Regenerative Medicine Minnesota
Progress Report
Due: 3/31/2018

Grant Title: Identification of Novel Regulators of Heart Regeneration

Grant Number: RMM 102516 009

Principal Investigator: Jop van Berlo, MD, PhD

Project Timeline: 4/1/2017 - 3/31/2019

Progress to Date:
For each specific aim in your proposal, please describe progress and obstacles and/or achievements. Please address any deviations from estimated timelines and limit ***total*** response to 2000 words. Figures and tables are allowed.

The original proposal had 2 specific aims. In the past year we have been working hard to address these specific aims and below we list our progress for each.

Aim 1. Determine the ability of identified regulators of cardiomyocyte proliferation to give rise to new cardiomyocytes

The goal of this aim was to validate in a follow up assay which of the genes that we originally identified as novel regulators of cardiomyocyte proliferation were indeed giving rise to new cardiomyocytes. The preliminary data measured DNA synthesis based on EdU incorporation. The main limitation of the orginal assay was that it was based on an n=1 experiment, given the high-throughput nature of tested all genes. We decided that the best approach to assess new cardiomyocyte formation was to 1. repeat the same assay of culturing fetal murine cardiomyocytes, infect them with a lentivirus that inhibits the identified genes, one per well, but with newly generated lentivirus at an n=3, and again incorporate EdU 4-5 days after plating these primary cardiomyocytes. This is actually a very stringent assay, to expect to see cardiomyocytes proliferate 4-5 days after isolation. This new experiment that was carried out for all hits from the original screen reduced the number of hits that affected cardiomyocyte proliferation down to ~75. Next, we performed phospo Histone H3 (ser10) staining on cultured fetal cardiomyocytes 5 days after plating. We measured a pHH3 percentage of 2.6% in the negative controls and 32.3% in the positive control. There were 56 hits with a pHH3 percentage above 5.6%, which we considered high enough to have important biological meaning. Certainly, many more genes were significantly different if tested against the negative control one by one, but a two-fold increase was considered the cutoff. The negative control ended up being rank 222 of the various conditions, so there were 40 treatments that did not increase pHH3 at all. Importantly, of the 75 hits that confirmed an EdU incorporation rate >5%, 48 showed pHH3 staining at that level. We attempted to stain for Aurora Kinase B, but the frequency of positive staining was too low to have any quantitative meaning in the 96 well format that this experiment was performed at. We will therefore repeat this staining for the few genes that we select to study further. To make the selection of those genes even more stringent, we are currently developing an assay that is even more rigorous and demanding, to stimulate cardiomyocyte proliferation that were isolate from neonatal mice. We have performed neonatal cardiomyocyte isolation now numerous times, and the EdU percentage in the negative control condition is consistently at 0.2% (compared to about 2% in fetal cardiomyocytes). Clearly

neonatal cardiomyocytes are much more difficult to stimulate to proliferate. Therefore, we anticipate that any short-hairpin RNA that is capable of stimulating proliferation of neonatal cardiomyocytes is more likely to have translational potential. Especially since the goal of this research project is to develop a therapeutic strategy for neonates with hypoplastic left heart syndrome.
In conclusion, we have completed SA 1.1 according to the anticipated timeline. The only deviance from the timeline is that we were not able to do the Aurora Kinase B staining. We did encounter some setbacks in the validation experiments that we performed to be able to successfully perform SA1.2. We have successfully done live cell imaging of neonatal rat cardiomyocytes, and we identified p21 inhibition as a successful approach to enhance cardiomyocyte proliferation in these cells. However, the variability of this assay turns out to be too high due to the fact that we have to use primary cells. There is simply too much variability in the amount of cell death from well to well. We nevertheless performed all validation experiments, and are getting ready to submit a manuscript on our initial findings in rat neonatal cardiomyocytes. Similar to the Aurora Kinase B staining we plan to do this assay for the few genes that we select to pursue to specific aim 2. We therefore believe we are well within the plan that was set out at the beginning of the project.

Aim 2. Assess which genes improve cardiac regeneration using an innovative treatment strategy.

The goal of the second aim was to develop a strategy that would allow testing the effect of knock-down of individual genes in vivo in wild type mice. To that end, we adopted a technique of apical resection as a surgical model of the relative lack of cardiomyocytes that exists in the left ventricle of patients with hypoplastic left heart syndrome. We reasoned that if we could show stimulation of cardiomyocyte proliferation under these conditions, it might likely result in a therapeutic strategy. The original plan was to use a gel-based application of adenovirus that would result in transduction of the myocardium to allow gene knockdown.

Since the method of anesthesia for the apical resection surgery is hypothermia, we optimized the gelation temperature to be below 10°C, but above 0°C. We tested various permutations of these types of gels to see which formulation would result in a workable gel that could be applied to the minuscule neonatal mouse heart during a surgical intervention. After optimizing the gelation temperature, we next tested the ideal level of stickiness. During the first couple of tests, we noticed that the gel would not really adhere to the wet and bloody heart. Therefore, we added a component to the gel that would increase the stickiness. Unfortunately, this also affected the gelation temperature, so we had to reassess the ideal mixture to obtain gelation at the desired temperature. With the newly formulated gel, we now had a workable solution that could readily be applied during the surgical intervention and that would adhere to the heart. Next, we tested if this formulation would allow for sufficient transduction efficiency. We loaded the gel with adenovirus that expresses green fluorescent protein (GFP), and first assessed whether the virus would infect cells if the gel was applied to cultured cells. This turned out not to be the case, and we assessed the possible problems. It turned out that the sticky component addition, also caused acidification of the gel, and we reasoned that this may have reduced overall virulence of adenoviruses. We therefore neutralized the gel and retested transduction efficiency on cultured cells, which now showed a dose-dependent transduction efficiency. We were now ready to test the gel in vivo and applied it to the neonatal mouse heart immediately after apical resection. Although the formulation of the gel was easy to work with, and the gel adhered to the heart, we did not observe transduction of the myocardium. Instead, we noticed transduction of the epicardium, all the way around the ventricles and atria. Based on previous literature, we reasoned that the addition of trypsin might allow for a better penetration of the virus into the myocardium, and we assessed this as well. This turned out to be a balancing act. We did observe better penetration of the myocardium with 1.0 – 1.5% trypsin included in the gel. However, especially the higher percentages of trypsin appeared to have a negative effect on mouse survival. We are not entirely sure what caused this reduction in survival, and haven't performed sufficient surgeries to definitively claim a certain mortality. However, even with the high percentage of trypsin the transduction efficiency was relatively low.

Based on these findings, we are currently reassessing whether gel-based application of a virus is indeed the best approach for our purposes. We are considering a number of alternatives. However, all of these would increase the duration of the project and significantly increase the cost beyond the available budget. The main strategy we are considering that might allow for good transduction efficiency and reasonable cost is the administration of Adeno-Associated Virus (AAV). At first, we discarded this as a possibility, because it had been shown in young adult mice that it takes about 14 days before the AAV becomes expressed at measurable levels. This is not consistent with any timeline of performing surgeries in neonatal mice. However, more recently, we have learned that if the AAV is administered at the day of birth, expression is measurable already 5-7 days later. This timeline would be consistent with the timeline we planned to follow. We could infect neonates on the day they are born, and perform apical resection a week later, when mice are no longer regenerative. If we could enhance cardiomyocyte proliferation at that stage, we would likely improve the regenerative ability of the heart. Unfortunately, we are not aware of any AAV cores within the state of Minnesota that could provide us with an AAV of sufficient titer to use in vivo. Therefore, we are currently exploring commercial and non-commercial sources of AAV that we could use.

In the past year, we have validated that we can perform apical resection in neonatal mice at day 1 or at day 7 after birth, and that this indeed results in complete regeneration if performed on the day after birth, and results in scar formation if performed 1 week after birth.

The alternative approach would be to generate genetic mouse models to assess an in vivo regenerative response. Unfortunately, this falls well beyond the available budget, and also the available timeline. Nevertheless, we could opt to generate 1 mouse line, or maybe try to obtain a genetic mouse model that was already generated for other purposes. The main downside of this approach is that we are limited by what mice have already been generated by other groups, and that this approach requires a fair amount of mouse breeding. Nevertheless, we are pursuing 1 genetic mouse model in particular that was available through a collaborator. This is a lox-P targeted mouse line for one of the genes that was a positive hit for the initial screen, but fell just below cutoff levels in follow up assays (3.6% EdU incorporation and 3.9% pHH3 positivity). We didn't know that this gene would fall just below levels we would consider positive when we initiated mouse breeding strategies. We have currently cross-bred these loxP targeted mice with a cardiomyocyte-specific, tamoxifen-inducible Cre recombinase expressing mouse model, and are awaiting the first offspring of homozygous loxP targeted mice with the Cre recombinase. We are anticipating that this genetic cross may produce preliminary evidence for further grant funding applications, if genetic deletion of this gene from cardiomyocytes indeed results in enhanced cardiomyocyte proliferation.

Please list any of the following that have resulted from the Minnesota Regenerative Medicine grant funding:
We have not published or submitted manuscripts based on the data derived from the Minnesota Regenerative Medicine grant funding, but are currently preparing 3 separate manuscripts that result from the work covered by this grant. The manuscript working titles and authors are listed below:

1. Dirkx E, Raso A, el Azzouzi H, Cubero RJ, Olieslagers S, Sorensen D, Huibers MM, de Weger R, Siddiqi S, Moimas S, Torrini C, Zentillin L, Braga L, da Costa Martins PA, Zacchigna S, **van Berlo JH**, Giacca M, De Windt LJ. A microRNA program controls the transition of cardiomyocyte hyperplasia to hypertrophy and stimulates mammalian cardiac regeneration.

2. Yucel D, Pengo T, Saucerman JJ, **van Berlo JH**. Live cell imaging identifies p21 as a critical regulator of cardiomyocyte proliferation.

3. Caronia J, Sorensen D, **van Berlo JH**, Azarin S. Development of gel-based viral gene delivery.

We have not filed for patents
We have not applied for additional grant funding based on findings from this grant

Budget Update:
There were no deviances from the proposed budget >20%.

Reporting to all Minnesotans:
Briefly and using lay language, please describe your overall progress and how it is significant to the patients in need of regenerative medicine therapies in Minnesota. This will be used on the RMM website to demonstrate how funds are being used to advance the health of all Minnesotans.

Identification of Novel Regulators of Heart Regeneration
The heart has a limited ability to repair itself, which is in part due to its inability to increase the number of contractile muscle cells. Based on studies in animals, we believe that if we could stimulate the contractile muscle cells of the heart to increase in number, this might lead to better repair of heart injury. One example of a disease that is in part caused by a lack of contractile heart muscle cells is a condition called hypoplastic left heart syndrome. Patients that are born with this congenital heart defect have, among other problems, an underdeveloped left chamber of the heart. This requires multiple surgeries in the first couple of years of life to allow these children to survive. We believe that if we could stimulate the contractile muscle cells of the heart to divide and increase in number, we could develop a therapy that would improve the outlook for these patients. Unfortunately, we don't know why contractile muscle cells stop dividing soon after birth. Therefore, the goal of this grant was to test almost every gene for a stimulatory effect on division of these contractile muscle cells. Based on this large-scale experiment, we identified 250 genes that could stimulate contractile muscle cells to divide, and we are currently working on further narrowing this list down to the most promising genes that could then be used in a therapeutic strategy, first in research animals, and then for patients with hypoplastic left heart syndrome.