell proliferation through the NRG1-ERBB3 pathway. NRG1 induction deactivates the components of the Hippo pathway resulting in activation of YAP by dephosphorylation (He, 2015). However, the specific mechanism by which the Hippo pathway switches to inactivated state remains unclear. With this in mind, some studies have proposed that the Hippo pathway may be connected to the PI3K pathway. Within the PI3K pathway, PDK1 was shown to form a complex with the components of the Hippo pathway when this pathway is activated (Fan, 2013; Xia, 2018; Zhao, 2018). However, this complex dissociates when EGF interacts with EGFR resulting in activated PDK1 kinase (Fan, 2013). More specifically, activation of PIK3CA and PIK3CB (class 1 PI3Ks) leads to PDK1 activation, which may indirectly inhibit the activity of LATS1/2 through some other downstream target within this pathway (Zhao, 2018). This shows that PDK1 can act as a regulator of YAP1 activity by indirectly targeting the components of the Hippo pathway. In addition, activated YAP1 was shown to be positively regulated by AKT via an indirect mechanism (Zhao, 2018). AKT was investigated further to see if it interacts with LATS in the Hippo pathway but LATS1 and LATS2 were not affected by co-expression of AKT (Zhao, 2018). This data showed that AKT does not affect YAP1 activation through the Hippo pathway, but acts directly on YAP1 through an unknown mechanism. In our human neonatal Islet-1+ CPC model, we showed a significant increase in expression of NRG1, ERBB3, PIK3CD, and YAP1 at 72 hrs. Class 1 PIK3CD-PDK1 pathway negatively regulated the Hippo pathway leading to

activation of YAP1. We further demonstrated a gradual increase in YAP1 protein expression from 96 hrs to 144 hrs in human neonatal Islet-1+ CPCs associated with dephosphorylation of YAP1 protein by Western blot. Activation of YAP1 was induced and maintained NRG1 expression, allowing NRG1 to rebind to the ERBB3 receptor on the cell surface in an autocrine loop effect (Fig.2).

Conclusion

Our findings support the involvement of the ERBB3 pathway in cardiovascular repair post-infarction via activation of NRG1. Activated ERBB3 relays its signal downstream to the PIK3CD pathway (in humans) or PIK3C2B (in sheep) stimulating elevated expression of intranuclear YAP1. As a result, NRG1 becomes upregulated where it participates in an autocrine loop effect with ERBB3 receptor on the cell surface. Activation of the NRG1 induced ERBB3 pathway contributes to cardiomyocyte proliferation, dedifferentiation, growth, and survival. Understanding the role of ERBB signaling after cell-based treatment and the mechanistic basis by which this pathway interacts with and stimulates YAP1 will provide new treatment options in patients suffering from heart failure.

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