Summer 2011

AAB CARDIOVASCULAR RESEARCH INSTITUTE
Department of Medicine

CVRI Beat

UNIVERSITY of ROCHESTER MEDICAL CENTER
MEDICINE of THE HIGHEST ORDER

Upcoming Scientific Events:

Scientific Sessions of the American Heart Association in Orlando FL on Nov 12-16, 2011.
American Society of Hematology (ASH) annual meeting in San Diego, CA on December 10-13, 2011

Director's Column

As summer draws to an end, we reflect back on many beautiful days here in the greater Rochester area. From the Blue Angels in June to the Dinosaur Barbecue Picnic this past month, we have had one of the most sunny and warm summers in recent memory. We also are fortunate to have dodged Hurricane Irene which followed a rare east coast earthquake. This time of year also marks the anticipation of a new school year, both for our children leaving for higher education across the county and abroad as well as the students and faculty here at the University of Rochester. As the days get shorter we ponder what the cool days of winter will bring to our Aab CVRI campus (ice skating and hockey anyone?).

Since the last issue of the CVRI Beat there have been many important developments here at the Aab CVRI. First, three shelled rooms in our vivarium were fully outfitted and will soon be bustling with new procedures and an expanded colony of mice for our important studies relating to atherosclerosis, thrombosis, and heart failure. Second, the University approved the construction of a new and improved roof over the entire building. This roof is much like the roofs installed over other buildings across the University and will provide decades of protection from Rochester's inclement and unpredictable weather. Completion of the final phase of this construction is scheduled for mid-late September. We are grateful to the University of Rochester and Alberti & Associates for their continued commitment to make the Aab CVRI a premiere research institute.

We are very pleased to welcome Dr. Eric M. Small, who is our second major recruitment since 2009 (Dr. Craig Morrell was first). Dr. Small received his B.S. in Cellular and Molecular Biology from the University of Michigan and his PhD in Molecular Biology with Paul Krieg (*Xenopus laevis* development) at the University of Texas at Austin. Following a brief stint with Dr. Benoit Bruneau at the Hospital for Sick Children in Toronto, Dr. Small traveled south to the University of Texas Southwestern with Dr. Eric Olson, one of our esteemed Scientific Advisory Board members. During his tenure in the Olson lab, Eric contributed to a number of important discoveries relating to myocardin and several microRNAs in the cardiovascular system. He brings with him his wife, Erin, a beautiful baby boy, named Dean, and some scientifically exciting projects that complement our existing strengths in cardiac and vascular pathobiology. You can read more about Dr. Small's research program in this issue's "In Focus" section.

We would like to remind everyone of the upcoming Scientific Advisory Board meeting to be held on September 16th here in the Aab CVRI. We look forward to many of our SAB members returning to Rochester and offering their sage advice about our research progress and future directions. Each year we ask one of the Scientific Advisory Board members to speak about their research: this year we have invited Dr. Ed Fisher to be our featured speaker. Dr. Fisher, Professor of Cardiovascular Medicine at New York University, is a world expert in lipoprotein metabolism and atherosclerosis. Please join us for his lecture on "HDL and Atherosclerosis: Effects on Macrophages and Vascular Smooth Muscle Cells" which will be held in the first floor conference room on Friday, September 16th at 3:00 PM.

-- Charlie Lowenstein, MD
--Joseph Miano, PhD

Technical Corner

So, you want to study a mouse carrying a knockout of your favorite gene? If you are contemplating such an endeavor, you are probably wondering whether to look for an existing knockout mouse, use a company, or possibly make your own mouse. Of course, if the knockout mouse is widely available and is a "true" knockout, then it is wise to consider obtaining such a mouse through a

Technical Corner continued…

collaborator. On the other hand, if the knockout does not exist or is not a true knockout then you are left with either paying a company to make it or generating it yourself. The company route can be quite expensive (10s of thousands of dollars) and there may be some strings attached (eg, intellectual property ownership). Moreover, the approach to generating the knockout may not be robust and could even cloud your interpretation of data (eg, hypomorphic alleles arising from so-called gene trap knockouts). The good news is there is local expertise right here in the Aab CVRI, and the University employs one of the most skilled knockout mouse generators in the world (Dr. Lin Gan has personally made over 200 knockouts!).

Joseph Miano, PhD

Generating a knockout mouse is a useful learning experience (especially for graduate students) and provides the owner with a unique tool for scientific discovery. But where does one begin?

The most important consideration in making a knockout mouse is the design of the targeting vector. These days, the default approach is to design a conditional knockout where two 34 base pair sequences known as *lox*P sites are strategically placed to flank a region of a gene locus that, upon excision with the bacteriophage-derived Cre recombinase placed under control of a cell-restricted promoter, results in loss of gene function in the cells where the Cre recombinase is active. The question of where to place the *lox*P sites is one that requires thoughtful planning. Accordingly, it is vitally important to know the gene locus of interest in as much detail as possible. Some of the more important questions include: Is there a single promoter? Are there alternative splice variants and would splicing around the deleted region result in a retained open reading frame? Is the gene actually a chimera of two adjacent transcripts (a crazy phenomenon that has recently come to light)? Is there a non-protein coding RNA (such as a microRNA) embedded within an intron or (rarely) exon? Would excision of the region flanked by *lox*P sites result in loss of a critical regulatory element to another gene either adjacent to or remote from your gene of interest? While seemingly daunting, many of these questions can be reasonably addressed through the use of algorithms at the NCBI workstation and/or such genome browsers as the one out of the University of California, Santa Cruz (http://genome.ucsc.edu/). Here, one can quickly dial up a gene of interest and examine the gene structure, alternative promoters, internal genes and the like. Then, using programs in comparative genomics (such as the commonly used VISTA program from Eddy Rubin's group), highly homologous sequences representing potentially important non-protein coding RNAs or regulatory elements can be identified. It is also useful to know the functional domains of your protein and where they are within the exonic structure of the gene.

Investigators have different ideas as to where the *lox*P sites should be placed. If the gene is small enough (meaning 5-15 kb in size) it may be advantageous to "flox" the entire locus. There are however two important caveats to this design strategy. First, there is the possibility that upon excision of the gene, the episomal DNA will remain stable in the nucleus and generate a protein anyway. This was an intriguing yet, undoubtedly, frustrating discovery made by one of our fellow vascular biologists, Dr. Luisa Iruela-Arispe (see *Genetics* **186**: 959-67, 2010). The second concern is that the chances of deleting functionally important sequences such as non-protein coding RNAs or remotely acting regulatory elements increases as the size of the floxed DNA increases. One idea we strongly favor is to flox the proximal promoter and first exon, assuming the first exon is a protein coding exon. We have done this with the *Srf* locus (*PNAS* **101**:17132-37, 2004) and are planning a similar strategy with a new SMC-restricted gene. This design strategy generates true knockouts and avoids the frequent problem of hypomorphic knockouts, that is, partial loss in gene function. The latter is problematic with many conditional knockouts where the *lox*P sites are placed around an internal exon with a well-known functional domain. The Miano lab has a detailed SOP for generating a conditional knockout mouse where the promoter and first exon are floxed.

The next step is the actual "building" of the targeting vector through sequential molecular cloning of PCR products that correspond to two homology arms and the knockout arm. Before this can be done however, there is the question of how correct targeting will be verified following electroporation of embryonic stem cells. This step, the all important genotyping strategy, is a close second to the design of the targeting vector in terms of overall importance. In fact, final design of a targeting vector actually hinges on how the targeting vector will be genotyped. Since 100 or more ES cell clones will be genotyped, the most efficient method of genotyping is via Southern

blotting with DNA probes that are ***external*** to the homology arms. It is absolutely imperative that at least one, and preferably more than one, DNA probe yield an unambiguous, clean Southern result. Once this step is complete, one can begin PCR cloning the homology arms and the intervening sequence that is to be deleted upon Cre-mediated excision (so-called knockout arm). The actually building of the targeting vector is fairly straightforward though careful consideration must be given as to the restriction sites used for cloning and the order in which each cloning step is done. Indeed, the restriction sites used for cloning are deliberately chosen to optimize conditions for unambiguous genotyping. Once the targeting vector is built and checked for sequence fidelity around cloning junctions as well as across both *lox*P sites, it should be linearized and delivered to a Knockout Core for electroporation, selection, expansion, and validation by Southern blotting and Cre-mediated excision (to verify that the *lox*P sequences are present and working). It may also be important to perform a karyotype of the ES cells to ensure a normal diploid genome. When two independent clones are so validated, a Core will inject them independently into mouse blastocysts (C57Bl/6) for the generation of chimeric mice that are then bred for germline transmission (agouti coat color and/or black eyes if mating to albino C57Bl/6 mice, highly recommended).

Germline transmission results in mice heterozygous for the floxed allele. Interbreeding heterozygous mice is then done to generate homozygous floxed mice. These mice will then be bred to a Cre driver mouse to fix the Cre allele on the floxed background (same strain!) so that resulting heterozygous floxed/Cre positive mice may be bred to homozygous floxed mice.

Some final points worth noting:

1. Most knockout mice are initially of a mixed genetic background. Thus, a phenotype observed early could be lost as mice are successively bred to the parental strain. Such back-crossing is often required to generate a near pure bred strain of mouse. There are a lot of thoughts over the nuances of breeding mice and one should consult the literature for more details related to this critical point (JAX Labs has outstanding literature).
2. Always check for proper expression of your gene (mRNA and protein) in homozygous floxed mice.
3. The FRT flanked neo cassette should be removed by breeding to a FLP recombinase mouse to avoid potentially untoward effects of Neo on endogenous gene expression (hence need for step 2 above).
4. Test the fidelity of your Cre driver by crossing with a reporter mouse (such as R26R lacZ).
5. Unless you are 100% certain as to where the Cre allele integrated (which is rare), such mice should be maintained in a hemizygous state, that is only one copy of the Cre allele; mice are not bred to homozygosity. The reason for maintaining hemizygous transgenic mice relates to the fact that some 15% of transgene integrations result in defective phenotypes when bred to the homozygous state. In fact, since most of the genome is transcribed into non-protein coding RNA of ill-defined function, it is likely the actual percent is much higher!
6. We generally maintain the Cre allele in male floxed mice and breed to homozygous floxed females because of quirky Cre activity in the oocyte that can result in the loss of one allele in all tissues of offspring. While this unpredicted activity has been reported in the male germ cells (see *Physiol. Genomics* **35**:1-4, 2008) it appears to occur for some reason more often in female germ cells (see references in *Nat. Immunol.* **8**:665-68, 2007). One should be aware of these peculiarities and address them through careful genotyping and recordkeeping.
7. Rapidly determine whether deletion of your gene results in developmental arrest by genotyping neonates (this does require UCAR approval).
8. Tamoxifen is fine to use (for inducible Cre drivers) so long as the dose is less than 40 μg/g weight.
9. Finally, please consider sharing your mice and possibly submitting them to JAX Labs in order to safeguard the knockout for long term use.

Making a knockout mouse can be a very rewarding experience with years of potential usage. The key things to remember along the way are fastidious planning, diligent validation of sequence fidelity, Cre-mediated excision, sage breeding (avoid genetic drift by “refreshing” breeder colonies at least once every 10 generations) and careful record keeping of your line.

“In Focus”

The focus of my research is the regulation of gene expression during development of the cardiovascular system and pathological remodeling of the heart. Specifically, I am interested in answering the question: How does the cell respond to changes in its environment by regulating the level of a signal-responsive gene program? To address this question my laboratory

In Focus continued…

will focus on understanding how the regulation of transcription by serum response factor (SRF) and its co-factors, myocardin-related transcription factor (MRTF) - A and MRTF-B, impinge upon normal development and how MRTF-A and MRTF-B promote a response to stress in the heart. Recent studies demonstrate that MRTF-A and MRTF-B, which are broadly expressed in the embryo and adult, respond to various cues that result in their nuclear accumulation and the activation of SRF target genes. As such, MRTFs represent exciting regulators of organogenesis and stress responsive gene activation.

Eric Small, PhD

We have recently found that MRTF-A promotes the development of pathological fibrosis in the heart following myocardial infarction by inducing activation of the myofibroblast, the primary cell type responsible for extracellular matrix deposition following injury. We are currently determining the mechanism responsible for MRTF-A activation and nuclear translocation during cardiac remodeling and how MRTF-A promotes myofibroblast differentiation. We have also recently identified a subset of microRNAs, which act to "fine-tune" the expression of target genes that are directly regulated by MRTF-A and may control various aspects of cardiovascular homeostasis. Finally, we are in the process of characterizing pharmacological agents that can modulate MRTF-A activity and myofibroblast activation. Understanding how MRTFs and microRNAs regulate gene expression during normal and diseased states using cell biological approaches and mouse genetics is the main focus of my laboratory's research, and has the potential to lead to exciting new therapies for the treatment of cardiac remodeling, the leading cause of morbidity and mortality in the United States.

Recent Publications

Yamakuchi, M., Yagi, S., Ito, T., Lowenstein, C.J. MicroRNA-22 Regulates Hypoxia Signaling in Colon Cancer Cells. PLoS One. 6(5):e20291. 2011

Shi, F., Sottile, J. MT1-MMP regulates the turnover and endocytosis of extracellular matrix fibronectin. J. Cell Sci. 2011. (in press)

Morrell, C.N., Maggirwar, S.B. Recently recognized platelet agonists. Current Opinion in Hematology. 2011

Morrell, C.N., Srivastava, K., Swaim, A., Lee, M.T., Chen, J., Nagineni, C., Hooks, J.J., Detrick, B. Beta interferon suppresses the development of experimental cerebral malaria. Infection and Immunity. 79(4):1750-8. 2011

Baldwin, W.M. 3rd, Kuo, H.H., Morrell, C.N. Platelets: versatile modifiers of innate and adaptive immune responses to transplants. Current Opinion Organ Transplantation. 2010

Ramesh, S., Morrell, C.N*., Tarango, C., Thomas, G.D., Yuhanna, I.S., Giradi, G., Herz, J., Urbanus, R.T., de Groot, P.G., Thorpe, P.E., Salmon, J.E., Shaul, P.W., Mineo, C. Antiphospholipid antibodies promote leukocyte-endothelial cell adhesion and thrombosis in mice by antagonizing eNOS via β2GPI and apoER2. Journal of Clinical Investigation. 121(1):120-31. 2011

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Shi, G., Morrell, C.N. Platelets as initiators and mediators of inflammation at the vessel wall. Thrombosis Research. 127(5):387-90. 2011

Belmonte, S.L., Blaxall, B.C. G-Protein Coupled Receptor Kinases as Therapeutic Targets in Cardiovascular Disease. Circ Research. 109(3):309-19. 2011

Shi, X., Yan, C., Nadtochiy, S.M., Abe, J., Brookes, P.S., Berk, B.C. p90 ribosomal S6 kinase regulates activity of the renin-angiotensin system: A pathogenic mechanism for ischemia-reperfusion injury. J Mol Cell Cardiol. 51(2):272-5. 2011

World, C., Spindel, O.N., Berk, B.C. Thioredoxin-Interacting Protein Mediates TRX1 Translocation to the Plasma Membrane in Response to Tumor Necrosis Factor-{alpha}: A Key Mechanism for Vascular Endothelial Growth Factor Receptor-2 Transactivation by Reactive Oxygen Species. Arterioscler Thromb Vasc Biol. 31(8):1890-7. 2011

Aguilar, F., Belmonte, S.L., Ram, R., Noujaim, S.F.,Dunaevsky, O., Protack, T.L., Jalife, J., Todd, Massey, H., Gertler, F.B., Blaxall, B.C. Mammalian enabled (Mena) is a critical regulator of cardiac function. Am J Physiol Heart Circ Physiol. 300(5):H1841-52. 2011

Albinsson, S., Skoura, A., Yu, J., DiLorenzo, A., Fernández-Hernando, C., Offermanns, S., Miano, J.M., Sessa, W.C. Smooth muscle miRNAs are critical for post-natal regulation of blood pressure

and vascular function. PLoS One. 22;6(4):e18869. 2011

Zhao, J., Wang, W., Ha, C.H., Kim, J.Y., Wong, C., Redmond, E.M., Hamik, A., Jain, M.K., Feng, G.S., Jin, Z.G. Endothelial Grb2-associated binder 1 is crucial for postnatal angiogenesis. Arterioscler Thromb Vasc Biol. 31(5):1016-23. 2011

Xie, W.B., Li, Z., Miano, J.M., Long, X., Chen, S.Y. Smad3-mediated myocardin silencing: a novel mechanism governing the initiation of smooth muscle differentiation. J Biol Chem. 29; 286(17): 15050-7. 2011

Streb, J.W., Long, X., Lee, T.H., Sun, Q., Kitchen, C.M., Georger, M.A., Slivano, O.J., Blaner, W.S., Carr, D.W., Gelman, I.H., Miano, J.M. Retinoid-induced expression and activity of an immediate early tumor suppressor gene in vascular smooth muscle cells. PLoS One. 6(4):e18538. 2011

Park, C., Hennig, G.W., Sanders, K.M., Cho, J.H., Hatton, W.J., Redelman, D., Park, J.K., Ward, S.M., Miano, J.M., Yan, W., Ro, S. SRF-dependent microRNAs regulate gastrointestinal smooth muscle cell phenotypes. Gastroenterology. 141:164-175, 2011.

Long, X., Miano, J.M. TGFβ1 utilizes distinct pathways for the transcriptional activation of microRNA 143/145 in human coronary artery smooth muscle cells. J.Biol.Chem. 286:30119-30129, 2011.

Benson, C.C., Long, X., Zhou, Q., Miano, J.M. Method of identifying single nucleotide polymorphisms in the human CArGome. Physiological Genomics. In Press

Long, X., Slivano, O.J., Cowan, S.L., Georger, M.A., Lee, T-H., Miano, J.M. Smooth muscle calponin: an unconventional CArG-dependent gene that antagonizes neointimal formation. Arterioscler.Thromb.Vasc.Biol. In Press

Jons, C., O-Uchi, J., Moss, A.J., Reumann, M., Rice, J.J., Goldenberg, I., Zareba, W., Wilde, A.A., Shimizu, W., Kanters, J.K., McNitt, S., Hofman, N., Robinson, J.L., Lopes, C.M. Use of mutant-specific ion channel characteristics for risk stratification of long QT syndrome patients. Sci Transl Med. 3(76):76ra28. 2011

Pang, J., Xu, X., Getman, M.R., Shi, X., Belmonte, S.L., Michaloski, H., Mohan, A., Blaxall, B.C., Berk, BC. G proteincoupled receptor kinase 2 interacting protein 1 (GIT1) is a novel regulator of mitochondrial biogenesis in heart. J Mol Cell Cardiol. 2011

Small, E.M., Olson E.N. Pervasive roles of microRNAs in cardiovascular biology. Nature. 469, 336-342. 2011

Small, E.M., O'Rourke, J.R., Moresi, V., Sutherland, L.B., McAnally, J., Gerard, R.D., Richardson, J.A., Olson, E.N. Regulation of PI3 Kinase/Akt signaling by muscle-enriched microRNA-486. Proc Natl Acad Sci USA. 107, 4218-4223. 2010

Small, E.M., Thatcher, J.E., Sutherland, L.B., Kinoshita, H., Gerard, R.D., Richardson, J.A., DiMaio, J.M., Sadek, H., Kuwahara, K., Olson, E.N. Myocardin-related transcription factor-A controls myofibroblast activation and fibrosis in response to myocardial infarction. Circ Research. 107, 294-304. 2010

Small, E.M., Sutherland, L.B., Rajagopalan, K., Wang, S., Olson, E.N. MicroRNA-218 regulates vascular patterning by modulation of Slit-Robo signaling. Circ Research. 107, 1336-1344. 2010

New Grants/Awards

Dr. Burns Blaxall received The Inaugural URMC CTSI "Incubator" Grant for interdisciplinary work to develop small molecules with common molecular targets in both heart failure and HIV-mediated neurodegeneration.

Dr. Jun-ichi Abe was awarded funding by the NIH for his R01 entitled, *Ubiquitin-like protein modification in diabetic cardiomyopathy.*

Dr. Nhat Tu Le, a postdoc in the Abe Lab was a chosen finalist for the 2011 AHA scientific sessions, Melvin L. Marcus Young Investigator Award in Cardiovascular Sciences for her article, *p90RSK Inhibits C Terminus of Hsc70-interacting Protein (CHIP) Ubiquitin E3 Ligase Activity and Promotes Cardiac Apoptosis Through ERK5-Ser 496 Phosphorylation and Association;*
By Le, N.T.*, Takei, Y., Shishido, T., Woo, C.H., Chang, E., Heo, K.S., Lee, H., Lu, Y., Morrell, C., Oikawa, M., McClain, C., Wang, X., Tournier, C., Molina, C.A., Taunton, J., Fujiwara, K., Yan, C., Patterson, C., Yang, J., Abe, J. Congratulations, Nhat Tu!!

Dr. Craig Morrell was awarded the Provost Multidisciplinary Award from the University of Rochester

Dr. Craig Morrell was awarded funding by the NIH for his R01 entitled, *Platelet and T-cell Interactions.*

Comings and Goings

Welcome to our newly hired Aab CVRI Personnel:

The Berk Lab is happy to welcome back Dr. Elaine Smolock who was appointed Research Assistant Professor on July 1st.

Dr. Eric Small, Assistant Professor, will join the CVRI on September 1st.

Hannah Cushman
Lab Technician
Abe Lab

Dr. Sam Majumder
Postdoctoral Research Associate
Berk Lab

Dr. Lian Wang
Postdoctoral Research Associate
Berk Lab

Sara Ture
Lab Technician
Morrell Lab

Best Wishes and a Fond Farewell to:

Dr. Padma Baskaran (Berk Lab) relocated to Wyoming to be with her husband who recently obtained a faculty appointment at the University of Wyoming

Eugene Chang, pre-doctoral student (Abe Lab) completed his PhD this spring and is now attending Medical School at the University of Iowa.

Dr. Amit Dhamoon, Postdoctoral Research Associate (Lowenstein Lab) relocated to Syracuse to be closer to his family

Dr. Jin Ouchi (Lopes Lab) completed his postdoctoral training and has taken a faculty position in the Center for Translational Medicine at Thomas Jefferson University in Philadelphia.

Dr. Abha Sahni (Alexis Lab) relocated to Houston to be with her husband who recently obtained a faculty appointment at the University of Texas.

Xi Shi, pre-doctoral student (Berk Lab) completed her PhD in June and will temporarily remain in the Berk Lab as a postdoc.

Congratulations!

Well, Aab CVRI hockey players continue to thrive even in the heat of summer with an astounding championship win at the 1st annual Juvenile Diabetes Tournament held all day at Thomas Creek Park Ice Arena on July 9th. Burns Blaxall, Joe Miano and Craig Morrell strapped on the skates and played 5 consecutive games to win 1st overall. It was grueling day, but they helped raise over $1,700 for a very good cause.

Special Thanks!

Many thanks to Sara Ture (Morrell Lab), Orazio Slivano (Miano Lab), Christine Christie (Berk lab), Kyung Ke Ao (Micro-Surgery Core) and Deb Haight (Taubman/White lab) for their willingness to extend their time and effort to cleaning out the animal procedure rooms in preparation of the AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care)** visit at the CVRI. We are happy to say that the University was re-accredited by AAALAC! These techs are strong proponents of keeping our facility operating smoothly and efficiently. We also thank Alberti & Associates for completing many repairs in the vivarium in preparation for the AAALAC visit.

**When animals are used, AAALAC works with institutions and researchers to serve as a bridge between progress and animal well-being. This is done through AAALAC's voluntary accreditation process in which research programs demonstrate that they meet the minimum standards required by law, and are also going the extra step to achieve excellence in animal care and use.
The AAALAC International accreditation program evaluates organizations that use animals in research, teaching or testing. Those that meet or exceed AAALAC standards are awarded accreditation. After an institution earns accreditation, it must be re-evaluated every three years in order to maintain its accredited status.