DEVELOPMENTAL BIOLOGY OF THE CARDIOVASCULAR SYSTEM

Organizers: Judith L. Swain and Bernardo Nadal-Ginard

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Developmental Biology (Joint)

P 001 THE ROLE OF PAX GENES IN MAMMALIAN DEVELOPMENT, Peter Gruss, Max Planck Institute of Biophysical Chemistry, Am Faßberg, D-3400 Göttingen/Germany.

In order to study molecular mechanisms underlying the development of the nervous system we have utilized mouse paired box containing genes (Pax genes) which were cloned on the basis of their homology to the *Drosophila* segmentation gene "paired". Seven members of this family are expressed in a spatial and temporal specific manner in the developing and adult nervous system. In particular, *Pax-3, 6,* and *7* are active in cells of the ventricular zone of the developing neural tube. They respond to a notochord-floorplate induction cascade in a concentration or position-dependent manner. Therefore, since the pattern is set-up by a notochord-floorplate effect in cells of the ventricular zone which subsequently generate the terminally differentiated cells in a ventral to dorsal progressive manner these genes are good candidates which could control a part of this genetic gram. In order to study the role of these genes we have examined loss-of-function mutations. *Pax-3* was correlated with a pre-existing mutant named "*splotch*" (*Sp*). This mutant shows exencephalus, spina bifida and partial lack of spinal ganglia. One of the advantages of working with the mouse as a model system is the relative ease to find homologous genes in the human system. For this particular gene a human homologue was identified in individuals with "Waardenburg Syndrom I". Several mutations have been discovered in individual cell imposing the DNA-binding of the mutated Pax-3 gene. Mutants with mutations in these genes in conjunction with mutational analyses will allow us to gain insight into molecular mechanisms underlying the development of the nervous system.
Our current research is directed towards establishing the network of control events. Experiments which shed light on the molecular function of these genes will be reported.

P 002 A COMMUNITY EFFECT IN MUSCLE DEVELOPMENT, J.B. Gurdon, E. Tiller and K. Kato, Wellcome CRC Institute, Cambridge, U.K.

In amphibia, and probably in most other animals, muscle is first formed as a result of a mesodermal induction. In Xenopus, vegetal blastula cells are believed to release growth factor-like substances which redirect animal hemisphere cells from an ectodermal into a mesodermal pathway of differentiation. We have used single cell transplantation of mid-gastrula mesoderm cells to determine the time in development when these cells are fully committed to a muscle fate. We find that single muscle precursor cells are not fully committed until the late gastrula to early neurula stage, that is long after the time (early gastrula) when cells can no longer emit or receive the mesoderm inducing factors. We conclude that a second type of cell interaction takes place during middle and late gastrulation and that this is required for cells to complete the process of commitment to muscle differentiation. Evidence for an interaction between cells of like type, that is among those taken from the dorso-lateral region of a gastrula, comes from experiments in which cells are transplanted singly or in aggregates into ectodermal sandwiches. We find that the muscle differentiation marker XMyoD is expressed only when cells are surrounded by like neighbours, and we have termed this a community effect. This type of cell interaction among cells from the same region is different from the mesoderm-forming induction and takes place during gastrulation. We will describe a range of cell transplantation and reaggregation experiments which characterise this new type of cell interaction in muscle development.

P 003 SPONTANEOUS ACTION POTENTIAL ACTIVITY AND THE PATTERNING OF CONNECTIONS DURING CNS DEVELOPMENT, C.J. Shatz, Division of Neurobiology, Department of Molecular and Cell Biology, University of California, Berkeley, CA.

Neural connections in the adult central nervous system are highly organized. In the visual system for example, retinal ganglion cells (RGCs) send their axons to target neurons in the LGN of the thalamus in such a way that axons originating from the two eyes terminate in adjacent but non-overlapping eye-specific layers. During early development, however, inputs from the two eyes are intermixed, and the adult pattern emerges gradually as axons from the two eyes sort out to form the layers. How do these layers form? Evidence does not favor the existence of specific molecular cues that designate left and right eye LGN zones. Rather, experiments suggest that the sorting out process, even though it occurs before vision, requires specific patterns of action potential activity and involves synaptic competition between axons from the two eyes for LGN neurons.

Experiments indicate that the machinery necessary for an activity-dependent competition is present during the relevant developmental times. RGC axons form synapses even prior to the onset of layer formation; some of these synapses, later eliminated, are initially located in territory that will ultimately belong exclusively to the other eye. Physiological studies demonstrate that about 90% of LGN neurons initially recieve convergent excitation from both optic nerves, whereas after the layers have formed, only about 10% do (1). Moreover, experiments have demonstrated that RCGs can generate action potentials spontaneously even in utero (2). To investigate whether activity is necessary for layer formation, we blocked it by infusing tetrodotoxin. Layers did not form, and in fact, RGC axons were highly abnormal in shape (3): axons formed terminal arbors across the entire LGN without regard for their normal laminar boundaries. These observations suggest that the segregation of RGC axons from the 2 eyes to form the set of eye-specific LGN layers requires the formation and selective elimination of functioning synaptic connections between RGC and LGN neurons.

Activity per se is not sufficient to promote the formation of eye-specific layers in the LGN; correlations in the firing of neighboring retinal ganglion cells are required (4). Using a multielectrode array, physiological recordings were made from up to 100 ganglion cells simultaneously in fetal cat or neonatal ferret retinas in vitro (5). Results showed that cells can fire spontaneously generated action potentials that are synchronized with each other in time and space: the pattern of RGC firing is wavelike, with nearest neighbors firing in near synchrony. Waves of activity sweep across the retina at a velocity of about 100 µm/sec. These waves are of the appropriate pattern and at the appropriate developmental times to be useful in providing essential information to postsynaptic LGN neurons concerning the location and eye of origin of the presynaptic ganglion cell axons. However, LGN neurons must be able to detect these correlations and strengthen or weaken these synaptic inputs accordingly. To study such synaptic mechanisms, we (6) made whole cell recordings from slices of neonatal ferret LGN in vitro. Tetanic stimulation of RGC axons produced marked and maintained (up to 2 hours) enhancement of synaptic transmission, indicating that retinogeniculate synapses can indeed undergo activity-dependent changes in strength. Thus, such changes may underlie the process of synaptic rearrangement occurring during the formation of layers within the LGN. Since spontaneously generated activity is present elsewhere in the developing CNS, these observations suggest a general role for neural activity in promoting the formation of precise connections. Supported by NSF IBN 9212640, NIHMH 48108, and The March of Dimes.

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Cardiac Induction/Determination

P 004 ZEBRAFISH HEART DEVELOPMENT: PROSPECTS FOR GENETIC ANALYSIS, Didier Stainier, Robert Lee , Mark C. Fishman, Cardiovascular Research Center, Massachusetts General Hospital, and Harvard Medical School, Boston, MA 02159

The zebrafish, *Brachydanio rerio*, offers several advantages for study of the developing cardiovasculature. The embryo is transparent so that individual cells of the heart and blood vessels are visible. The adult is small, hardy and fertile, so genetic screens for cardiac mutations are feasible. We have analyzed the cell lineage and fashioning of the heart, and isolated several cardiac mutants as a first step in the dissection of this system. We find that cardiac precursors arise in a predictable location of the blastoderm, and, by injection of lineage tracers, we can identify them by the 512-cell stage. Even before gastulation distinct lineages are evident for different chambers and for endocardium or myocardium. By two days most of the major morphogenetic milestones have been passed, including the generation of the endocardial and myocardial tubes, separation into chambers separated from each other by valves, looping of the heart to the right, initiation of the heart beat, and establishment of a functional circulation. We have isolated and begun to analyze several recessive lethal mutants that perturb different components of this process.

P 005 CELLULAR AND MOLECULAR CONTRIBUTIONS OF THE NEURAL CREST IN CARDIOVASCULAR DEVELOPMENT, Margaret L. Kirby and Michelle Rhodes, Medical College of Georgia, Augusta, GA

Normal development of the heart and great arteries requires the participation of cells derived from the neural crest located on the neuraxis between the mid-otic placode and the caudal border of somite 3. This region of the neural crest has been designated the cardiac neural crest because of its importance in cardiovascular development. The cells from the cardiac neural crest migrate to pharyngeal arches 3, 4 and 6 via the circumpharyngeal region. Once in the pharyngeal arches, they provide an ectomesenchymal sheath for the pharyngeal arch arteries. Unlike the more cranial pharyngeal arteries that disappear early, the arteries derived from the caudal three pharyngeal arches will persist into adulthood as the bilaterial brachiocephalic arteries, the single ascending aorta and its arch, and the bilateral proximal pulmonary arteries. Some of the ectomesenchymal cells derived from the neural crest will migrate from the pharyngeal arches into the outflow tract of the developing heart and participate in outflow septation. Removal of the cardiac neural crest results in two types of malformations of the heart: outflow septation defects where the truncus arteriosus persists as a single outflow vessel, and alignment defects in which the aorta and pulmonary artery are separate but arise inappropriately from the left and right ventricles. These two major classes of defects have at least two different etiologies.[1] Division of the aorta and pulmonary trunk is disturbed when a critical number of ectomesenchymal cells from the cardiac neural crest does not reach the outflow tract. Alignment is altered by an as yet unknown mechanism that is transmitted from the pharyngeal arches upstream to the heart during the period of looping. Development of the pharyngeal arch arteries and pharyngeal arch mesenchyme are altered early in embryogenesis, and these changes are coupled with hemodynamic abnormalities in the heart. In this situation the heart either does not loop correctly or unloops to result in defective alignment. Several studies have shown that the cardiac neural crest is unique in its ability to support cardiovascular development. This may be due to determination of segmental identity by a variety of homeotic genes.[2] We have been interested in unique proteins produced by the neural crest in the pharyngeal region, that would be candidates for supporting normal development of the pharyngeal arch arteries. Several proteins can be identified on two dimensional gels of pharyngeal regions of stage 14 and 18 embryos with the normal complement of cardiac neural crest, that are missing from the pharyngeal regions of embryos lacking the cardiac neural crest. Interestingly, these differences are lost by stage 21 of development[3] In order to characterize unique products of the cardiac neural crest in the pharyngeal region of stage 14 chick embryos, we have performed subtractive hybridizations of pharyngeal regions of stage 14 embryos with intact cardiac neural crest versus pharyngeal regions of embryos lacking the cardiac neural crest. One subtracted product found only in embryos with cardiac neural crest identifies a message of about 2 kb and can be conceptually translated to a protein that is 40-50 kD. One region of 45 residues of the putative protein shows striking homology with two bacterial heat shock proteins in the 47 kD class. The 3' region shows about 45% homology with the 3' untranslated region of a 47 kD chicken fibroblast heat shock protein identified by Takechi et al.,[4] which is a collagen-binding stress protein expressed during the differentiation of F9 teratocarcinoma cell, and assumed to be a molecular chaperone specific to collagen. Further characterization of this message will be presented.

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Supported by PHS grant HL36059.

Growth Factors and Development

P 006 TGF-ß SIGNALS GROWTH INHIBITION THROUGH A HETEROMERIC KINASE RECEPTOR. Joan Massagué, Liliana Attisano, Juan Cárcamo, Fernando López-Casillas, Jacqueline Doody, Jeffrey L. Wrana and Alejandro Zentella. Howard Hughes Medical Institute and Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Transforming growth factor-ß (TGF-ß) belongs to a large family of secretory polypeptides that are broadly multifunctional as regulators of cell growth, differentiation and tissue organization, expressed in many cell types, and highly conserved in evolution. Cell interaction with the three mammalian isoforms, TGF-ß1, ß2 and ß3, involves various membrane proteins including the ubiquitous high affinity receptors I and II, two type III receptors (betaglycan and endoglin), and other proteins. Betaglycan presents TGF-ß to receptor II. Receptor II belongs to a new family of transmembrane protein kinases,and signals TGF-ß responses in association with receptor I. Receptors I and II are interdependent components of a heteromeric kinase receptor complex: receptor I requires receptor II to bind TGF-ß, and receptor II requires receptor I to signal. This mode of operation points to fundamental differences between this receptor and the tyrosine protein kinase receptor family.

Activation of this receptor complex induces a delay in cell progression through the G1 phase of the proliferative cycle. This effect involves an inhibition of G1 cyclin-dependent protein phosphorylation events including Rb protein phosphorylation. The ability of TGF-ß to override the positive signals of growth promoting factors in mid-to-late G1 phase has two possible outcomes depending on the cell type. In some cells, TGF-ß action leads to a reversible growth arrest whereas in others such as L6E9 myoblasts, it triggers terminal differentiation with permanent withdrawal from the cell cycle.

P 007 MECHANISM OF ACTIVATION OF LATENT TGF-ß. Daniel B. Rifkin, Soichi Kojima, Mayumi Abe, John Harpel and Irene Nunes. Department of Cell Biology, New York University Medical Center, New York, NY 10016.

The proliferative response of the vascular system is under tight regulatory control. Endothelial cells can remain quiescent for years but then be stimulated to proliferate rapidly by various angiogenic stimuli. The proliferative response usually is eventually eliminated and the endothelium resumes a state of quiescence. A number of positive and negative regulatory molecules have been implicated in this process. One of the potential negative regulators is transforming growth factor ß (TGF-ß) that can block endothelial mitosis, migration, and invasive capacity. However, since TGF-ß appears to be initially released from cells in a latent form the generation of the active molecule is of potential importance. We have found that contact between endothelial cells and smooth muscle cells or pericytes stimulates the transformation of latent TGF-ß to TGF-ß. This process requires cell-cell contact, the surface generation of plasmin, and the participation of several proteins that serve to create a cell surface activation assemblage. These additional proteins are the cation-independent mannose-6-phosphate/IGF-II receptor, the latent TGF-ß binding protein and the type II tissue transglutaminase. Interference with anyone of these three proteins prevents TGF-ß formation in heterotypic cell cultures. TGF-ß can be formed in homotypic cultures after treatment of the cells by agents such as retinoids or basic fibroblast growth factor. The details of these processes and specific consequences will be discussed.

P 008 MOLECULAR MECHANISMS OF TGFβ SIGNAL TRANSDUCTION IN CARDIAC MUSCLE CELLS. Michael D. Schneider*†‡, W. Robb MacLellan*, Thomas Brand*, Maha Abdellatif*, Te-Chung Lee†, and Robert J. Schwartz†‡. Departments of *Medicine, †Cell Biology, and ‡Molecular Physiology & Biophysics, Baylor College of Medicine, Houston, TX 77030

TGFβ1, the prototype for a growth factor superfamily, selectively up-regulates a set of genes associated with embryonic myocardium in neonatal cardiac myocytes, resembling the fetal phenotype produced by load. TGFβ in adult heart has been suggested as an autocrine or paracrine factor mediating signals initiated by mechanical stress. Moreover, from findings with explants and ES cells, TGFβ1 and related peptides are thought to be positive or negative regulators of cardiac myogenesis itself. Molecular mechanisms for TGFβ signal transduction and transcriptional control are largely unknown.

Skeletal α-actin (SkA) is representative of genes for fetal isoforms up-regulated in myocardium both by load and TGFβ. The proximal 202 bases of the SkA promoter, which suffice for full cardiac-restricted transcription, contain three serum response elements (SRE), cognate sites for serum response factor (SRF), a member of the MADS-box transcription factor family. Previously characterized TGFβ-response elements include AP-1 and CTF/NF-I sites, neither of which is present in this region. The most proximal SRE (SRE1) confers tissue-specificity as an isolated element upstream from a neutral promoter. To determine what cis-acting sequences might cooperate with SRE1, which sequences impart TGFβ1 responsiveness, how these relate to motifs for tissue-specificity, and what trans-acting factors interact with them in cardiac myocytes, we have correlated a mutational analysis of the SkA promoter with defined changes in DNA-protein recognition. Whereas all three SREs were indispensable for transcription in skeletal muscle cells, basal cardiac-restricted transcription in Percoll-purified ventricular myocytes involved only SRE1. SRE1, -2, and -3 possessed equivalent affinity for SRF in cardiac myocyte nuclear extracts. SRE1, unlike the distal SREs, also bound a second factor, YY1, a C_2H_2 zinc finger trans-acting factor. Mutations were engineered to block recognition of SRE1 by SRF and YY1 independently. Two mutations that impair SRF binding virtually abolished basal and induced expression of the SkA promoter, whereas mutation of the YY1 binding site increased both basal and TGFβ1-dependent activity. Thus, disproportionate dependence on SRE1 cannot explained by differential affinity for SRF or by selective binding of YY1, a negative regulator of the gene. Mobility-shift experiments revealed that cardiac myocytes contain factors that recognize potential Sp1 and TEF-1 binding sites, proximal to SRE1, that were unimportant for SkA transcription in skeletal muscle: by contrast, these sites were obligatory, in concert with SRE1, both for efficient basal transcription in cardiac myocytes and for induction by TGFβ. Interaction of SRF with mesoderm-restricted homeodomain proteins such as MHox may also help resolve the paradox that SREs direct cardiac and skeletal muscle-restricted transcription of the SkA promoter.

Dominant-negative mutations provide a means to delineate the circuitry for TGFβ signaling. The recently cloned type II TGFβ receptor (TβRII) has a Ser/Thr kinase domain as a predicted signaling domain distinct from all growth factor receptors with Tyr kinase activity (RTK). To test whether a kinase-deficient TβRII can suppress signaling by wild-type receptor, as shown for kinase-defective RTKs, we truncated TβRII (ΔTβRII), omitting cytoplasmic residues 280-560. ΔTβRII served as a dominant inhibitor of TGFβ-induced SkA transcription, but did not interfere with basal cardiac-restricted expression. The N17 allele of c-H-ras, well-characterized as a dominant-inhibitor of RTK signal transduction, likewise blocked TGFβ induction of SkA. Thus: (1) Signal transduction for TGFβ, as for growth factors which work through RTKs, involves a ras-dependent pathway. (2) Kinase-defective TβRII is a dominant inhibitor of TGFβ effects, which should facilitate efforts to determine the function of endogenous TGFβs in cardiac hypertrophy or development.

Transcriptional Control

P 009 POSITIVE AND NEGATIVE REGULATORS OF THE CARDIAC MUSCLE GENE PROGRAM, K. Chien, H. Zhu, K. Lee, A. Brown, V. Nguyen, S. Navankasattussas, M.D. Zhou and Y. Zou, Department of Medicine, Center for Molecular Genetics and the AHA-Bugher Foundation Center for Molecular Biology, University of California, San Diego, La Jolla, CA 92093

The rat cardiac MLC-2 gene has served as a valuable model system to identify the cardiac muscle factors which control chamber specification during mammalian cardiogenesis. In independent lines of transgenic mice, a 250 bp MLC-2 promoter fragment confers ventricular-specific expression to a luciferase reporter gene, with negligible expression in other tissues. Mutations in the conserved HF-3 site leads to a marked up-regulation (> 100 fold) of luciferase activity in muscle sub-types which do not express the endogenous gene, e.g. uterus. Luciferase activity in non-muscle tissues (kidney, liver, spleen) is not increased over background, and is not decreased in the ventricular chambers. Thus, HF-3 serves as a negative regulatory element to suppress expression of cardiac genes in closely related muscle sub-types. Point mutations in the conserved HF-1a element result in background levels of reporter activity in ventricular myocardium, and > 100-fold increases in reporter activity in non-cardiac tissues (muscle and non-muscle (kidney, etc.)). Thus, depending upon cellular context, the HF-1a element may serve as both a positive and negative regulator of MLC-2 promoter activity. Mutations in the neighboring HF-1b site severely cripple ventricular-specific expression, with little effect on luciferase activity in non-cardiac tissues, suggesting that HF-1b serves primarily as a positive regulatory element. These studies in transgenic mice suggest that regional specification of the MLC-2 gene may be due to a unique combination of regulatory pathways that include positive regulatory elements (HF-1b); lineage restricted negative regulatory elements (HF-3); and an element (HF-1a) which can serve as a positive and negative regulator, depending upon the cell context. To dissect these individual pathways, we have isolated a novel SP-1 related zinc finger gene product (HF-1b), that activates the expression of the rat cardiac MLC-2 gene via an E-box independent pathway. Three independent criteria (binding site specificity, removal of the endogenous HF-1b binding activity with fusion protein antibodies, and site-specific transcriptional activation) indicate that this novel, tissue-restricted zinc finger protein mediates an E-box independent pathway for cardiac muscle specific expression of the MLC-2 gene. Analogous studies of the cardiac muscle factors which bind to the HF-3 and HF-1a regulatory elements are currently in progress.

P 010 TRANSCRIPTIONAL COMPLEXES IN HEART AND MUSCLE: ROLE IN MYOCARDIOPATHIES AND DIFFERENTIATION,
Larry Kedes, Elzbieta Biesada, Masahiko Kurabayashi, Jeyaseelan Raju and Vittorio Sartorelli,
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Investigations into the mechanisms of models of several myocardiopathies has uncovered selective and specific mechanisms of transcriptional dysfunction in response to chemotherapeutic agents and some HIV gene products. Doxorubicin (Dox, Adriamycin), a potent chemotherapeutic agent with a major side effect of cardiomyopathy, selectively inhibits muscle gene expression in cardiac muscle cells. Dox also completely prevents the expression of muscle-specific genes in skeletal muscle cells (C2 cells) without significantly altering non-muscle gene transcripts. Transient transfection of CAT reporter constructs indicated that Dox inhibits muscle-specific promoter/enhancer activity in a dose dependent manner. Both Dox and the HIV protein Tat inhibited the ability of MyoD to *trans*-activate muscle-specific reporter genes. MyoD cDNA was constitutively expressed in 10T1/2 cells. Such cells treated with Dox failed to turn on endogenous MyoD and myogenin genes. The Tat expressing cells failed to express transfected muscle specific promoters but Tat expression had no effect on the expression of non muscle specific promoters. Interestingly, Id RNA levels were significantly increased in Dox-treated C2 and LTR-MyoD 10T1/2 cells. Furthermore, the 5′-flanking region of the Id2 gene mediates the inducible expression by Dox through selective up-regulation of Id gene transactivators. Finally, over expression of E2-5, which can form inactive heterodimers with Id overcomes the Dox-induced suppression of MyoD *trans*activation activity in 10T1/2 cells. Thus Dox inhibits myogenesis by interfering with the function of MyoD protein through inducing Id gene expression. Since basic helix-loop-helix (bHLH) proteins play a prominent role in myocardial gene expression Id up-regulation in myocardium in response to Dox may be responsible, at least in part, for the myocardiopathy associated with its clinical toxicity. The molecular effects of Tat expression in this system are being investigated. Specific transcription factors are implicated in the expression of muscle specific genes. Required binding sites for four nuclear proteins, serum response factor (SRF), SP1, MyoD, and TUBF, are located between -110 and -35 base pairs upstream from the start of transcription of the human cardiac α-actin gene. A high molecular weight stable complex is formed when nuclear extracts from myogenic cells, but not from non-myogenic cells, are allowed to interact with the intact promoter. SRF, SP1 and MyoD are all required for complex stability and no intermediate complexes form. Site specific mutations in individual binding domains prevents complex formation. TUBF does not participate in the complex and its binding to the promoter appears to increase if the complex is disrupted. The organization of this complex and the role of protein-protein interactions in its formation and stability are being investigated.

P 011 MOLECULAR CORRELEATES OF *SKI*-INDUCED MUSCLE DIFFERENTIATION, Ed Stavnezer, Craig Richmond, Hong Chen, Guoxing Zheng, and Clemencia Colmenares, University of Cincinnati College of Medicine, Cincinnati, OH

The nuclear proteins encoded by v-*ski* and c-*ski* are capable of inducing both cellular transformation and muscle differentiation. Although lacking previously characterized oligomerization or DNA binding domains, these proteins have been found to bind DNA in association with other cellular proteins. One sequence bound by *ski*-containing complexes is the consensus binding site of the NF-1 family of transcription factors, suggesting that *ski* might associate with one or more of the NF-1 proteins. Affinity chromatography with GST fusion proteins, and antibody supershift experiments support this idea and indicate that only a subset of NF-1 proteins interact with *ski*. Surprisingly, *ski* proteins also bind specifically to a second sequence that has not been previously identified as a transcription factor binding site. This sequence is present in the regulatory regions of several muscle-specific genes. When multiple copies of this sequence are placed upstream of a reporter gene, cotransfection with *ski* results in 10-50 fold activation. Using muscle-specific elements containing this sequence it appears that *ski* cooperates with myogenic regulatory proteins to activate gene expression. Physical studies indicate that pure *ski* protein is capable of binding this sequence specifically and that the binding species is either a homo-dimer or a homo-tetramer. Additional binding studies with GST fusions and in vitro translated proteins have mapped the *ski* domains involved in DNA binding and protein associations

Genetics of Cardiovascular Disease

P 012 MOLECULAR BASIS OF HCM AND ITS IMPLICATIONS FOR CARDIAC PATHOPHYSIOLOGY, Robert Roberts, Ali J. Marian, Qun-Tao Yu, Linda Bachinski, Adolph Mares, Molecular Cardiology Unit, Baylor College of Medicine, Houston, Texas, 77030

Familial hypertrophic cardiomyopathy (FHCM) is generally recognized as restricted to the heart. The recent documentation of mutations in beta-myosin heavy chain (βMHC) gene, being responsible for this disease, at least in those families linked to chromosome 14, raises several perplexing but intriguing problems as to the mechanism whereby the particular phenotype is induced. Beta-MHC is by far the predominant myosin of the heart with the αMHC being present in only 1% or 2% while the remainder being βMHC which is distributed throughout both the right and left ventricles and is the single most predominant protein of the myocardium. Furthermore, βMHC is a predominant myosin of skeletal muscle and in slow twitch fibers, which make up anywhere from 50% to 60% of skeletal muscle, it is by far the predominant contractile protein. A major concern then is why the hypertrophy in most individuals is localized primarily to the ventricular septum. We recently obtained skeletal and cardiac biopsies from a patient with FHCM known to have the missense mutation in exon 13 of the βMHC gene. A skeletal biopsy was also obtained from a normal individual. The exons 12 to 14 were amplified via polymerase chain reaction and the product digested with DdeI restriction endonuclase. Electrophoretic analysis of normal skeletal muscle showed two fragments of 181 bp and 140 bp while cardiac and skeletal samples from affected individuals showed four fragments of 181 bp, 149 bp, 140 bp, and 32 bp, the latter due to the mutation in exon 13. Thus, the abnormality (mutation) in affected individuals is expressed in the mRNA of skeletal as well as cardiac muscle. To our knowledge there has not been detected any significant abnormality in function or morphology in skeletal muscle of individuals which this disease. This further perplexes the problem as to why it is localized to the septum in the heart. It has the implication that this abnormality in some way interacts with the environment and that the hypertrophy is a secondary phenomenon. There have now been a total of at least 11 missense mutations together with one deletion that have been identified in families with FHCM and the predominant phenotype appears to be the same for all mutations. Secondly, it is now recognized that there are at least one other locus other than that on chromosome 14 and yet the phenotype in these families is the same as that manifested in families localized to chromosome 14. All of the missense mutations are in the globular head of the myosin molecule and the deletion involves the tail end of the rod. None of the known domains have yet shown up in mutated form in part perhaps because they may be lethal. The nature of the mutations and how they relate to the amino acid charge and other features will be discussed and put into a perspective in terms of the implications for further *in vivo* structure function analysis.

P 013 GENETIC CONTROL OF RESPIRATORY CAPACITY IN CARDIAC AND SKELETAL MUSCLE, Rhonda S. Bassel-Duby, Kang Li, William J. Parsons, George A. Ordway, Randall W. Moreadith, Cynthia S. Smagula, Maria Hernandez, Jason M. Grayson, and R. Sanders Williams, University of Texas Southwestern Medical Center, Dallas, TX 75235.

Specialized subtypes of striated muscle (e.g. cardiac vs. fast skeletal vs. slow skeletal) differ markedly in their capacity for ATP production via mitochondrial respiration. We have examined genetic control mechanisms that respond to developmental or environmental cues to modulate mitochondrial biogenesis and expression of myoglobin in striated muscle. Recent studies of the human myoglobin gene have defined sequence motifs within the 5' flanking region that are essential for expression in both cardiac and skeletal muscle, as assessed by transient transfection in myogenic cell lines, direct injection of plasmid constructs into the myocardium of intact rats, and generation of germline transgenic mice. Muscle-specific enhancer function can be reconstituted with only three distinct elements: a TATA box, an A/T rich region that resembles but is distinct from binding sites for MEF-2 in other genes, and a cytosine-rich sequence motif (CCAC box). A cDNA clone encoding a protein that binds specifically to this latter element was selected from a λgt11 expression library. This protein has a limited region of sequence homology to nuclear proteins involved in liver-specific gene expression in mammals and to a class of homeotic genes in Drosophila. Other studies have focused on transcriptional regulation of MRP-RNA, a small RNA subunit of a mitochondrial RNA-processing enzyme hypothesized to be rate-limiting to mitochondrial DNA replication. Expression of this gene, which is transcribed by Pol III, is increased up to 20-fold in striated muscle by stimuli that augment mitochondrial biogenesis. In cultured myocytes, transcription from the MRP-RNA promoter requires an upstream region that includes octamer and Sp1 binding motifs. Both myoglobin and MRP-RNA are expressed during embryonic and postnatal development of the mouse heart but exhibit distinctive patterns of gene activation. These results will be discussed in the context of current models for control of gene expression in the heart.

Ion Channels (Joint)

P 014 SODIUM CHANNEL DEFECTS IN HUMAN NEUROMUSCULAR DISORDERS. Stephen C. Cannon, Department of Neurology and Howard Hughes Medical Institute, Massachusetts General Hospital, Boston, MA 02114.

The electrical excitability of muscle and nerve is mediated by voltage-gated ion channels. Several inherited neuromuscular disorders are caused by altered excitability of the sarcolemmal membrane and the underlying ion channel defects are now being elucidated at the molecular and functional levels. Hyperkalemic periodic paralysis (HPP) is inherited autosomal dominantly and is characterized by recurrent episodes of flaccid paralysis in association with raised serum K (5.5 to 7.5 mM range; normal 3.5 to 4.5). Attacks last minutes to hours and are not associated with sensory symptoms or alterations in consciousness. During an attack affected muscle fibers are depolarized and refractory to firing action potentials. Voltage-clamp studies of biopsied fibers showed that a non-inactivating TTX-sensitive current causes the depolarization. A related disorder, paramyotonia congenita (PC), presents as cold-induced stiffness due to electrical hyperexcitability of the sarcolemma with repetitive action potentials and impaired relaxation (myotonia). In some families, patients with HPP also have myotonia.

In all families tested to date, both HPP and PC are linked to the gene encoding the α-subunit of the skeletal muscle sodium channel (SCN4A) on 17q. Two mutations in SCN4A have been identified in families with HPP and 6 others have been found in association with PC. In all cases the mutation is a transversion of a single nucleotide which causes a substitution at one amino acid residue. Most of the substitutions interchange one neutral residue for another and most are located at the cytoplasmic face of the membrane, in transmembrane segments adjacent to the putative pore-forming region.

We have investigated the functional consequence of the HPP mutations. Sodium currents were measured in human myotubes cultured from a patient with HPP. The functional defect is a K-induced disruption of inactivation. Single-channel conductance and activation are not affected. At low [K] normal and mutant channels are indistinguishable. In 10 mM [K] a small proportion of mutant channels have prolonged open times and open repetitively throughout the depolarization. The steady-state open probability is only about 0.02 to 0.05, but this is 10 to 50 times larger than that observed for normal Na channels. These HPP myotubes contained the Met1592→Val mutation. We showed that this mutant and the Thr704 → Met mutation are sufficient to disrupt inactivation by using site-directed mutagenesis in the normal rat Na channel and expression in HEK cells. A toxin-based animal model and computer simulation were used to show that a small proportion of non-inactivating sodium current (Popen of 0.015 to 0.05) is sufficient to produce the myotonic and paralytic phenotypes.

The physiology explains the mechanism of dominant inheritance. As serum [K] increases, from exercise or ingestion, Popen approaches 0.01 to 0.02. The steady inward current slightly depolarizes the cell towards threshold and repetitive action potentials occur. With higher [K], Popen increases to 0.02 to 0.05 and the persistent current now causes a large depolarization that inactivates the normal Na channels. This renders the fiber refractory from firing action potentials and causes paralysis.

P 015 MOLECULAR CHARACTERIZATION OF CALCIUM RELEASE CHANNELS: GENE REGULATION, STRUCTURE AND FUNCTION, Andrew R. Marks, Molecular Medicine Program, Department of Medicine, Brookdale Center for Molecular Biology, Mount Sinai School of Medicine, New York, NY 10029

Intracellular calcium release signals numerous biologic processes including muscle contraction, neurotransmitter release, cell differentiation and growth. A family of intracellular calcium release channels (ICRCs) have been described. Two types of ICRCs are ryanodine receptors (RyRs) predominantly on the sarcoplasmic reticulum of cardiac and skeletal muscles, and the inositol 1,4,5-trisphosphate receptor receptors (IP_3Rs) on the endoplasmic reticulum of most cell types. The RyRs and IP_3Rs share limited homology in terms of primary structure but have similar ultrastructural properties characterized by four-fold symmetry. The RyRs are comprised of four identical 565,000 MW subunits, and the IP_3Rs contain four 313,000 MW subunits. The RyR genes are developmentally regulated in muscle, exhibiting increased expression at birth. The RyR gene is downregulated by fibroblast growth factor in a myogenic cell line (skeletal), during end-stage heart failure in humans (cardiac), by doxorubicin treatment in rats (cardiac), and by phenylephrine in cultured cardiocytes (cardiac). The IP_3R gene is up-regulated in cardiac myocytes during end-stage heart failure suggesting that a hormonally-sensitive pathway may become important in regulating cardiac contractility and/or hypertrophy in disease states. We have characterized ICRC expression in cardiac myocytes and in skeletal muscle demonstrating distinct patterns of expression for both the RyR and IP_3R. IP_3R expression in some muscle types may provide an alternative pathway for modulating cytoplasmic calcium levels (as opposed to membrane depolarization which activates ryanodine receptors via the voltage-gated calcium channels on the plasmamembrane). The cloned expressed skeletal RyR yields a caffeine-sensitive ICRC in *Xenopus* oocytes. We have described an association between the RyR and FKBP-12, the binding protein for the immunosuppressant FK506 and rapamycin. Rapamycin, but not FK506, blocks calcium dependent proliferation in a myogenic cell line. We have also detected RyRs in endothelial cells and in vascular smooth muscle where their role in modulating cytoplasmic calcium concentration remains to be elucidated. The interaction between RyR and IP_3R may provide a means for positive and/or negative feedback between the two divergent pathways for intracellular calcium release in cells in which both types of channels are expressed.

Manipulation of the Mammalian Genome

P 016 TARGETED MUTATION OF TGF-ß1 IN THE MOUSE GERMLINE. Tom Doetschman[1], Marcia Shull[1], Ilona Ormsby[1], Sharon Pawlowski[1], Mike Eis[1], Ann Kier[2], and Ron Diebold[1], [1]Department of Molecular Genetics, [2]Department of Pathology, University of Cincinnati College of Medicine, Cincinnati.

TGF-ß1 elicits diverse cellular responses depending on cell type, state of differentiation, and culture conditions. The biological actions of TGF-ß1 include regulation of cell proliferation, control of extracellular matrix protein production and degradation, and modulation of cellular differentiation. *In vitro* studies with TGF-ß1 have demonstrated inhibition of adipogenesis and myogenesis, inhibition of proliferation of hematopoietic progenitor cells, stimulation of chondrogenesis and osteogenesis, and modulation of immune and inflammatory responses, including inhibitory effects on proliferation and function of B lymphocytes, thymocytes, and natural killer cells; inhibitory and stimulatory effects on T cell proliferation; and enhancement of monocyte migration and function and granulation responses. The effects of these activities in the development and functioning at the whole animal level are unknown. To investigate the role of TGF-ß1 *in vivo*, we disrupted one of the TGF-ß1 alleles in ES-D3 cells by homologous recombination. At approximately weaning age TGF-ß1-deficient mice develop an acute, multifocal, mixed inflammatory cell infiltration that can variably affect the heart, lung, skeletal muscle including diaphragm, stomach, liver, pancreas, salivary gland, and brain. This disease leads to organ failure and death. All of the mutant animals have myocarditis and a significant portion die of heart failure (Shull et al, 1992, *Nature* 359:693-699). To determine the usefulness of this animal as a model for human cardiac disorders, we are determining the age at which the infiltration begins and the type of inflammatory cells in the affected heart. This information is necessary to develop therapies to block the inflammation. Only in this way can we separate the inflammatory response from other potential abnormalities such as improper control of the switch from hyperplastic to hypertrophic growth in the post-natal heart, abnormal MHC class II presentation of muscle-specific antigens, or graft-vs-host reactions in the heart. Results from experiments designed to separate disorders of this type from the inflammatory response will be presented.

P 017 CARDIOMYOCYTE GROWTH IN TRANSGENIC ANIMALS. Adil I. Daud, Ellen Katz, Gou Y. Koh, Michael G. Klug, Mark H. Soonpaa, Mark E. Steinhelper and Loren J. Field, Krannert Institute of Cardiology, Indiana University School of Medicine, Indianapolis, Indiana 46202-4800.

Cardiomyocytes in the adult mammal exhibit little if any capacity to undergo cell division. Consequently, myocardial loss due to injury or disease is irreversible. To determine if adult cardiomyocytes can re-enter the cell cycle, we have generated several transgenic mouse models in which expression of the SV40 large T-Antigen (T-Ag) oncogene was targeted to the heart. These animals heritably develop cardiac tumors in the atrium and the ventricle. The transgenic tumors are comprised of differentiated, proliferating cardiomyocytes.

A basic mechanistic understanding of T-Ag induced cardiomyocyte transformation would likely also provide invaluable insight into cell cycle regulation of normal cardiomyocytes. Recently it has been shown that the transforming activities of DNA tumor virus oncogenes is due in part to the formation of stable complexes with cellular proteins involved in cell cycle regulation. We have exploited this approach to identify cellular targets for T-Ag in cardiomyocytes. Three associated proteins have been identified to date. They are (1) p193, a novel protein which forms a tertiary complex with T-Ag and p53; (2) p107, a previously identified protein which is structurally and functionally related to the retinoblastoma gene product; and (3) p53, a well known tumor suppressor.

In addition, several proteins which cross reacted with either anti-T-Ag or anti-p53 antibodies were identified. Two of these proteins, p250 and p110, were only observed in cardiomyocytes. Expression of a third cross reacting protein, p180, was dependent upon the rate of cell proliferation.

Efforts are currently underway to isolate and clone the T-Ag associated and cross-reacting proteins identified in our transformed cardiomyocyte cultures. In addition, several new transgenic models expressing other oncogenes will be discussed.

P 018 TARGETED DEVELOPMENTAL OVEREXPRESSION OF CALMODULIN INDUCES PROLIFERATIVE AND HYPERTROPHIC GROWTH OF CARDIOMYOCYTES IN TRANSGENIC MICE. Carol L. Gruver[1], Francesco DeMayo[2], Margaret A. Goldstein[2], and Anthony R. Means[1]; [1]Duke University Medical Center, Durham, NC 27710, [2]Baylor College of Medicine, Houston, TX 77030.

Calmodulin (CaM) levels are developmentally regulated in the mouse heart[1]. During late gestational and early postnatal stages, CaM levels decline several-fold in close temporal association with the declining population of proliferating cardiomyocytes[2]. This correlation suggests that CaM may influence cardiomyocyte cell cycle activity, particularly since CaM is implicated in cell cycle control in several eukaryotic non-muscle cells[3]. To test this possibility, nucleotides -500 to +77 of the human atrial natriuretic factor (hANF) gene were linked to a chicken CaM minigene to establish two pedigrees of transgenic mice which express 3- to 5- fold increased levels of CaM in cardiomyocytes. Developmental overexpression of CaM in mouse cardiomyocytes produced a markedly exaggerated cardiac growth response characterized by the presence of cardiomyocyte hypertrophy in regions demonstrated to overexpress CaM and by cardiomyocyte hyperplasia, apparent at early developmental stages. A 50% increase in DNA synthesis is observed in the hearts of transgenic mice at embryonic day 14.5 but has returned to the control level by embryonic day 16.5. During this proliferative phase we have preliminary evidence for chronic activation of the mutifunctional Ca^{2+}/CaM dependent protein kinase (CaM kinase II) and of the mRNA for c-fos. Early postnatal suppression of fusion gene expression in the cardiac ventricles correlated with regression of the ventricular growth response in transgenic relative to nontransgenic mice between 3 days-of-age and 6 to 10 weeks-of-age but was not apparent in the cardiac atria where levels of CaM remained constitutively elevated until advanced stages. Morphological changes and alteration of electrical activity in atrial cells of adult transgenic mice are similar to those observed in the early adaptive phase of cardiac hypertrophy. To test the possibility that increased cytosolic Ca^{2+} buffering contributes to the growth response induced by CaM overexpression, two additional lines of transgenic mice were generated using the same hANF promoter to target expression of a CaM mutant (amino acids 75-82 deleted) in cardiomyocytes. This mutant has previously been shown to bind Ca^{2+} with kinetic properties similar to wild-type CaM but was unable to activate CaM-dependent target enzymes *in vitro*[4]. Despite high level expression of the CaM mutant, no growth response was apparent in the hearts of transgenic relative to nontransgenic mice, suggesting that increased Ca^{2+} buffering is unlikely to contribute to the growth response induced by CaM overexpression. Taken together, these findings reveal that cardiomyocyte growth regulation is specifically influenced by CaM concentrations in transgenic mice.

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P 019 TRANSGENIC ANALYSES OF CARDIAC-SPECIFIC TRANSCRIPTION, Arun Subramaniam, Hansjörg Rindt, James Gulick, Stephanie Knotts and Jeff Robbins, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0575

There are two myosin heavy chain (MHC) genes, α and β, that are expressed in the mammalian heart. These genes are part of a large, multigene family whose members are expressed in a muscle type- and developmental stage- specific manner. The two genes that are expressed in the heart are closely linked (β→α, in the 5'→3' orientation) and are separated by ~4.5 kb of intergenic region DNA. The expression of these two genes in the cardiac compartment is tightly regulated. In the small rodent, such as the rat or mouse, the embryonic and fetal ventricles contain predominantly β-MHC transcripts but, at birth, there is an antithetic switch in ventricular MHC gene expression and the β-MHC mRNA is replaced by the α-MHC gene's transcripts. This switch is mediated at the level of transcription and numerous, *in vitro* assays have been used to define the cis-acting structural elements that underlie the cardiac myosin genes' activation/repression. We have utilized a transgenic approach to determine the regions that are necessary and sufficient to drive high levels of transcriptional activity in a tissue and developmental stage-specific manner. The role of two putative, *cis*-acting thyroid hormone responsive elements, TRE_1 and TRE_2, located at -129 to -149 and -102 to -120 respectively on the murine α-MHC gene, has been investigated. These motifs are present in a 4.5 kb fragment lying upstream of the mouse α-MHC gene's transcriptional start site: this fragment directs appropriate expression of a reporter gene in transgenic mice.[1] Within the context of this active promoter, which consists of the entire intergenic region, independent mutations of the TRE_1 and TRE_2 elements by base substitution have been made. The resultant mice were analyzed for transgene expression in different muscle and non-muscle tissues including the atria and ventricles. Normal levels of transgene expression were observed in euthyroid mice carrying a mutation in TRE_1. Both cardiac compartment- and developmental stage-specific expression of the transgene was maintained at levels approximately equal to those observed in the wild type lines in euthyroid animals. In contrast to these results, mice in which TRE_2 was mutated showed reduced levels of CAT activity in both the atria and ventricles. Further analyses identified a previously undefined upstream region on the α-MHC promoter that is involved in mediating thyroid hormone action. The promoter of the other MHC gene that is expressed during cardiac development was also studied. We isolated the 5' upstream region of the murine β-MHC gene, defined the exon/intron organization of the 5' untranslated region, and determined the transcriptional start site. Subsequently, three classes of transgenic mice were generated. The constructs contained approximately 7000, 2500, and 600 base pairs of β-MHC upstream sequence fused to the *cat* reporter and were termed β7, β2.5, and β.6, respectively. Muscle-specific expression was observed with all three constructs. However, only the β7 lines directed high levels of muscle-specific transgene expression. Expression driven by the two shorter constructs was two to three orders of magnitude lower. These data suggest that a distal positive element directs high levels of gene expression in the ventricle and in slow skeletal muscles.

[1]Subramaniam, A., Jones, W. K., Gulick, J., Wert, S., Neumann, J., and Robbins, J. (1991) *J. Biol. Chem.* **266**, 24613-24620

P 020 THE DEVELOPMENTAL ROLES OF MYOGENIC HLH TRANSCRIPTION FACTORS REVEALED BY GENE TARGETING IN MICE, Michael A. Rudnicki[1,2], Thomas Braun[3], Shuji Hinuma[2], Hans-Henning Arnold[3], and Rudolf Jaenisch[2]. [1]Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton, Canada. [2]Whitehead Institute, Cambridge, USA. [3]Institute für Biotechnologie und Biochemie, Abt. Zell und Molekularbiologie, Braunschweig, Germany.

The myogenic basic helix-loop-helix (HLH) transcription factor family of genes, consisting of MyoD, myogenin, Myf-5 and Myf-6, are thought to be involved in regulating skeletal-muscle differentiation. These genes are activated sequentially during embryonic development suggesting specific functions for each protein. However, *in vitro* experiments have failed so far to reveal significant functional differences between individual myogenic factors. To understand the role of MyoD and Myf-5 in myogenesis, we have introduced targeted mutations in these genes into the germ line of mice. Histological examination of skeletal muscle in newborn mice lacking MyoD or Myf-5, by light- and electron-microscopy, failed to reveal any morphological abnormalities. Northern analysis with a panel of skeletal-muscle specific probes indicated that normal levels of muscle-specific mRNAs accumulate in mutant mice. Mice lacking a functional Myf-5 gene die perinatally due to severe rib abnormalities that prevent normal respiratory function of the lung. This phenotype ascribes a unique developmental role to Myf-5 which cannot be substituted by the other myogenic factors. We have hypothesized that Myf-5, being the first developmentally activated myogenic HLH factor, may be indispensible for the induction of myotomal fibers, which in turn, might play a crucial role in sustaining proliferation of the rib rudiments. later induction of primary myoblasts may be dependent on the expression of other myogenic HLH factors, and these myoblasts might well be capable of forming an apparently normal skeletal muscle system. Surprisingly, mice lacking MyoD are viable and fertile. Significantly, Myf-5 is abundantly expressed in muscle of postnatal mutant MyoD mice, and MyoD levels are reduced in newborn mutant Myf-5 mice. Normally, Myf-5 mRNA levels are markedly reduced at day 12 of gestation about the time MyoD mRNA first appears. This result suggests that Myf5 expression is repressed by MyoD and that MyoD expression is induced by Myf-5. The simplest interpretation of these experiments is that MyoD and Myf-5 are dispensable for skeletal muscle development in mice. Thus, some degree of functional redundancy may exist in the control of the skeletal myogenic developmental program. Clearly, the phenotype of skeletal muscle of mice lacking both MyoD and Myf-5 genes will be highly informative as it directly addresses the question of mutual functional substitution of these two myogenic transcription factors in myogenesis. Resent results describing the phenotype of these mice will be presented.

Myogenic Determination I

P 021 ISOLATION AND EXPRESSION OF A XENOPUS CARDIAC TRANSCRIPTION FACTOR, Heather Blumberg[1], Clair Kelley[2], Leonard Zon[2], and Todd Evans[1], [1]Dept. Biological Sciences, University of Pittsburgh, Pittsburgh, PA, 15260, and [2]Children's Hospital, Harvard Medical School, Boston, MA, 02115

We have isolated and characterized cDNA clones for a new transcription factor that represents an early marker for the developing Xenopus heart. The cDNA clones encode a protein we have designated GATA-4, as it is now the fourth identified member of the GATA family of cell-type restricted DNA-binding transcriptional activators. The GATA-4 protein contains the highly conserved DNA-binding domain which characterizes this family and it binds specifically to GATA consensus cis-elements. GATA-4 gene expression is restricted to a distinct sub-set of tissues in the adult frog. Expression levels are highest in heart and gut; lower levels of RNA are also detected in various endodermally derived tissues. *In situ* hybridization to sections of adult stomach show that expression in this tissue is primarily restricted to epithelium. Similar experiments performed using adult heart did not reveal expression in the myocardium; results we describe below (Kelley et. al.) studying GATA-4 transcription in developing embryos suggest instead that GATA-4 is expressed in endocardium. Western blots demonstrate that the GATA-4 protein is present in adult heart tissue, but not in skeletal muscle. The GATA-4 gene is also regulated during development. Similar to the other known GATA factors, GATA-4 RNA is first detected during gastulation at stage 11 of Xenopus development. It is therefore not likely to be an immediate target of mesoderm induction. We find GATA-4 is first expressed during embryogenesis in cells of the developing heart and therefore provides an exciting new marker for studying the inductive processes which lead to the formation of the cardiovascular system.

P 022 MOUSE GATA-4: A RETINOIC ACID-INDUCIBLE GATA-BINDING TRANSCRIPTION FACTOR EXPRESSED IN HEART AND ENDODERMAL DERIVATIVES, Robert. J. Arececi[1], Alexandra A. J. King[1], M. Celeste Simon[1], Stuart H. Orkin[1,2], and David B. Wilson[3], [1]Boston Children's Hospital and Dana Farber Cancer Inst., [2]Howard Hughes Medical Inst., [3]Division of Pediatric Hematology-Oncology, Washington University School of Medicine, St. Louis, MO 63110.

We report the cDNA cloning and characterization of mouse GATA-4, a new member of the family of zinc finger transcription factors that bind a core GATA motif. GATA-4 cDNA was identified by screening a 6.5 day mouse embryo library with oligonucleotide probes corresponding to a highly conserved region of the finger domains. Like other proteins of the family, GATA-4 is approximately 50 kDa in size and contains two zinc finger domains of the form C-X-N-X-(X_{17})-C-N-X-C. Co-transfection assays in heterologous cells demonstrate that GATA-4 *trans*-activates reporter constructs containing GATA promoter elements. Northern analysis and *in situ* hybridization show that GATA-4 mRNA expression is restricted to atrial and ventricular cardiomyocytes, small intestine epithelial cells, primitive endoderm, germ cells, and to a lesser extent liver. Retinoic acid-induced differentiation of mouse F9 cells embryonal carcinoma cells into visceral or parietal endoderm is accompanied by increased expression of GATA-4 mRNA and protein. *In vitro* differentiation of embryonic stem cells into embryoid bodies is also associated with increased GATA-4 expression; this induction coincides with the appearance of rudimentary myocardial and endodermal tissue in the embryoid bodies. We conclude that GATA-4 is a tissue-specific, retinoic acid-inducible, and developmentally-regulated transcription factor. Based on its tissue distribution, we speculate that GATA-4 plays a role in gene expression in heart, intestinal epithelium, primitive endoderm, germ cells, and liver, although the target genes for this transcription factor are presently unknown.

P 023 GATA-4 IS AN EARLY MARKER OF CARDIOVASCULAR DEVELOPMENT AND IS EXPRESSED IN ENDOCARDIAL CELLS. Clair Kelley[1], Heather Blumberg[2], Todd Evans[2], Leonard Zon[1]. [1]Children's Hospital, Harvard Medical School, Boston, MA 02115, [2]Dept. of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260.

The progenitors for cardiovascular endothelium are initially localized during gastrulation in two bilateral regions of the inner marginal zone mesoderm. The progenitors migrate ventrally and meet at midline during the early tailbud tadpole stage, forming a long trough which is envisioned to be the vascular tube. Because of a lack of early cardiac markers, this process has not been studied at a molecular level. Whole embryo in situ analysis of Xenous embryos has demonstrated that GATA-4 is expressed in presumptive cardiac ventral mesoderm at the time of fusion of the bilateral progenitor populations. GATA-4 is therefore the earliest molecular marker of cardiogenesis which has been characterized. The expression extends anteriorly from stage 18 to stage 30. By stage 30, the cardiac mesoderm expressed GATA-4 mRNA in an organization reminiscent of the developing atria and ventricles. By stage 36, the heart expresses high levels of GATA-4 RNA, and cross-sections demonstrate that GATA-4 is expressed in the endocardium, but not the muscle layer. By stage 40, the expression of GATA-4 extends to the great vessels, inferior vena cava, and liver. The expression of GATA-4 is initially present throughout the marginal zone, and later, partially localized to the ventral marginal zone. GATA-4 is also detected at high levels in endodermal explants from the vegetal region of stage 8 embryos, consistent with its later expression in small intestine. In mesoderm induction experiments, GATA-4 expression is not induced in animal caps treated with activin. The factors which regulate the induction of GATA-4 will likely be critical for cardiovascular formation.

Receptors/Signal Transduction

P 024 THROMBIN RECEPTOR STRUCTURE AND FUNCTION. Scott Soifer, Kevin Peters, Julie O'Keefe, Nicolas Nelken, Thien-Khai Vu, and Shaun Coughlin, Cardiovascular Research Institute, UCSF, San Francisco.

Thrombin, a multifunctional serine protease generated at sites of vascular injury, has actions on a variety of cell types. Thrombin's potent activation of platelets is very likely critical for hemostasis and thrombosis. Its actions on endothelial and smooth muscle cells and leukocytes have been defined largely in vitro and may be important in mediating proliferative and inflammatory responses to injury. The recent characterization of the cellular receptor for thrombin is providing tools for defining the role of thrombin in various cellular events in vivo. The receptor is a member of the seven transmembrane domain family of receptors, but is activated by a novel mechanism. Thrombin binds to and cleaves its receptor's relatively long extracellular amino terminal extension, unmasking a new amino terminus. This new amino terminus then functions as a tethered peptide ligand, binding to an as yet undefined domain withing the body of the receptor to effect receptor activation. A synthetic peptide mimicking this new amino terminus was a full agonist for receptor activation, providing a new tool for activating the receptor independent of thrombin and thrombin's protease activity. This peptide has been used to define the role of thrombin receptor activation in a variety of cellular activities. The cDNA clone has been used to define receptor expression in vivo by in situ hybridization. In the adult, low level receptor mRNA expression was seen in vascular endothelial cells and in renal glomeruli, and robust expression was found in circumscribed areas of the central nervous system, in particular, Purkinje neurons. Robust expression was also noted within human atherosclerotic plaques, suggesting a possible for thrombin receptor activation in sclerotic processes in vivo. In contrast to the adult, widespread receptor expression was seen in mouse embryos, with particularly high expression noted in endocardial and endothelial cells. Thus the role of thrombin and its receptor in a variety of settings outside of platelet activation and thrombosis deserves exploration.

P 025 **SIGNAL TRANSDUCTION PATHWAYS OF MECHANO-TRANSCRIPTION COUPLING IN CARDIAC MYOCYTES *IN VITRO***

Seigo Izumo and Jun-ichi Sadoshima. Molecular Medicine Unit, Beth Israel Hospital, Harvard Medical School, Boston, MA 02215.

It is well known that external load plays a critical role in determining cardiac muscle mass and its phenotype, but little is known as to how mechanical load is transduced into intracellular signals regulating gene expression. To address this question we performed a systematic analysis to identify the "mechano-transcription" coupling process using an in vitro model of load-induced hypertrophy. Neonatal rat cardiocytes were grown on silicon substrate in serum free media. Stretching cardiocytes results in a rapid induction of immediate-early (IE) genes followed by an increase in *protein synthesis* and activation of the "fetal genes, a pattern very similar to that of in vivo hypertrophy by hemodynamic stress. DNA transfection experiments using *the c-fos gene promoter* constructs have revealed that the "stretch response element" maps to the 5' outer palindromic arm of the serum response element (SRE). Biochemical and pharmacological analyses have indicated that stretch causes a rapid activation of multiple signal transduction pathways simultaneously. The main pathway appears to involve activation of tyrosine kinases, p21^{ras} phospholipase C, protein kinase C, MAP kinases and ribosomal S6 kinase, but activation of phospholipase D, phospholipase A2 (particularly P450 pathway) may also contribute to the stretch response. On the other hand, cAMP and cGMP pathways do not seem critical for the stretch-induced IE gene expression. Three putative mechanotransducers, stretch-activated ion channels, microtubules and microfilaments, are not necessary for the strech-induced IE gene expression. The stretch response may involve autocrine or paracrine mechanism, because the "stretch-conditioned media," when transferred to non-stretch cardiocytes, mimic the effect of stretch. These results suggest that mechanical stretch may cause a release of growth factor(s) which in turn activate(s) multiple intracellular signal transduction pathways, leading to the induction of IE genes and hypertrophy of cardiac myocytes.

P 026 INTRACELLULAR TRANSPORT AND TARGETING OF ADRENERGIC RECEPTORS,

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G protein-coupled receptors are responsible for approximately 80% of transmembrane signal transduction. A remarkable outcome of the application of molecular biology to the study of G protein-coupled receptors is the identification of multiple closely related receptor subtypes having a high degree of structural homology and essentially indistinguishable functional properties. For example, there are three subtypes of alpha 2 adrenergic receptors, all of these receptors are activated by both epinephrine and norepinephrine, and activation of all three alpha 2 receptor subtypes leads to inhibition of adenylyl cyclase. These observations raise the question of whether other functional parameters, apart from ligand binding and G protein coupling, may differentiate receptor subtypes. Simultaneous immunocytochemical co-localization of beta 2 and epitope-tagged alpha 2 adrenergic receptor subtypes expressed in the same cells revealed that these receptors differ from one another in their subcellular targeting and in the regulation of their intracellular trafficking by agonists. Alpha 2A and alpha 2B receptors co-localize with beta 2 receptors in the plasma membrane, but can be distinguished by differences in agonist mediated internalization. Like the beta 2 receptor, the alpha 2B receptor is rapidly internalized into intracellular vesicles following exposure to agonist for 15 min. In contrast, the a2A receptor remains on the plasma membrane following agonist exposure. The alpha 2C receptor differs from alpha 2A and alpha 2B receptors in that it is localized predominantly in intracellular vesicles even in the absence of agonist exposure. By co-expressing the alpha 2C and beta 2 receptors in the same cell, we observe that the vesicle compartment containing the alpha 2C receptor is different from the compartment containing the agonist internalized beta 2 receptors. These results indicate that structurally similar adrenergic receptors are sorted in a subtype-specific manner, and that subtype-specific sorting comprises another parameter, in addition to ligand binding affinity and G protein-coupling specificity, which may functionally differentiate receptor subtypes.

Gene Therapy

P 027 GENE TRANSFER INTO CARDIAC AND SKELETAL MYOCYTES IN VIVO,

Eliav Barr[1], Hua Lin[2], Steven Bolling[2], Michael S. Parmacek[1], Karen Kozarsky[2], James Wilson[2], Sandeep Tripathy[4], Gary Engleman[3], and Jeffrey M. Leiden[1], [1]University of Chicago, Chicago, IL 60637, [2]University of Michigan, Ann Arbor, [3]Loyola University, Chicago.

The ability to program recombinant gene expression in cardiac and skeletal myocytes in vivo holds promise for the treatment of a number of acquired and inherited diseases. We have explored several methods for programming recombinant gene expression in muscle cells, including (i) direct injection of plasmid DNA into cardiac muscle, (ii) the use of genetically modified skeletal myoblasts to deliver recombinant proteins to the systemic circulation, and (iii) the use of replication defective adenoviruses to deliver recombinant genes to cardiac myocytes following catheter-based delivery into the coronary sinus or coronary arteries. The results of these studies can be summarized as follows:

(i) Injection of plasmid DNA into the myocardium in vivo results in recombinant gene expression in approximately one percent of cells in the area of injection. Gene expression is limited to cardiac myocytes and is stable for periods of as long as six months. This technique has been used to program expression of several recombinant angiogenesis factors in the left ventricular wall.

(ii) Murine myoblasts have been stably transfected in vitro with a series of eukaryotic expression vectors encoding human growth hormone, simian erythropoietin, and human factor VIII. In all cases the genetically modified myoblasts produce and secrete high levels of recombinant proteins in vitro. Following intramuscular injection these genetically modified myoblasts can be used to stably deliver physiological concentrations of recombinant proteins to the systemic circulation. Transfection of primary myoblasts with the same eukaryotic expression vectors has revealed that primary cells secrete 5-10 times more recombinant protein than to the genetically modified primary murine myoblast cell lines. We are currently in the process of reimplanting genetically modified myoblasts into animals to study their ability to fuse into endogenous myofibers in vivo and to secrete recombinant gene products into the systemic circulation.

(iii) We have developed a novel technique, percutaneous coronary gene transfer (PCGT) to program recombinant gene expression in both coronary vessels and myocardium. In this method doubly replication-defective (E1- and E3-deleted) adenovirus vectors expressing the LacZ gene under the control of the CMV promoter have been infused using catheters into the coronary sinus and the coronary arteries. Following infusion of $2x10^9$ infectious particles, efficient LacZ expression was observed both in the vessel infused with virus and in the surrounding myocardium. Thus, PCGT can be used to program recombinant gene expression both in the coronary vasculature and, regionally in the ventricular myocardium.

P 028 **GENE TRANSFER INTO THE ADULT MAMMALIAN HEART IN VIVO,** L.A. Leinwand, R.N. Kitsis, P. Buttrick, R. Sindhwani, and F. Ismail-Beigi, Albert Einstein College of Medicine, Bronx, New York 10461.

The heart has a remarkable ability to change its patterns of gene expression both dramatically and rapidly in response to a wide variety of normal and abnormal stimuli. Until recently, it has been difficult to characterize the molecular events that dictate cardiac gene expression, partly because of the lack of appropriate cell culture models for the intact adult heart. We have developed a method of direct hypodermic needle injection of naked DNA into the intact rat heart for the purpose of studying cardiac myosin heavy chain (MHC) gene regulation. Various stimuli have been shown to shift the myosin composition of the rodent heart from α to β and *vice versa*. We have studied the contribution of transcriptional regulation to the final MHC mRNA composition of the rat ventricle by injecting DNA constructs linking various sequences upstream of the rat α and β MHC genes to the luciferase reporter into the intact adult heart in various thyroid states and quantitating reporter gene activity. For the α MHC gene, we have identified both positive and negative elements contributing to its regulation. Interestingly, behavior of DNA constructs *in vivo* is distinct from that seen *in vitro* from transfection into dissociated fetal or neonatal cardiocytes. In addition to transcriptional regulation, through analysis of steady state RNA populations, we have uncovered two RNA processing events occurring in α MHC gene expression. One is alternative splicing resulting in the inclusion or exclusion of a glutamine residue toward the carboxyl terminus while the other event involves alternate 3'end formation from a single polyadenylation signal. In addition to RNA processing, we have determined that sequences in the 3' untranslated region of the βMHC gene regulate MHC RNA stability in response to various thyroid states. While DNA injection has already proven very useful for analysis of quantitative aspects of cardiac gene regulation, the utility of this approach for modifying phenotype remains to be demonstrated. Only striated muscle cells take up and express directly injected DNA. In the rat, heart is far superior to skeletal muscle as a target organ for direct gene transfer. Injected genes appear to remain episomal, and following about 2 weeks, 20-30% of animals express injected genes long-term. A single injection into the apex of the heart results in the transfection of about .02% of the myocytes of the heart, but 98% of transfected cells are located within 2mm of the injection site. In an attempt to biologically modify the heart through direct DNA injection, we asked whether we could transcriptionally transactivate a gene normally expressed only in skeletal muscle by introducing MyoD into the heart by gene injection. MyoD expression constructs and a construct with the skeletal muscle myosin light chain 1 promoter and enhancer linked to the CAT gene (from N. Rosenthal) were coinjected into rat hearts. Significant transcriptional transactivation of the MLC 1,3 sequence was achieved and was shown to be mediated through an E-box. In addition, coinjection of an Id expression construct inhibited transactivation. To explore the prospects of the heart as a target organ for gene therapy, two additional avenues are currently being pursued. In the first, DNA constructs encoding ectopic gene products such as growth hormone are being introduced into hearts of mice and rats. In the second, modified adenoviruses are being introduced into mice and rats with sequences that target expression of recombinant genes to the heart.

Myogenic Determination II

P 029 NOVEL REGULATORY ROLE FOR 3' UNTRANSLATED REGIONS IN DIFFERENTIATION, Helen M. Blau, Farzan Rastinejad, Department of Pharmacology, Stanford University School of Medicine, Stanford, CA 94305.

Differentiation of skeletal muscle entails the activation and continued expression of a battery of genes for tissue-specific function. To date, efforts to identify regulators of myogenesis have yielded DNA-binding transcription factors. In order to explore novel regulatory pathways in myogenesis, we have used a genetic complementation approach. A differentiation defective myoblast mutant (NMU2) was isolated that expresses MyoD, but lacks regulators necessary to activate α-cardiac actin and myogenin promoters. NMU2 cells with stably transfected muscle-specific promoter constructs were transfected with a cDNA expression library. Four cDNAs activated the promoter constructs as well as the endogenous myogenin gene. Three activating cDNAs were identified as muscle structural genes. The activity mapped to the 3' untranslated region (3'UTR) of the cDNAs. These data suggest that 3'UTRs act in trans to mediate a feedback loop that promotes and stabilizes the differentiated state of muscle.

P 030 TRANSCRIPTION FACTORS INVOLVED IN THE SPECIFICATION AND MAINTENANCE OF THE CARDIAC CELL LINEAGES. V Mahdavi, V Andres, Y Lee, S Kaushal, D Laheru and B Nadal-Ginard, Harvard Medical School, Boston.

Major outstanding questions in cardiac biology involve the origin of the cardiac cell lineage and the production as well as the maintenance of the differentiated phenotype. As an approach to these questions we have undertaken the isolation and characterization of transcription factors that specify gene expression in the myocardial tissues. We have cloned a novel homeoprotein (Clox). The homeodomain of Clox is closely related to that of the Drosophila cut gene product, a determinant of neural cell specification. In addition, three 73-amino acid repeats, the so-called cut repeats are also conserved between Cut and Clox. This conservation suggests that the cut repeat motif may define a new class of homoeproteins. Both cloned and endogenous Clox proteins are nuclear DNA-binding proteins with similar sequence-specificity. Western blot analysis revealed distinct Clox protein species in a variety of tissue and cell types, including cardiac and skeletal muscles and brain. The relative abundance of these proteins is regulated during mouse development and cell differentiation in culture. The ~180-190 Kd Clox proteins, which predominate in several tissues of early embryos, are absent in the adult. These proteins, which are not detected in 10T1/2 cells, are up-regulated upon 5-azacytidine treatment and in committed myoblasts and chondrocytes, but down-regulated upon terminal differentiation. Clox DNA-binding activity is correlated with the abundance of these 180-190 Kd proteins. In contrast, the larger Clox proteins species (~230-250 Kd), detected mainly in adult tissues and in terminally differentiated cells, do not bind to DNA. Thus, Clox, like their Drosophila counterparts, are candidate regulators of cell-fate specification in diverse programs. Cotransfection experiment indicate that Clox can repress tissue-specific gene transcription. Specifically, Clox can prevent transactivation of the β-MHC enhancer by the muscle-specific transactivator MEF2. In Drosophila, determination of neural precursor cells by the proneural genes daughterless (da) and achaete-scute complex (AS-C), both of which are bHLH proteins, occurs prior to cut function. However, because Clox gene expression is detected before that of MyoD in myoblasts, it is tempting to postulate that lineage-specific transcriptional regulators, together with Clox, cooperate in establishing commitment and differenciation in a number of precursor cells.

We have cloned a family of transcription factors that interact with an A/T rich motif found in the regulatory region of several genes expressed in cardiac and skeletal muscles, termed MEF2. The MEF2 proteins belong to the MADS gene superfamily and are encoded by four genes, each of which generates several protein variants by alternative splicing. Although MEF2 mRNAs are detected in most tissues, the expression of the corresponding protein is strictly restricted to cardiac, skeletal and smooth muscles, as well as to the neural cells of the brain. These tissues, where different isoforms are expressed in a developmentally regulated manner, display MEF2-DNA binding activity and have saturating levels of endogenous MEF2 trans-activators. In non-muscle cells, transfection of MEF2 is sufficient to induce the expression of MEF2-responsive reporter genes, demonstrating that the cloned sequences correspond to the positive trans-acting factors. Interestingly, there is a close functional correlation between the bHLH myogenic factors and the MEF2 proteins. Expression of MyoD1 in various non-muscle cells is sufficient to induce MEF2 DNA-binding and transactivation functions. MEF2, in turn, activate myogenin expression. In addition, MEF2 interact in vivo and in vitro with MyoD and myogenin and, together, these two types of factors activate target genes in a cooperative fashion. It is thus tempting to postulate that functionally equivalent transcription regulators might also modulate expression and function of the MEF2 proteins in cardiac and neural tissues.

P 031 Regulatory Circuits Controlling Myogenesis, Eric N. Olson, Ph.D. Department of Biochemistry and Molecular Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030.

Transcription of muscle specific genes during myogenesis is regulated by combinatorial interactions among cell type-specific and widely expressed transcription factors, and is influenced by extracellular signals that regulate complex intracellular pathways of signal transduction. Myogenin is a muscle-specific helix-loop-helix protein that acts as a molecular switch to induce myogenesis. The *myogenin* gene is activated in embryonic somites and limb buds at the time of myogenic lineage commitment. *cis*-acting sequences and *trans*-acting factors that impart muscle specificity, developmental regulation, and growth factor responsiveness have been identified within the 5' flanking region of the *myogenin* gene. The myogenin protein serves as a target for a variety of growth factor signaling pathways which silence its transcription-activating functions. Among these is the protein kinase C pathway which leads to direct phosphorylation of the myogenin DNA binding domain at a site that is conserved in all myogenic HLH proteins. Activation of the myogenic program by myogenin is dependent on other cell type-specific as well as ubiquitous factors. One such factor is the mesodermally-restricted homeodomain protein Mhox. Regulatory interactions between myogenin, Mhox, and the cellular circuits that control muscle cell proliferation will be discussed.

Late Abstract

MYOGENIC HELIX-LOOP-HELIX REGULATORS IN THE MOUSE EMBRYO, Margaret Buckingham, Christine Biben and Shahragim Tajbakhsh, Department of Molecular Biology, CNRS URA 1148, Pasteur Institute, Paris, France.

Targetting of the myf-5 gene with introduction of a lacZ marker sequence under the control of the endogeous regulation of the locus has permitted us to examine more closely, at a cellular level, the sites of expression of this gene during mouse embryogenesis. Myf-5 is the first of the four myogenic regulatory sequences of the MyoD family to be transcribed during skeletal myogenesis in vivo (1). However in addition to labelling of cells in the early somite and subsequently in the limb bud and in other pre-muscle masses, we note expression in certain cells of the nervous system in vivo. When embryonic stem cells, carrying the lacZ marker in the myf-5 gene, are induced to form embroid bodies in vitro, some neurogenic as well as myogenic precursors become positive. No labelling of cardiac cells is observed at any stage.

The mouse cardiac actin gene is expressed in the developing and adult heart and in embryonic skeletal muscle. In the BALB/c mouse the gene is transcriptionally less active; skeletal actin accumulates in the hearts of these mice. This mutant mouse carries an insertion, with a partial duplication of the gene, several kilobases upstream of the cardiac actin promoter (2). A DNaseI hypersensitive site analysis demonstrates the presence of two sites, one of which is displaced by the insertion. Both sites function as muscle specific enhancers when tested in transfection experiments in cultured cells. A transgenic analysis shows that the proximal promoter alone is not sufficient to give detectable levels of expression, although multiple copies of this transgene show low muscle specific activity in vivo, as already demonstrated for the human gene in vitro (3). Upstream sequences are necessary to target high level expression to the heart and to embryonic skeletal muscle in transgenic mice. Molecular analysis of the distal enhancer demonstrates the presence of multiple E-boxes, potential binding sites for myogenic factors, within the active region. Binding activity is present in differentiated muscle cells, but the MyoD family is not principally involved. Transactivation of the enhancer by these sequences is not observed. It is therefore likely that other helix-loop-helix factors are involved in the regulation of this major cardiac gene.

(1) Buckingham, M.E. (1992) TIG 8:144-149
(2) Garner et al (1986) EMBO J. 5:2559-2567; Dev Biol (1989) 134:236-245
(3) Sartorelli et al (1990) Genes & Dev 4:1811-1822

Developmental Biology; Cardiac Induction/Determination; Manipulation of the Mammalian Genome; Myogenic Determination; Gene Therapy

P 100 THE MURINE PLATELET ENDOTHELIAL CELL ADHESION MOLECULE (PECAM-1) IS DEVELOPMENTALLY REGULATED DURING CARDIAC MORPHOGENESIS.

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PECAM-1 is a newly recognized immunoglobulin superfamily cell:cell adhesion molecule strongly expressed on human endothelial cells, endocardium, and platelets. The distribution of this cell adhesion receptor during early embryogenesis and its role in cardiac ontogeny is unknown. To characterize the murine PECAM-1 molecule and delineate its distribution during cardiac development, we screened a mouse heart cDNA library and isolated a 3.2 kb fragment containing the full length murine PECAM-1. Complete sequence analysis demonstrated an 80% homology at the amino acid level to the human PECAM-1 with conservation of a consensus glycosaminoglycan binding domain believed to be important for adhesion as well as a deletion of a portion of the cytoplasmic domain. Reverse Transcriptase Polymerase Chain Reaction experiments utilizing a "nested" primer technique from pre-somite (7.5 day), early somite (8 day), and straight heart tube stage embryos (8.5 days) confirmed the presence of PECAM-1 mRNA during the initiation of vascular development. Immunohistochemical studies of staged mouse embryos probed with a hamster anti-mouse PECAM-1 monoclonal antibody (2H8) demonstrated PECAM-1 expression on the endocardium and dorsal aorta during initial formation of the straight heart tube which persisted throughout development. However, PECAM-1 could not be detected on endocardial cells undergoing mesenchymal transformation (11 day embryo) in the atrioventricular canal or aorticopulmonary outflow tract suggesting it is down regulated during endocardial cushion formation. These data confirm that the structure of PECAM-1 is conserved across species, is the earliest marker of mammalian endocardial differentiation described to date, and is developmentally regulated during critical periods of cardiovascular development.

P 101 PCGT, CATHETER-BASED GENE TRANSFER INTO THE HEART USING REPLICATION-DEFICIENT RECOMBINANT ADENOVIRUSES,

Eliav Barr[1], Sandeep K. Tripathy,[1] Karen Kozarsky[2], James Wilson[2], John D. Carroll[1], Sanjeev G. Shroff[1], and Jeffrey M. Leiden[1], [1]Department of Medicine, University of Chicago School of Medicine, Chicago, IL 60637, and [2]Howard Hughes Medical Institute, University of Michigan, Ann Arbor, MI 48109

The expression of recombinant genes in the human myocardium and coronary vasculature holds promise for the treatment of a number of inherited and acquired diseases of the cardiovascular system. The development of somatic gene therapy techniques for myocardium requires both expression vectors that promote high level recombinant gene expression and a method for transducing recombinant DNA into adult cardiac myocytes, cells which cannot be grown *in vitro*. In this report, we describe Percutaneous Coronary Gene Transfer (PCGT), a method for the induction of recombinant gene expression in cardiac myocytes and coronary vessels following percutaneous, catheter based delivery of adenovirus into coronary vessels *in vivo*. A catheter was inserted into the right carotid artery or internal jugular vein of adult rabbits and advanced under fluoroscopic guidance to the left coronary artery or coronary sinus ostium, respectively. 2×10^9 plaque forming units of AdCMVβ-gal, a replication defective recombinant adenovirus containing the β-galactosidase (β-gal) gene under the control of the Cytomegalovirus Promoter/Enhancer, were infused into the coronary vasculature. Animals were sacrificed 5 days to 3 weeks following injection, and hearts were assayed histochemically for β-gal enzyme activity. Recombinant β-gal activity was efficient but patchy and was observed most significantly in coronary vessels and in myocardium distal to the injection site. No β-gal activity was observed in hearts injected with a control adenovirus preparation. PCGT represents a relatively non-invasive and efficient method of inducing stable recombinant gene expression in myocardium and coronary vasculature *in vivo* and may therefore facilitate the development of novel therapeutic approaches for a variety of cardiovascular diseases.

P 102 ENDOTOXIN INDUCES IN VIVO MYOCYTE HSP70 GENE EXPRESSION AND PROTECTION FROM CARDIAC ISCHEMIC INJURY

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Heat shock, cardiac ischemia, and cardiac hypertrophy after aortic coarctation have been related to increased expression of heat shock protein 70 (HSP70) mRNA in vivo. Like heat shock, sublethal endotoxemia has induced protection from isolated rat heart ischemia/reperfusion injury. We pretreated rats with endotoxin (ETX, 500 ug/kg/IP) and found the isolated heart (Langendorff, crystalloid buffer, global ischemia x 25min) was protected from ischemia/reperfusion injury 72h later (DP 76 ± 9 mmHg vs. 47 ± 6 mmHg for control). Though total heart protein content (Lowry assay of homogenate) was not increased after ETX, cyclohexamide treatment (500 ug/kg/IP) of the rat 3h before ETX prevented protection (DP 49 ± 8 mmHg vs. 47 ± 6 mmHg for control). HSP70 mRNA levels were increased in ventricles peaking 12h after ETX. In situ immunohistochemistry localized increased inducible HSP70 protein to myocytes and capillary endothelium. These results suggest that HSP70 gene upregulation may be a conserved response in myocardial adaptation to an altered environment and may be involved in the acquisition of a protected state after sublethal endotoxemia.

P 103 REGULATION OF HYPERTROPHY INVOLVES DIFFERENT TYPES OF POST-TRANSLATIONAL PROCESSING IN ISOLATED ADULT AND NEONATAL CARDIOMYOCYTES,

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There is an approximately 9-fold difference between the rates of protein synthesis in isolated adult and neonatal heart cells in long term culture. A more significant difference, however, between these different developmental stages appears to be related to post-translational mechanisms for maintaining protein balance in the cardiomyocyte. In embryonic and neonatal heart cells the kinetics of protein turnover may be closely approximated using single-compartment first-order rate equations. However, these same equations fail to account for observed patterns of amino acid incorporation in isolated adult feline cardiomyocytes. Rather labeling kinetics suggest that in the adult nascent proteins are segregated into kinetically distinct turnover compartments. Analysis of data from both pulse and continuous label incorporation over 32 days in culture indicated the following compartment parameters. The fast turnover component comprises 10% of total cellular protein and has a mean half-life of 21.9 hours. The slow component has a mean half-life of 15.6 days. Both the observed mean rate of label equilibration and the rate predicted from this two component mixture was best fit by a single exponential function with a fractional synthesis rate of 5.48% d^{-1} (mean protein half-life of 12.6 days). Further evidence of segregation of nascent peptides into different turnover compartments was provided in pulse-chase experiments. Using a dual label approach to distinguish nascent peptides from proteins labeled over the course of 7 days in vitro, the differential processing of nascent peptide pools could be evaluated both in the total protein compartment and in individual proteins separated by SDS-PAGE. Approximately 20% of the total myosin HC pool exists in a labile high turnover compartment with a half life of less than 24 hours. In contrast, the nascent to long-term decay rate of the 55 kDa intermediate filament proteins suggests that only 6% of these proteins are in a high turnover compartment. These results indicate that regulation of the levels of contractile and cytoskeletal proteins of adult cardiomyocytes involves not only transcriptional control, but also a post-translational component which regulates turnover of different classes of nascent peptides.

P 104 EXPRESSION OF CARDIAC SPECIFIC MYOSIN HEAVY CHAIN TRANSCRIPTS DURING EARLY *XENOPUS* DEVELOPMENT,

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In order to study the molecular aspects of cardiogenesis in *Xenopus laevis*, we have isolated an early cardiac-specific molecular marker. We describe here the isolation and developmental expression analysis of an Embryonic Cardiac Myosin Heavy Chain gene (ECMHC). A novel 3'-RACE RT-PCR technique and whole mount *in situ* hybridization was used to isolate and test the cardiac specificity of the ECMHC prior to cloning. The deduced amino acid sequence of the PCR amplified *X.l.* partial ECMHC gene cDNA has 85-90% homology to cardiac myosin heavy chain genes of other vertebrates. RT-PCR amplification was used to detect ECMHC and α-cardiac actin gene expression in a developmental staging series (st.14-42). ECMHC and α-cardiac actin transcripts were detected in the heart primordium as early as stage 14 (late gastrula). Both continue to be expressed in the heart at stage 42 (tadpole). ECMHC gene transcripts, however, were not detected in stage 15 (early neurula) through stage 40 (tadpole) somites. The expression of the ECMHC gene will therefore serve as a valuable cardiac-specific molecular marker to study cardiac induction and cardiac gene regulation in *Xenopus*. In addition to embryonic cardiac specificity, the ECMHC was found to be expressed in a subset of head muscles (e.g. orbitohyoideus, interhyoideus) in the stage 42 tadpole. These head muscles are also positive for MyoD expression. This implies a differential mechanism for the regulation of this ECMHC gene (expressed in both heart and some somitically derived muscles) may exist. The ectopic expression of ECMHC transcripts in embryos injected with regulatory factor mRNAs (e.g. MyoD) and in blastula animal caps exposed to various growth factors (e.g. activin) is currently being used to study ECMHC gene regulation and cardiac induction. This work is supported by a Pre-doctoral Fellowship (W.G.C.) and a Grant-In-Aid (A.W.N.) from the American Heart Association, Indiana Affiliate, Inc.

P 105 NEW MEMBERS OF THE GABP-BETA FAMILY OF TRANSCRIPTION FACTORS.

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Herpes simplex virus 1 (HSV1) immediate early genes are induced at the outset of lytic infection by the virion-associated protein VP16. VP16 induction of immediate early genes requires at least two distinct cis-regulatory elements. One element which is characterized by the sequence 5'-TAATGARAT-3' is recognized by a protein complex that includes VP16, Oct-1, and an additional host cell factor (HCF; Christie and Sharp, 1989; Stern and Herr, 1991). The second element required for induction by VP16is characterized by purine-rich sequences that interact with a protein complex called GA binding protein (GABP; LaMarco and McKnight, 1989). The protein complex consists of GABP-β (GABP-β), an ankyrin repeat containing polypeptide and GABP-α, an ETS-related DNA binding protein (LaMarco and McKnight, 1989; LaMarco et al., 1991; Thompson et al., 1991). We have used a standard molecular approach to identify and clone cDNAs of GABP-β-related proteins. We constructed a mouse DNA genomic library and screened it with probes representing the ankyrin repeats or the 3' dimerization region of GABP-β. Southern analysis of the library clones revealed one genomic clone that hybridized only weakly to the ankyrin repeats probe. This novel GABP-β-related fragment shows 80% sequence similarity with the region spanning the ankyrin repeats of GABP-β. The GABP-β-related genomic fragment was used as a probe to screen a mouse lung cDNA library. These analyses have identified cDNAs that encode distinct isoforms of an ankyrin repeat-containing GABP-β-related protein. The characterization of these putative GABP-β-related proteins is in progress. Analysis of novel GABP-β-like proteins will permit studies that address their association with ETS-related proteins such as GABP-α and the importance of this association with respect to transcription activation.

P 106 PROPERTIES OF A CLONAL CELL LINE DERIVED FROM CARDIOGENIC MESODERM OF THE JAPANESE QUAIL,

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A clonal cell line sharing many properties of cardiogenic mesodermal cells has been derived from 20-methylcholanthrene treated splanchnic mesoderm of the Japanese quail. The QCE-6 cell line resulted from the adaptation of a parental clone which had expressed the muscle proteins sarcomeric myosin heavy chain and desmin. However, during continual passage of the parental clone these proteins progressively disappeared. By the time of derivation of the QCE-6 cell line, the expression of these two proteins was no longer detected. However, immunochemical analysis demonstrated that these cells retained other proteins akin to cardiogenic cells, namely both smooth muscle and sarcomeric α-actins. Cardiogenic differentiation can be induced in QCE-6 cells with the addition of retinoic acid and growth factors, as demonstrated by the expression of the muscle proteins sarcomeric myosin heavy chain, desmin, muscle-specific α-actinin, titin, and the cardiac-specific isoform of troponin I (cTnI). In addition to the muscle-specific proteins, noninduced QCE-6 cells displayed positive staining with antibodies to cytokeratin, vimentin, cingulin and N-cadherin, whereas the induced cells also expressed desmoplakin I & II. In conjunction with morphological analysis these results indicate that the QCE-6 cells exhibit the phenotype of epithelial mesodermal cells, which is characteristic of their cardiac origin. Accordingly, these cells did not react to antibodies used as specific markers for endothelial, fibroblastic, smooth and skeletal muscle cells. The QCE-6 cell line demonstrates optimal growth and differentiation on a fibronectin substrate and has been maintained in culture for more than 2.5 years without morphological changes. It is anticipated that the QCE-6 cell line will serve as a useful model system to study cardiogenic differentiation.

P 107 RAT CARDIAC MYOCYTE CELL LINES: TOOLS FOR ANALYSIS OF HEART DEVELOPMENT.

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Ventricular myocyte development in the mammalian fetus is characterized by rapid, near-terminal proliferative events and initiation of cellular maturation. Analysis of growth and maturation of embryonic and fetal cardiomyocytes has been limited due to the lack of a suitable cell line which reflects the cardiac muscle lineage. We have recently developed three such cell lines by retroviral transformation and present data indicating that these cell lines can be further modified and used to assess heart development *in vivo*. BWEM and CLEM cell lines (v-*myc* generated) have been made G418 resistant and permanently express ß-galactosidase (ß-gal) by additional CXL-181 retroviral infection. Single cell cultures from these two lines were established by FACs sorting and multiple clones established. Two clones expressing high levels of ß-gal were selected for microinjection back into developing ventricular tissue of day-10 rat embryos. Results from several injected embryos incubated for an additional 24-36 hrs *in vitro* and stained en-block for ß-gal indicate that ventricular localization of the injected cell lines could be found. ß-Gal stained cells were only found in the ventricle, and appeared to be distributed in all three ventricular walls, with the septal region most avidly loaded with cells. An estimated 15-45 cells were injected per embryo. Because cells were injected into the heart prior to septation, the results suggest that normal morphogenetic changes of the endogenous myocytes occurred and the reintroduced cells did not modify these events. Co-staining of sections from the injected embryos with antisera are ongoing to determine if the ß-gal positive cells co-express muscle marker proteins within and surrounded by endogenous myocytes. The potential for these rat myocyte cell lines to assess cardiac lineage formation and ventricular myocyte growth and development *in vivo* can now be evaluated without formation of transgenic animals. Somatic cell therapy of developing rat embryos or infarcted adult animals as well as cell line-mediated over-expression studies of potential proteins impacting on myocyte development can now be addressed within the normal microenvironment of the developing heart. Supported by: HL-42218 (GLE); HL-42266 (MS); HL-45458 DAF); & AHA (TM)

P 108 EARLY MYOGENIC DIFFERENTIATION TRIGGERED BY ANTISENSE RNA TO ACIDIC FGF

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Acidic fibroblast growth factor (aFGF) and related family members regulate differentiation in various systems ranging from *Xenopus* to mammals. Previous findings that aFGF abundance in cultured myocytes decreases coordinately with differentiation suggests a role for aFGF in controlling myogenic development. In this study, we specifically block the production of aFGF in murine Sol 8 myoblasts using antisense RNA. The creatine kinase activity of the myocytes was used as a marker for differentiation. Sol 8 cells stably transfected with antisense aFGF differentiated earlier than Sol 8 cells stably transfected with the control vector. Exogenously supplied aFGF rescues this phenotype. Further results suggest that the effect of FGF on myogenic differentiation is mediated in part through inhibition of myogenin expression. These results demonstrate a direct role for endogenously synthesized growth factors in regulating myogenesis, and provide support for a general role for related proteins in mammalian development.

P 109 CHARACTERIZATION OF A CARDIAC - SELECTIVE AND DEVELOPMENTALLY UPREGULATED PROMOTER IN TRANSGENIC MICE,

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Based on *in situ* hybridization studies that indicated the expression of the myosin-light-chain-2 (MLC-2) gene in embryonic myocardium, a model system for selective targeting of genes to the heart of transgenic mice has been developed. A 2.1 kb DNA fragment of the 5' flanking region of the rat cardiac MLC-2 gene was fused to the firefly luciferase reporter gene and introduced into fertilized mouse oocytes. In four independent transgenic mouse lines, the expression of the MLC-2 luciferase fusiongene was found exclusively in heart muscle. In contrast, no luciferase activity was detectable in slow- or fast-twitch skeletal muscle, smooth muscle, or any non-muscle tissue of these transgenic lines. Between days 9 to 13 post conceptionem (p.c.), when the atrial trunk and ventricular loop have developed and the formation of inter-atrial and inter-ventricular septum takes place, the luciferase reportergene displayed a 10-fold elevation in activity, which gradually declined to a steady state level 15 days post-partum. Taken together, these results indicate that the 2.1 kb DNA fragment of the 5' flanking region of the cardiac MLC-2 gene contains specific regulatory elements (i) for selective gene expression in cardiac myocytes and (ii) for enhanced expression in early heart development. The 2.1 kb DNA sequence may serve as an important tool to identify heart muscel-specific DNA elements, transcription and/or repression factors, which are responsible for heart muscle differentiation. The transgenic model developed should aid in determining the influences of pathogenic gene products on developing and mature heart muscle to elucidate the etiology of myocardial diseases such as cardiomyopathies.

P 110 α_1-ADRENERGIC-MEDIATED HYPERTROPHY OF NEONATAL CARDIAC MYOCYTES DOES NOT INVOLVE INS 1,4,5-P_3-INDUCED INCREASES IN CYTOSOLIC FREE CA^{2+} CONCENTRATION,

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Activation of α_1-adrenergic receptors in neonatal cardiac myocytes alters contractile activity and induces hypertrophic growth. The mechanisms responsible for these diverse effects are not yet established, but presumably involve the associated α_1-adrenergic stimulation of phosphatidylinositol (PI) turnover. This study examined the relationship between α_1-adrenergic-mediated hypertrophic growth, α_1-adrenergic-stimulated PI turnover and Ins 1,4,5-P_3-induced Ca^{2+} mobilization in quiescent neonatal cardiac myocytes. Exposure to 10 uM norepinephrine in the presence of 2 uM propranolol for 48 hr resulted in a 40 % increase in the ratio of protein to DNA and a 350 % increase in release of atrial naturietic factor, indicating the induction of hypertrophic growth. During this same period, PI turnover remained persistently activated. In contrast, norepinephrine failed to increase cytosolic free Ca^{2+} levels in 85 % of the myocytes examined over this period. The few cell that could be characterized as responding had very small increases in Ca^{2+}. Direct generation of Ins 1,4,5-P_3 by photolysis of microinjected caged Ins 1,4,5-P_3 was also incapable of increasing cytosolic free Ca^{2+} levels. Interestingly, an anti-Ins 1,4,5-P_3 receptor antisera immunoprecipitated a protein of the same molecular weight as the cerebellar Ins 1,4,5-P_3 receptor. These results indicated that, despite α_1-adrenergic-generation of Ins 1,4,5-P_3 and the presence of Ins 1,4,5-P_3 receptors, Ins 1,4,5-P_3 was not capable of increasing cytosolic free Ca^{2+} levels in neonatal cardiac myocytes. Since, in these same quiescent cells, α_1-adrenergic stimulation induced hypertrophic growth, an increase in cytosolic free Ca^{2+} cannot be an important or necessary signal for the induction of cardiac myocyte hypertrophy. *Supported by an AHA Grant-in-aid (CAH), an AHA EI award (JDR) and NIH Grant (SJK).*

P 111 **TARGETTED MODIFICATION OF THE ALPHA-TROPOMYOSIN GENE IN MOUSE EMBRYONIC STEM CELLS,**

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In an effort to better understand the structure function relationships and the coordinate regulation of the various contractile protein isoforms incorporated into the cardiac muscle fiber, we are using gene targetting in ES cells to make specific mutations in the mouse alpha-tropomyosin gene. We have devised a two-step scheme whereby the striated muscle-specific exons are first replaced by a Neo^R/HerpesTK gene cassette using resistance to G418 as the selection protocol. This cassette is then removed or replaced by specifically altered genomic sequences by selecting for resistance to gancyclovir. The primary targetting has been completed and chimeric mice derived. *In vitro* differentiation is being used to examine the ability of targetted cells to form functional embryonic cardiac tissue and to determine the relative levels of cytoskeletal and striated isoforms of tropomyosin MRNA.

P 112 RAT α-TROPOMYOSIN GENE GENERATES THE STRIATED MUSCLE RNA ISOFORM THROUGH INTERNAL MUSCLE-SPECIFIC TERMINATION.

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Rat alpha-tropomyosin (TM) RNA is alternatively spliced to generate isoform diversity. One alternative choice involves the final three exons at the 3' end: 12, 13 and 14. Exons 12 and 13 are selected to provide the final sequence of striated muscle alpha-TM; exon 14 is used to produce nonmuscle and smooth *muscle transcripts*. The complete sequence of the rat alpha-TM gene involving these three exons and introns was determined, including the lengthy intron following exon 13 and the termination sequence after exon 14. Constructs were built that contained the smooth muscle cDNA for exons 1 to 11 and the genomic sequence for exons/introns 11 through 14. To investigate the possibility of obtaining striated muscle-specific termination, the generally accepted termination sequences following exon 14 were not included in these constructs. The constructs were stably incorporated into rat L6E9 (muscle) and R3T3 (fibroblast) cells. Southern blot analysis of DNA generated using RT-PCR from RNA produced by these cells shows that muscle-specific termination occurs after exon 13 in L6E9 but not in R3T3 cells. In addition, the results show that although high levels of muscle transcripts are produced, a small percentage of nonmuscle (non-internally terminated) transcripts are found, a situation which mimics normal *in vivo* alpha-TM production. The regulated production of these isoforms suggests the activity of a muscle-specific transacting factor which allows the region behind exon 13 to be used as a terminator exclusively in muscle cells.

P 113 THE DEVELOPMENTAL BIOLOGY OF VASCULAR SMOOTH MUSCLE (VSM) AS STUDIED WITH A NEW MONOCLONAL ANTIBODY DIRECTED AGAINST AN EMBRYONIC QUAIL VSM ANTIGEN.

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A critical event of vascular development is the proper recruitment of vascular smooth muscle (VSM) cells to the nascent endothelium, which comprises the initial embryonic vascular network. To date there are no available markers to embryonic VSM antigens. Markers to adult VSM are the only available means to identifying embryonic VSM, and are problematic because they are typically expressed by multiple cell types in the embryo. We have prepared a monoclonal antibody, 1E12, against a homogenate of 10 day old embryonic quail dorsal aorta. The 1E12 antigen is a marker for early VSM in the avian embryo. Immunofluorescence microscopy shows this antigen to be present in 3 day embryos through to hatchlings. In comparison to a smooth muscle α-actin monoclonal antibody (the most commonly used adult VSM marker), the 1E12 antibody labels a more restricted set of cells around the vascular endothelium, and does not label myotomal cells or cardiac myocytes in the embryo. (1E12 does label some smooth muscle cells of the developing gut as well.) We are currently attempting to characterize the 1E12 antigen and determine its distribution during early development.

P 114 HIGHLY EFFICIENT GENE TRANSFER INTO ADULT CARDIAC MYOCYTES BY RECOMBINANT ADENOVIRUS.

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The molecular dissection of mechanisms for establishment, maintenance, and modulation of the differentiated cardiac phenotype has, for cogent technical reasons, largely been undertaken in neonatal ventricular myocytes, which are relatively amenable to gene transfer. To circumvent the expected limitations of other methods, the present study was undertaken to determine whether replication-deficient adenovirus (ΔE1, lacking 1.0-9.8 mu) would provide a suitable means for high efficiency gene transfer to adult cardiac cells in culture. Adult ventricular myocytes were isolated from 250-300 g male Sprague-Dawley rats, plated at a density of 10^5 cells/35 mm dish, and subjected to primary culture by a modification of the methods of Piper and Borg. Twenty-four hr after plating, myocytes were infected with up to 2×10^8 PFU of an engineered adenovirus construct containing a CMV-driven lacZ gene (AdLacZ) or adenovirus without insert (Ad), generously provided by A. Bett and F. L. Graham. Forty-eight hr after infection, ventricular myocytes were examined histochemically to identify the efficiency of gene transfer in infected myocytes. The proportion of morphologically differentiated rod-shaped cells exceeded 80%. The frequency of lacZ+ rod-shaped myocytes was half-maximal at 4×10^5 PFU and exceeded 80% at 2×10^6 to 2×10^8 PFU. In contrast, uninfected cells and Ad-infected cells remained colorless at each viral titer examined. Quantitative analysis of β-galactosidase activity in infected myocytes concurred with the proportion of lacZ+ cells. Hence, expression of β-galactosidase activity was specifically contingent on transfer of the recombinant lacZ gene from virus into the adult cardiac myocyte and cannot be accounted for by endogenous lacZ activity. These data suggest that adult ventricular myocytes are amenable to gene transfer with recombinant adenovirus, at a efficiency one to two orders of magnitude greater than that achieved, even in neonatal cells, by plasmid-based techniques. Furthermore, the relatively uniformity for gene transfer by adenovirus should facilitate tests to determine the impact of putative regulators, including dominant-negative mutations, upon the endogenous genes and gene products of virally modified cardiac cells.

P 115 ABSENCE OF CARDIAC GROWTH IN AORTIC CONSTRICTED *c-myc* TRANSGENIC MICE.

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C-myc has been suggested to play a role in cellular proliferation and hypertrophy. Transgenic mice, which constitutively overexpress *c-myc* exclusively in cardiac myocytes beginning in embryonic life, demonstrate cardiac masses at birth which are 1.5 fold increased as compared with non-transgenic littermates (Jackson, T. *et al. Mol Cell Biol*, 1990. 10: 3709-3716). These hearts contain 2.3 fold more cardiac myocytes, the mean volume of which is decreased 40%. The number of cardiomyocytes appears not to increase postnatally. In response to T3 administration, the cardiac masses of these transgenic mice increase more markedly than those of non-transgenic controls (Robbins, R.J., and Swain, J.L. *Am J Physiol*, 1992. 262: H590-597), but the cellular basis of this growth has not been determined. To characterize the cardiac growth phenotype of *c-myc* transgenic mice in response to a hemodynamic load, we subjected these animals (myc) and wild type controls (wt) to 5 days of supra-renal abdominal aortic constriction (AC) or sham operation (S). We wished to determine whether this stimulus would result in cardiac myocyte hyperplasia, hypertrophy, both, or neither. In wt, AC resulted in a 28% increase in the mean left ventricular (LV) to body weight (BW) ratio (AC 3.61 +/- 0.11 mg/g, n = 4 vs. S 2.83 +/- 0.09 mg/g, n = 2; $p < 0.01$). In contrast, LV/BW did not change significantly in myc subjected to AC (AC 3.85 +/- 0.33 mg/g, n = 7 vs. S 3.65 +/- 0.21 mg/g, n = 8; p NS). The expression of ANF mRNA was induced by AC in the LVs of both myc and wt. These results demonstrate the absence of net LV growth in myc animals subjected to AC for 5 days. In addition, ventricular growth and induction of ventricular ANF expression are dissociated. The absence of LV growth in banded myc mice may reflect an intrinsic alteration in cardiac myocyte physiology due to the transgene. Alternatively, myc cardiac myocytes may be relatively unloaded in the basal state due to their increased number. Further experiments with isolated cardiocytes will attempt to distinguish between these possibilities.

P 116 CARDIOMYOCYTE DEVELOPMENT IN EMBRYOID BODIES DERIVED FROM MURINE EMBRYONIC STEM CELLS, Michael G. Klug and Loren J. Field, Krannert Institute of Cardiology, Indiana University School of Medicine, Indianapolis, IN 46202

The expression of cardiac specific markers in embryoid bodies derived from murine embryonic stem (ES) cells was examined. Pluripotent embryonic stem cells induced to differentiate into embryoid bodies (EBs) develop areas of spontaneous contractile activity, which has been associated with α and β cardiac myosin heavy chain expression. The proportion of actively contracting areas is variable between individual EBs. Additionally, chronological variation exists for the initiation of beating. To further characterize cardiomyocytes in EBs, frozen sections were examined immunohistochemically with known cardiac markers. Cardiac specific protein expression appears to correlate well with areas exhibiting spontaneous contractile activity. Reverse transcriptase PCR results concurred with the antibody studies. Upon prolonged culture, EBs loose the beating phenotype. Preliminary data suggests that this is also concomitant with a loss of myofibrillar gene expression. In conclusion, cultured EBs parallel some steps observed during embryonic cardiomyocyte differentiation. The transient nature of the cardiac phenotype presently limits the usefulness for cell culture experiments. Fortunately, ES cells are particularly amenable to the introduction of recombinant genes, which could be designed to alter cardiomyocyte development.

P 117 LONG-TERM SURVIVAL OF AT-1 CARDIOMYOCYTE GRAFT IN SYNGENEIC MYOCARDIUM, Gou Young Koh, Mark H. Soonpaa, Michael G. Klug and Loren J. Field. Indiana University School of Medicine, Indianapolis, IN 46202

The long term viability of cardiomyocyte grafts in the adult myocardium was tested. AT-1 cardiomyocytes, a differentiated tumor lineage derived from transgenic mice expressing an Atrial Natriuretic Factor-SV40 T Antigen fusion gene, were grafted directly into the myocardium of syngeneic animals. Viables grafts were detected as long as four months post-implantation. Thymidine uptake studies suggested that the grafted cardiomyocytes retained mitotic activity. The presence of AT-1 grafts, and the associated myocardial remodeling, *was not* accompanied by overt cardiac arrhythmia. Abundant desmosomes were observed between AT-1 cells and juxtaposed ventricular cardiomyocytes. This study indicates that the myocardium can serve as a stable platform for cells which have been manipulated in vitro, and suggests that cardiomyocyte grafts may provide a useful means for the local delivery of recombinant molecules to the heart. The long term survival of the AT-1 cells also raises the possibility that similar grafting approaches may be useful to replace diseased myocardium.

P 118 ISOLATION AND CHARACTERIZATION OF FOUR NOVEL TISSUE-SPECIFIC HOMEOBOX-CONTAINING GENES, Issei Komuro and Seigo Izumo, Molecular Medicine Unit, Beth Israel Hospital/Harvard Medical School, Boston, MA 02215

Homeobox-containing genes have been shown to play important roles on determining the patterns of body development in mammals as well as in *Drosophila*. A homeobox gene whose expression is restricted to specific tissue would be of particular interest as a candidate for a "tissue specification" gene. We isolated four novel homeobox-containing genes from mouse genomic and embryonic cDNA libraries by low stringenecy hybridization and PCR. Their homeobox sequences are quite divergent from those of *Antennapedia*-type *Hox* genes and they do not cosegregate with the known *Hox* cluster loci. Each of four genes is expressed in a tissue-specific manner, in contrast with the position-specific expression of the *Hox* class genes. Expression of one of four genes, *Gtx*, is restricted in glial cells of the central nervous system, including forebrain, and the germ cells of testis. *Gtx* is a sequence-specific transcriptional repressor and the preferred target sequence of GTX is different from that of *Ant*-type homeodomains. Two other genes (*Kbx*, *Imx*) are localized tandemly on the same chromosome. *Kbx* is expressed in adult kidney and brain. *Imx* is expressed in several tissues including the heart. The forth gene, *Csx*, is expressed only in the heart from 8 day embryo. It is also expressed during *in vitro* differentiation of embryonic stem cells from three days before the expression of α-myosin heavy chain gene. Thus, these tissue-specific homeobox-containing genes may play important roles in tissue differentiation and cell-type specificcation.

P 119 CARDIAC-SPECIFIC AND TGFβ1 INDUCIBLE TRANSCRIPTION OF SKELETAL α-ACTIN IS MEDIATED BY SERUM RESPONSE FACTOR, TEF-1 AND SP1. W. Robb MacLellan*, Te-Chung Lee†, Robert J. Schwartz†‡, and Michael D. Schneider*†‡. *Medicine, †Cell Biology, ‡Molecular Physiology & Biophysics, Baylor College of Medicine , Houston, TX 77030

The α-actins are a family of sarcomeric proteins that display developmental and tissue-restricted expression. Notably, skeletal α-actin (SkA) is expressed in the embryonic ventricle, is down-regulated at birth, and is upregulated in myocardium in response to mechanical and trophic signals including transforming growth factor beta (TGFβ1). Unlike skeletal muscle, where transcription of many muscle-specific genes is dependent on tissue-restricted factors of the MyoD family, no similar cardiac specific factor has been identified. A truncated SkA promoter comprising the proximal 394 bases has full basal and inducible activity and is expressed at ~1000 fold higher levels in cardiac myocytes versus cardiac fibroblasts. We undertook a mutational analysis of the SkA promoter to identify elements important for mediating basal and TGFβ1-induced transcription in rat neonatal cardiac myocytes. In contrast to skeletal muscle where transcription was equally dependent on all three serum response elements (SRE), we found a disproportionate dependence on the proximal SRE (SRE1), despite equal affinity of these elements for cardiac SRF. Using mobility shift assays we showed that cardiac myocyte extracts contain two factors that bind to SRE1, serum response factor (SRF) a member of theMADS-box family and YY1, a functionally diverse zinc finger transcription factor, postulated to be a negative regulator of SkA transcription. Mutations which block SRF binding abolished basal and TGFβ1-inducible expression. Inhibition of YY1 binding increases basal and induced expression, supporting its role as a negative regulator of SkA transcription. Disruption of potential TEF-1/MCAT and SP1 sites that were unimportant in skeletal muscle resulted in a dramatic decrease in the activity of SkA transcription. Thus, cardiac-specific and TGFβ1 inducible expression of SkA both rely on the co-operative interaction of at least three nominally ubiquitous factors. Whether these factors alone are sufficient to explain cardiac-restricted expression or whether recruitment of other tissue-restricted accessory factors is necessary remains to be detrmined.

P 120 DEVELOPMENTALLY REGULATED EXPRESSION OF AUTOCRINE GROWTH POTENTIAL, GROWTH FACTOR RESPONSIVENESS, AND THE cAMP-DEPENDENT TRANSCRIPTIONAL ACTIVATION PATHWAY IN VASCULAR SMOOTH MUSCLE CELLS. Phillip E. Schwartz, Colleen C. Cook, Mary C. M. Weiser, and Richard A. Majack, Department of Pediatrics and Cell and Structural Biology, University of Colorado Health Sciences Center, Denver, CO.

Aortic smooth muscle cells (SMC) were cultured from embryonic (E13-E17), fetal (E18-E21), neonatal and adult rats. All cultures exhibited the characteristic "hill-and-valley" growth pattern, expressed significant levels of elastin and α-actin messages, and stained uniformly positive for smooth muscle-specific α-actin. Embryonic and early fetal (E13-E18) SMC displayed a significant autocrine growth potential *in vitro* which disappeared by day 20 of intrauterine life. Embryonic/fetal SMC were refractory to known SMC mitogens such as PDGF isoforms, FGFs, EGF, and IGF. Unresponsiveness to mitogenic stimulation was not coupled to autocrine growth potential and appeared to be a separate and independently regulated developmental phenotype. SMC replication during development therefore appears to be self-driven and self-regulated; this may insure proper vascular morphogenesis. To identify potential molecular mechanisms involved in the developmental control of SMC phenotype we first examined the expression of the cAMP-response element binding protein (CREB). CREB belongs to the leucine zipper transcription factor family, is phosphorylated and activated by protein kinase A (PKA), and mediates transcription from CREs found within promoters of cAMP-responsive genes. CREB transcripts were undetectable by PCR in SMC until day 1 of postnatal life. PKA subunit gene expression was also examined by PCR in autocrine (E13), non-autocrine fetal (E19), and adult SMC. DNA sequencing of PCR products revealed that adult SMC express the Cα, RIα, and RIIα isoforms of the catalytic and regulatory subunits of the PKA holoenzyme. Additionally, PKA subunit genes were found to be developmentally regulated: E13 SMC expressed no detectable levels of message for either the catalytic or regulatory subunits; E19 SMC expressed small amounts of Cα and RIα mRNAs; adult SMC abundantly expressed Cα, RIα, and RIIα transcripts. Our results suggest potentially important developmental switches in expression of the molecular network responsible for cAMP-mediated gene transcription; these changes may contribute to alterations in autocrine growth potential and growth factor responsiveness observed during aortic SMC maturation.

P 121 EXPRESSION OF THE MOUSE 5-HT2C RECEPTOR IN DEVELOPPING BRAIN AND HEART, Luc Maroteaux, Sylvain Loric*, Jean-François Colas, and Jean-Marie Launay°, Strasbourg Medical School, LGME-CNRS, U184-INSERM, 11, rue Humann - 67085 Strasbourg Cedex - France *Institut Pasteur, 25 rue du Dr Roux, 75724 Paris Cedex 15 France, °Hôpital St Louis, 1 av C. Vellefaux, 75475 Paris Cedex 10 France

A novel member of the family of G protein-coupled receptors has been isolated from a mouse brain cDNA library by screening with polymerase chain reaction (PCR) generated fragment of mouse genomic DNA amplified using degenerated primers. Sequence comparison demonstrates that the encoded protein sequence shows the highest homology to the 5-HT2 family of receptors. The pharmacological profile of membranes from COS-7 cells transfected with this cDNA, corresponds to a new 5-HT2-like receptor that we propose to call 5-HT2C. Its major sites of expression are in the adult mouse intestine and heart, also with detectable expression in brain and kidney. Expression of the 5-HT2C receptor is detected before 8 days of mouse embryonic development, in the neural crest, and then in the heart rudiment. Expression of this receptor is also detected in DDT1 MF-2 smooth muscle cells as well as in teratocarcinoma derived cell lines 1C11. This receptor could account, at least in part, for the "atypical" functions attributed to the 5-HT1C/5-HT2 receptors. Therefore, we propose that some of the peripheral actions of 5-HT, specifically on smooth muscles, are mediated by the 5-HT2C receptor. In addition, the recent finding that serotonin, known to have a vasodilating effect on normal human coronary arteries, has a direct vasoconstricting effect via 5-HT2 like receptors when the endothelium is damaged, (as in coronary artery disease), makes it urgent to characterize in more detail the receptors involved.

P 122 CHRONIC ETHANOL EXPOSURE INDUCES AN ALPHA- TO BETA-MYOSIN HEAVY CHAIN TRANSITION IN RATS. Julia M. Meehan, Susan W. Kelley, and John M. Kennedy. Department of Physiology, University of Illinois, Chicago, IL 60640.

Adult male Sprague-Dawley rats were administered ethanol for 3 months by way of the Lieber-DeCarli liquid diet containing 8% ethanol. Control rats were pair-fed by replacing the ethanol with dextran-maltose to maintain an isocaloric diet. The ethanol-treated rats consumed approximately 3-5 g. of ethanol each day (11-15 g. ethanol/kg. body weight). Blood samples were taken periodically and the average blood ethanol levels were found to be 150 mg.%. The body weight of rats in both groups increased by 59% over the treatment time course. Cytochrome oxidase activity was determined as a measure of the ventricular oxidative capacity and was found to be unchanged by ethanol exposure. The myosin heavy chain isoform population in control and ethanol-exposed ventricles was examined in Coomassie blue-stained SDS-polyacrylamide gels and following Western blotting with an anti-MHC antibody. The relative abundance of alpha- and beta-MHCs was determined by densitometric analysis of stained gels and blots. Control ventricles contained almost exclusively the alpha-MHC isoform. Chronic ethanol exposure resulted in the accumulation of the beta-MHC isoform. Ethanol-exposed rat ventricles demonstrated a 3.1-fold or 2.3-fold increase in the relative abundance of the beta-MHC isoform when determined from Coomassie-stained gels or anti-MHC-stained blots, respectively. It is possible that this transition in the MHC phenotype may play a role in the decreased myocardial contractile force which has been reported following chronic ethanol exposure. This work was supported by grants from the NIAAA (1R29AA08716) and the NIH (T32HL07692).

P 123 AN *IN VITRO* MODEL OF VENTRICULAR SPECIFICATION OF THE MYOSIN LIGHT CHAIN-2 GENE DURING THE DIFFERENTIATION OF EMBRYONIC STEM CELLS INTO EMBRYOID BODIES

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The recent availability of an *in vitro* model of cardiogenesis now offers the opportunity to address issues regarding cardiac gene regulation in the context of cardiac development. The aspects of cardiogenesis, recapitulated during the *in vitro* differentiation of embryonic stem cells and reminiscent of embryonic development in the mouse heart, have been investigated to provide insight into the signalling mechanisms which activate regional specific expression of muscle genes during heart formation and throughout development. To determine whether activation of a cardiac program occurs in this system in a manner temporally and sequentially consistent with the activation *in vivo*, studies were performed to detect the transcriptional and translational products of cardiac muscle markers in differentiated ES cells. The study demonstrated the transcriptional regulation of the myosin light chain-2v (MLC-2v) and atrial natriuretic factor (ANF) genes during embryoid body (EB) cardiogenesis as investigated by reverse transcriptase - linked polymerase chain reaction and RNase protection analyses. MLC-2v and ANF transcripts were initially expressed on day 8 of EB differentiation, concomitant with the appearance of spontaneous contractile activity in localized regions of the EB's. The mRNA levels corresponding to these two genes were gradually up-regulated as the cultures increased in complexity (days 8 through 21). Translational products of these cardiac muscle markers were also detected by immunofluorescence studies in dispersed cells from beating EB's.
Regional patterning of the heart tube occurring at the earliest stages of cardiogenesis has been documented by the restricted expression of the MLC-2v gene to the ventricular region of the heart tube prior to septation and the development of distinct cardiac chambers. The finding of the expression of a ventricular specific marker (MLC-2v) in this *in vitro* system provides evidence for cardiac regional specification independent of positional cues contributed by the primitive heart tube. The demonstration of the expression of appropriate, developmental stage-specific muscle transcripts in differentiated ES cells, and particularly, of a ventricular specific marker, provides further support for the role of this system as a model of cardiogenesis and suggests a promising approach in the investigation of regional cardiac specification.

P 124 Single-stranded-DNA and E-box binding proteins interact with the α-myosin heavy chain proximal regulatory element A. Jeffery D. Molkentin and Bruce E. Markham, Department of Physiology, Cardiovascular Research Center, Medical College of Wisconsin, Milwaukee, WI 53226.

Investigations into the molecular mechanisms responsible for control of the α-myosin heavy chain gene have shown that sequences from -420 to -39 are sufficient for directing cardiac-specific expression. Deletion of sequences from -420 to -284 and -89 to -39 resulted in a loss of expression in cardiomyocytes. This analysis led to the definition of two important sequence elements, PRE-B (-318 to -284) and PRE-A (-92 to -54). The sequence element PRE-A contains both an E-box motif and "CArG-like" sequence. Characterization of PRE-A by EMSA has demonstrated the presence of six nuclear factors that specifically interact with this element. Four of these nuclear factors bind single-stranded PRE A, while the remaining two interact with the double-stranded site. The coding strand of PRE-A interacts with two factors. One is a ubiquitous factor and the other appears to be cardiac-specific. Competition and DEPC footprint analysis show that both of these interactions are specific for the E-box portion of PRE-A. The non-coding strand of PRE-A interacts with two ubiquitous factors, one of which appears to be the previously described MF-3 factor. Competition and footprint analysis of the two double stranded interactions show that one of these factors is specific for the E-box sequence and the other is specific for the "CaRG-like" sequence. The significance of both double and single-stranded interactions, occurring within the same element, is being evaluated. However, both types of interactions occur simultaneously when double-stranded PRE-A is used as a probe in EMSA.

P 125 THROMBIN INDUCES RAPID PROTEIN TYROSINE PHOSPHORYLATION AND MITOGENESIS IN CULTURED RAT AORTIC SMOOTH MUSCLE CELLS VIA ACTIVATION OF THE THROMBIN "TETHERED-LIGAND" RECEPTOR, Christopher J. Molloy, Helen Weber, David S. Taylor, and Steven M. Seiler, Dept. of Cardiovascular Biochemistry, Bristol-Myers Squibb Pharmaceutical Research Institite, Princeton, NJ 08543.

Using a primary rat aortic smooth muscle (RASM) cell culture model, the mitogenic effects of α-thrombin (α-T) and TR_{42-48} (SFLLRNP), a peptide that directly activates the thrombin "tethered-ligand" receptor, were analyzed. Both agonists stimulated DNA synthesis in a concentration-dependent manner as measured 48 h after addition to quiescent RASM cells. However, in control experiments, an inactive N-terminal acetylated analog of TR_{42-48} was ineffective as a mitogen. Maximal levels of DNA synthesis induced by α-T and TR_{42-48} were approximately 10-15-fold above that observed in serum-starved RASM controls. For α-T, maximal DNA synthesis was observed at a concentration of 0.3 - 1.0 nM. Maximal TR_{42-48}-stimulated DNA synthesis was observed at 100 μM. In signal transduction studies, we found that α-T and TR_{42-48} each stimulated rapid tyrosine phosphorylation of proteins with molecular weights of 120-, 75-, 44- and 42-kDa. The latter two proteins were identified as the mitogen-activated protein kinases (*mapk*) $p44^{mapk}$ and $p42^{mapk}$, respectively. Taken together these results indicate that activation of specific "tethered-ligand" thrombin receptors may have an important role in the stimulation of vascular smooth muscle proliferation. α-T-stimulated protein tyrosine phosphorylation and protein kinase activation represent early intracellular signaling events that may be critical to these proliferative responses.

P 126 TARGETED DISRUPTION OF THE SKI PROTO-ONCOGENE IN MURINE ES CELLS. Randall Moreadith, George Albright and Kathy Graves. Molecular Cardiology Laboratories, University of Texas Southwestern Medical Center, Dallas, Texas 75235.

We have initiated a series of investigations into the functions of the mouse *ski* and *sno* proteins. Previous studies have addressed the role these proteins may play in regulating the development of muscle, and have shown that avian *ski* is both myogenic (converts quail embryo fibroblasts to muscle) and can mediate selective hypertrophy of skeletal muscle in transgenic mice. Much less is known about the normal role of these genes in mammals. Studies in this laboratory, as well as others, have shown that c-ski (and c-sno) are widely expressed in the adult with multiple mRNA species. In order to further investigate their role in the animal we have undertaken the strategy of "gene-targeting" in embryonic stem (ES) cells in anticipation that creation of animals which lack *ski* and *sno* protein may more clearly elucidate their function. Both of these genes have been cloned and mutations (deletions plus PGK-neo casettes) have been engineered into exon I to allow for positive selection with G418. Attempts to target the ski locus with neo selection alone were unsuccessful (no targeted clones of >400 screened); however, constructs which employed the HSV-TK gene for negative selection were successful. The targeting frequency at the ski locus was approximately 1:20 (with an overall homology in the targeting construct of 7.5 kb, nonisogenic DNA). Targeting constructs for the related gene, sno, are currently being tested. These targeted ES clones have been injected into C57Bl6 blastocysts and high percentage chimeras have been obtained; matings to determine germline transmission are underway. The preliminary results of these studies will be discussed.

P 127 **Physical and Transcript Mapping of 11p15: a strategy for the localization and identification of the gene(s) responsible for the Long QT syndrome.** David J. Munroe, Eva Bric, Ralf Lobbert, Tania Whitton, Vince Stanton, *Alan Buckler, and David E. Housman. Center for Cancer Research, MIT, Cambridge, MA 02139, and *Molecular Neurogenetics, Massachusetts General Hospital, Charlestown, MA 02129.

The Long QT syndrome (LQT) is an infrequent disorder in which affected individuals present with electrocardiographic QT interval prolongation, a propensity to ventricular tachyarrhythmic syncope, recurrent fainting, and sudden death. Recent genetic linkage analysis, in a large multi generation family predisposed to LQT, has established tight linkage of the LQT locus to H-*ras*-1 located on chromosome 11 band p15.5. We have employed an overlapping set of strategies aimed at localizing and identifying the gene(s) responsible for this disorder through the generation of a high resolution physical and transcription map of 11p15.5-.4. The physical map has been established as a YAC clone contig encompassing 11p15.5-.4. This clone contig was assembled from the 1991 CEPH YAC library (average insert size 430 kbp) using a set of hybridization filters containing interspersed repeat element (IRE)-based PCR products derived from pools of clones from within the aforementioned library. A large set of anonymous markers generated with an anchored-IRE-PCR based cloning strategy are being ordered at high density along this contig. At the same time cDNAs specific to 11p15.5-.4 are being isolated and mapped within the context of the YAC contig. These cDNAs are being isolated from a set of arrayed cDNA libraries designed for high throughput screening by PCR or hybridization. Primers and probes for screening these cDNA libraries include a large set of 11p15.5-.4 specific exons isolated with an exon trapping scheme (PNAS 88: 4005 (1991)) as well as a set of complex hybridization probes generated from YACs and cosmids which map to this region.

P 128 **TROPOMYOSIN EXPRESSION DURING MURINE DEVELOPMENT**, Mariappan Muthuchamy, Laura Pajak, Philip Howles, Thomas Doetschman, and David Wieczorek, Department of Molecular genetics, University of Cincinnati Medical Center, Cincinnati, OH 45267.
To understand the developmental regulation of tropomyosin (TM) genes (α, β, TM30, and TM4), we have analyzed the expression of various TM isoforms in the pre- and postimplantation mouse embryo by employing a RT/PCR technique. Results demonstrate that striated muscle-specific α-TM is initially detected in the day 7.5 postcoitum (pc) embryo, whereas striated muscle β-TM is detected in the preimplantation embryo (day 4.5 pc). The β-TM isoforms, β-TM2 and β-TM3, are initially detected in the 8.5 day pc embryo and continue to be expressed throughout embryonic development. Also, brain-specific α-TM transcripts are detected in the day 7.5 pc embryo. Interestingly, both α- and β-TM smooth muscle isoforms and the nonmuscle TM isoforms, TM30nm and TM4, are expressed constitutively in both pre- and postimplantation embryos.
In addition, we have conducted a quantitative molecular analysis of TM isoform synthesis in embryonic, neonatal, and adult cardiac tissue. S1 nuclease analyses demonstrate that the α- and β-TM striated muscle transcripts are present in the early functional stages of the heart (day-11 embryonic heart), and these TM isoforms are identical to those present throughout cardiac development. Although the transcript levels of both α- and β-TM isoforms are increased during embryonic cardiac development, the ratio of striated α- to β-TM mRNAs changes from 5:1 to 60:1 during the embryonic to adult transition. Thus, our study provides an essential basis for measuring the potential effects following gene-targeted modification of cardiac muscle-specific genes.

P 129 ANALYSIS OF CONTRACTILE PROTEIN GENE EXPRESSION IN DEVELOPING EMBRYOID BODIES.
Laura Pajak, Mariappan Muthuchamy, Philip Howles, Thomas Doetschman and David F. Wieczorek, Ph.D., Dept. of Mol. Gen., Biochem. and Micro., Univ. of Cincinnati Medical School, Cincinnati, Ohio 45267
We examined contractile protein gene expression during early cardiogenesis employing the *in vitro* system of embryonic stem (ES) cells differentiating to embryoid bodies (EBs). Tropomyosin (TM) isoform expression (alpha, beta, and cytoskeletal) was determined, and a molecular analysis of atrial myosin light chain and cardiac troponin T and C transcript production was done. Results demonstrate that at least 12 different TM isoforms are expressed, including 4 striated, 2 smooth, and 1 brain-specific transcript. Also, results demonstrate that both the atrial myosin light chain and cardiac troponin C genes are expressed constitutively throughout differentiation of EBs from ES cells. Transcripts encoding the embryonic cardiac troponin T (cTnT) isoform are transiently expressed in undifferentiated ES cells, re-expressed on day 4 in EB development and remain synthesized thereafter. Conversely, the adult isoform of cTnT is detected only during late EB development (day 22). This interesting profile of TnT isoform expression prompted us to analyze the expression of the murine cTnT gene in isolated hearts from both embryonic and neonatal mice. Results demonstrate the switch from the embryonic to the adult isoform of cTnT begins by day 14 *in utero* and is essentially complete by birth. This pattern of murine TnT isoform transition is temporally distinct from the pattern ascertained in rats which initiate the TnT transition at birth. Furthermore, the temporal profile of contractile protein gene expression as it occurs in ES to EB differentiation reflects *in vivo* patterns, suggesting thin filament production precedes thick filament production in the developing myocardium.

P 130 GENETIC HETEROGENEITY OF CARDIAC DISEASES,
Olivier Pascal*, Sophie Peletier+, Odile Herbert*, Hervé Le Marec+, Jean Paul Moisan*, Jean-Brieuc Bouhour+. *: Genetic laboratory - +: Cardiological department ; University Hospital of Nantes, France.
During the last three years two major cardiac genetic diseases have been localized : **Hypertrophic cardiomyopathy** on chromosome 14q1 (cardiac Myosin Heavy chain gene seems to be directly involved), and **QT long syndrome** on chromosome 11 (closely linked to Harvey Ras 1 gene). In order to confirm this linkage, we have analyzed families, and surprisingly, no close linkage was observed between the diseases and the loci described.
Four families of Hypertrophic cardiomyopathy were analysed, using probes CRIL329, CRIL436 and pSC14. For each family, lod scores < -2 obtained at 1 % recombination with CRIL436, indicating that the defect involved in our families is not linked to Myosin Heavy chain. A family of QT long syndrome (associated with congenital sinus node dysfunction) was analysed. QT long syndrome diagnosis was clearly established with Electrocardiograms arguments. Lod score -∞ were obtained at 0 % recombination, indicating that, in opposition with Keating's families, our family is not closely linked with Hras gene. This result was confirmed by using the Insulin locus probe (located at 4 % recombination of Hras locus).
In conclusion, these results show a genetic heterogeneity in some cardiac diseases ; The same clinical symptoms could be caused by different mechanisms involving different genes.

P 131 A PEPTIDE WHICH ANTAGONIZES α-THROMBIN BUT POTENTIATES OTHER AGONISTS IN PLATELET AGGREGATION, Ulla B. Rasmussen, Christian Gachet*, Yasmin Schlesinger, Daniel Hanau*, Philippe Ohlmann*, Ellen Van Obberghen-Schilling*, Jacques Pouyssegur*, Jean-Pierre Cazenave*, and Andrea Pavirani. Dept. of Molecular and Cellular Biology, Transgene, 11 rue de Molsheim, F-67082 Strasbourg, France. *Centre Regional de Transfusion Sanguine, INSERM U-311, F-67085 Strasbourg, France. *Centre de Biochimie, CNRS-INSERM, Parc Valrose, F-06108 Nice, France.
We have designed a peptide, YFLLRNP, which induces a state of partial activation of the thrombin receptor in human platelets, but is unable to fully activate the receptor. Platelets exposed to YFLLRNP show immediate shape change (pseudopod formation) and potentiation of the ADP and PAF response, but no Ca^{++} mobilization or P47 (pleckstrin) phosphorylation. However, YFLLRNP is an antagonist to low concentrations of α-thrombin and the thrombin receptor agonist peptide (SFLLRNP), demonstrating that it interacts specifically with the thrombin receptor. Furthermore, when the platelets have been prestimulated with adrenaline (10 μM), YFLLRNP behaves as an agonist and induces aggregation, but only in the presence of added fibrinogen. We also found that PGI_2 can inhibit the peptide-induced shape change, but not EDTA, aspirin or apyrase (ADP scavenger).

Platelet shape change seems to occur independent of Ca^{++} mobilization and protein kinase C activation. The thrombin receptor in platelets may therefore communicate, independent of Ca^{++} mobilization or P47 phosphorylation, with factors modulating the cytoskeleton structure and factors involved in potentiation of other platelet agonist receptors.

Thus, YFLLRNP may be useful for differentiating between several possible activation states of the thrombin receptor and it may provide a template for developing more efficient antagonists targeted to the thrombin receptor.

P 132 MOLECULAR GENETIC MAPPING OF THE *IV* MUTATION Donna Rounds*, James McGrath#, Dave Ward*#, Art Horwich# and Martina Brueckner@ Departments of,*Molecular Biophys and Biochem., #Genetics, @Pediatric Cardiology, Yale University School of Medicine, New Haven, CT 06510

Inherited single gene defects in both mice and humans can lead to a loss of control over the direction of cardiac looping and the development of left-right visceral asymmetry. One example of such a mutation in the mouse, situs inversus viscerum (iv), is recessively inherited and results in randomization of left-right asymmetry; approximately 50% of the homozygous, liveborn mice have situs solitus and levocardia while 50% have situs inversus and dextrocardia. Linkage of *iv* to the Igh-C locus (immunoglobulin heavy chain constant region) was determined by RFLP analysis in a backcross of *+/iv* to *iv/iv* mice. In-vivo confirmation that *iv* lies distal to Igh-C was accomplished by mating *iv/iv* mice with T31H translocation mice. The latter bear a balanced translocation with a breakpoint distal to Igh-C. Specifically, monosomy for the segment of chromosome 12 distal to the T31H breakpoint permitted the hemizygous expression of the recessive *iv* phenotype. In order to isolate the Iv gene we have utilized an affinity capture technique combined with a cosmid walk, and have obtained approximately 70 kB of genomic DNA proximal to the telomere. Single-copy probes from these cosmid clones were used to generate a pulsed field map of the most telomeric 450 kB of chromosome 12.

P 133 LRK-1 AND LRK-2: PUTATIVE RECEPTOR-LINKED TYROSINE KINASE GENES EXPRESSED IN EMBRYONIC VASCULAR ENDOTHELIAL CELLS. Thomas N. Sato and Ying Qin, Department of Neurosciences, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110

Vascular endothelial cells differentiate from presumptive angioblasts in early mammalian development. Differentiated endothelial cells divide and migrate to form the mature vascular network. The molecular mechanisms underlying these two processes, vasculogenesis and angiogenesis have not been determined, especially in early mammalian development. Here we report cloning two genes, lrk-1 and lrk-2, that are specifically expressed in endothelial cells of blood islands, endocardium, and dorsal aorta at E8 of mouse embryo. The expression pattern of both genes in early embryonic vasculature is almost identical, although lrk-2 is more abundant, and the expression of both genes is dramatically reduced in adult vascular endothelial cells. The deduced amino acid sequences of lrk-1 and lrk-2 predict a multi-domain structure for the extracellular region and a split-type tyrosine kinase for the cytoplasmic domain. The extracellular domains of both lrk-1 and lrk-2 consist of two immunoglobulin-like structures flanking three EGF-repeats, followed by three fibronectin type III repeats. Based on the sequence similarity and the chromosomal location, lrk-2 may be a mouse homolog of the recently cloned human *tie* (Partanen J. *et al.* 1992, Mol. Cell. Biol. 12, pp.1698) and lrk-1 encode a closely related but a novel receptor protein. *Lrk* and *tie* may form a new class of receptor linked tyrosine kinase genes important for the embryonic vasculature development.

P 134 A NOVEL SUBTYPE OF HUMAN ANGIOTENSIN II RECEPTOR: cDNA CLONING, EXPRESSION AND CHARACTERIZATION, Shun'ichi Kuroda, Hiroaki Konishi, Miki Okishio, Yoshiyuki Inada and Yukio Fujisawa, Discovery Research Labs. II, Takeda Chemical Ind. Ltd., Yodogawa, Osaka, 532 JAPAN

Angiotensin II (AII) is a major regulator of cardiovascular function and fluid homeostasis. Recently, the gene for an AII type 1 receptor (AT_1R) was cloned from rat, bovine and human. In rat, at least two subtypes of AT_1R gene ($AT_{1a/b}R$ genes) were expressed in a tissue-specific fashion. To search for the novel subtype of human AT_1R ($AT_{1b}R$), we amplified several cDNAs by PCR using primers based on the consensus aa sequence of AT_1Rs. The product (267 bp) from human placental cDNA was distinct from the human $AT_{1a}R$, although the products from human heart and liver cDNA encoded the same sequence of human $AT_{1a}R$. By screening a human placental cDNA library with the 267-bp fragment, a full-length cDNA clone (1563 bp) encoding a novel $AT_{1b}R$ was obtained. The $AT_{1b}R$ cDNA encodes a 41-kDa protein (359 aa) with 97.2% aa homology to the human $AT_{1a}R$. All of the aa replacements between human $AT_{1a}R$ and $AT_{1b}R$ reside within the carboxy-terminal half of AT_1R. Distinct pharmacological differences between the human receptors were observed in comparative binding studies of $AT_{1a}R$ and $AT_{1b}R$ expressed in COS-7 cells. AII (IC_{50}, 0.07nM) and AIII (0.85nM) were more potent at the $AT_{1b}R$ than at the $AT_{1a}R$ (0.18 and 26.9nM, respectively). While DuP753 was more potent at the $AT_{1a}R$ (7.8nM) compared with $AT_{1b}R$ (33.1nM), CV-11974 (active form of TCV-116) was most potent at both receptors (about 0.30nM). The AT_2R-specific antagonist PD123177 did not inhibit binding to $AT_{1b}R$ even at micromolar concentrations. Expressed $AT_{1a}R$ and $AT_{1b}R$ in CHO cells showed both a transient increase of intracellular Ca^{2+} and a rapid stimulation of phosphatidylinositol hydrolysis in response to AII interaction. However, these responses at $AT_{1b}R$ were reduced at high AII concentrations (>1000nM), analogous to the rat $AT_{1b}R$. Two AT_1Rs exhibited different responses in the cAMP transduction cascades. $AT_{1a}R$ mediated the accumulation of cAMP formation, whereas $AT_{1b}R$ displayed an inhibitory action on the forskolin-stimulated cAMP accumulations.

P 135 ASSESSMENT OF THE MOLECULAR DEFECT IN FAMILIAL HYPERTROPHIC CARDIOMYOPATHY, Anthony J. Straceski, Anja Geisterfer-Lowrance, Ted Love, Hugh Watkins, Christine E. Seidman, J.G. Seidman, and Leslie A. Leinwand, Departments of Medicine and Microbiology & Immunology, Albert Einstein College of Medicine, Bronx, NY 10461 and Departments of Genetics and Medicine, Harvard Medical School, Boston, MA 02115

Seven distinct missense mutations of the beta myosin heavy chain (MHC) gene have been recently identified in patients with familial hypertrophic cardiomyopathy (FHC). To identify potential molecular defects of these mutant myosins, we have utilized transfections into muscle and nonmuscle cells in culture to assess: (1) protein stability; (2) filament formation; (3) solubility; (4) sarcomere incorporation. With a view toward developing an animal model for this disease, we have chosen for analysis the alpha cardiac MHC, the major ventricular MHC of the adult rat heart. Expression constructs encoding wild type alpha MHC and the seven corresponding FHC mutants were transfected into a mammalian nonmuscle cell (COS). A full length protein of 220kD was identified by immunoblot for each mutant, suggesting that gross alterations in protein stability are not caused by these mutations. Wild type alpha MHC, when transfected into these cells, forms structures previously shown to be thick filaments by several criteria, including electron microscopy. Surprisingly, up to 29% of these cells transfected with the FHC mutants failed to form filamentous structures. Wild type myosin has a characteristic pattern of insolubility in low ionic strength with increasing solubility at high ionic strength. The solubility of a partially purified FHC mutant myosin (R249 to Q) was altered relative to wild type in that it was solubilized at a lower ionic strength. To examine the ability of the mutant polypeptide to incorporate into nascent sarcomeres, the FHC mutant constructs were transfected into a primary culture of human skeletal myoblasts (HuSK). When induced to differentiate, these cells fuse to form myotybes and express four skeletal sarcomeric MHCs. Two FHC mutants (R249 to Q and R403 to Q) were able to incorporate into sarcomeres when examined by immunofluorescence 24-48 hours after myotube formation. We conclude that although the FHC mutant myosins can participate in sarcomere formation in a muscle cell, their ability to form filaments may be impaired.

P 136 *Abstract Withdrawn*

P 137 ENDOTHELIN-1 EVOKED PHOSPHOINOSITIDE TURNOVER IN CARDIOMYOCYTES: LONG-LASTING BUT AT AN ATTENUATED RATE, Han A.A. van Heugten, Karel Bezstarosti, Jos M.J. Lamers, Department of Biochemistry, Faculty of Medicine and Health Sciences, Erasmus University Rotterdam, 3000DR Rotterdam, The Netherlands

Endothelin-1 (ET-1) is known to induce hypertrophy in cultured cardiomyocytes and the response is likely to be signalled through the phosphoinositide (PI) cycle. In the present study ET-1 dose-dependently evoked generation of inositol phosphates (IP_n) and led to increased 1,2-diacylglycerol levels in cultured neonatal rat ventricular myocytes. After 30 min of continuous stimulation with 10^{-8} M ET-1 the velocity of IP_n production decreased dramatically resulting in low but sustained signal transduction after 2 hrs of stimulation. This desensitization of the ET-1 evoked IP_n response was shown not to be caused by depletion of the phospholipase C (PLC) substrate phosphatidylinositol-4,5-bisphosphate. Stimulation of the cardiomyocytes with the α_1-agonist phenylephrine (PHE, 10^{-4} M) also activated PLC but did not result in fast desensitization. After the ET-1 evoked IP_n production was down-regulated the PI pathway could again be activated by PHE but not by ET-1. Activation of protein kinase C (PKC) by incubation with phorbolester (1 μM) inhibited ET-1 as well as PHE-induced PI turnover. However, inhibition of PKC with staurosporine (1 μM) could not prevent desensitization of the ET-1 induced PI turnover, suggesting that PKC was not involved in the attenuation of the ET-1 response. Continuous presence of ET-1 was not required for induction of desensitization as 15 min stimulation of cardiomyocytes with 10^{-8} M ET-1 resulted in desensitization as described above. Recovery from the desensitization as induced by a 15 min pulse with ET-1 took longer than 5 hrs. These data indicate that PI turnover after ET-1 stimulation is rapidly attenuated resulting in long-lasting signal transduction at a low level. This long-lasting effect of ET-1 is maintained after removal of the hormone but renders the cells insensitive to new increases in ET-1 levels. (Supported by the Netherlands Heart Foundation)

P 138 THE ROLE OF THE CONNEXIN-PROTEIN FAMILY IN THE REGULATED CONTRACTION OF THE DEVELOPING MYOCARDIUM. **M.J.A. van Kempen, D. Gros*, E. Lommerde, A. Wessels, M.W.M. Markman, W.H. Lamers, A.F.M. Moorman. Department of Anatomy & Embryology, University of Amsterdam, Amsterdam, The Netherlands.*Laboratoire de Biologie de le Differenciation Cellulaire, Universite d'Aix Marseille, Marseille, France.**

The myocardium of the heart tube is characterized by a slow propagation of the depolarizing impulse, which leads to a peristaltic pattern of contraction. As the heart starts to loop, regional differences in conduction velocity appear. The relatively fast conducting atrial- and ventricular segments are flanked by the slower conducting inflow tract, atrioventricular canal and outflow tract, respectively. With an antibody, raised against gap-junction protein connexin43 (CX43) a developmental distribution profile during rat development was made. The distribution of the CX43 can explain the alternation of fast- and slow-conducting segments. In the adult, the slower conducting segments have become confined to the sinus node (SAN) in the inflow of the atrium and the AV-node (AVN), while the outflow tract has disappeared. In the SAN and the AVN no CX43 protein could be detected. Surprisingly, no CX43 could be detected in the fast conducting AV-bundle and top of the bundle branches. In situ hybridisation studies of the distribution of the CX43 mRNA show no CX43 mRNA either, indicating that the expression of the CX43 protein is primarily regulated at a pretranslational level.

Recent observations in the neonatal and adult heart of rat and pig seem to indicate that connexin40 protein is expressed in the AV-bundle, the bundle branches and the Purkinje network, whilst it seems not expressed in the SAN and AVN. These preliminary results may indicate that the presence of connexin40 in the AV-bundle, the bundle branches and the Purkinje network may contribute to the fast conducting properties of the ventricular conduction system.

P 139 THE SKI PROTO-ONCOGENE : TRANSCRIPTIONAL ACTIVATOR, REPRESSOR, OR BOTH? Michael Venincasa, George Albright, Walter Funk, Theo Pelzer, Woodring Wright and Randall Moreadith, Molecular Cardiology Laboratories, University of Texas Southwestern Medical Center, Dallas, Texas 75235.

The ski proto-oncogene appears to be a novel new member of the family of genes that may control the developmental fate of muscle. It is both myogenic (converts fibroblasts into muscle) in quail embryo cells and causes selective hypertrophy of skeletal muscle in transgenic mice. Indirect evidence suggests it may be a transcription factor, and the above results imply a potential role in the regulation of muscle genes. We have initiated studies of the murine ski and sno genes to investigate the possible role these genes may play in the developmental biology of muscle. We have fused full-length cDNAs of mouse ski and sno to the DNA-binding domain of the yeast protein GAL4 in an attempt to map potentially functional domains of the protein. Transfection of these fusion constructs into both muscle (C2C12) and nonmuscle (10T1/2, NIH3T3) cell lines revealed that both proteins encode a domain that mediates transcriptional repression of a reporter plasmid (UAS-luciferase). These fusion constructs caused a dose-dependent inhibition of the potent transcriptional activator GAL4-E1A (or GAL4-VP16); this repression persisted even when the transcriptional activation domain of E1A was fused to the domain of *ski* which mediates this repression. These results seemed paradoxical given *ski's* known effects in transgenic mice. To further investigate this, potential interactions of *ski* with the known family of HLH proteins which appear to control the development of skeletal muscle (eg. myogenin) were performed. CASTing studies have shown that myogenin can cooperate with factors recognizing a consensus E-box juxtaposed to an NF-1 binding site. Since *ski* appears to bind to an NF-1 site (E. Stavnezer, Keystone 1991) the potential interaction between these two proteins was investigated. Fusion of E-box and NF-1 binding sites to a minimal promoter (HSP-TATA box linked to luciferase) followed by cotransfection into both C2C12 and 10T1/2 cells revealed that *ski* promotes transcriptional activation in an E-box **dependent** and NF-1 **independent** manner. Attempts to detect direct interaction between *ski* and myogenin (by the GAL4 "two hybrid" system) revealed no detectable interaction at the HLH domain. Immunoprecipitation of hemmaglutinin epitope-tagged *ski* with *in vitro* translated members of the HLH family are presently being investigated. The significance of these results will be discussed.

P 140 ASSOCIATION OF PHOSPHATIDYLINOSITOL (PI) 3-KINASE WITH G-PROTEINS: A NOVEL REGULATORY MECHANISM IN HUMAN NEUTROPHILS,

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Treatment of human neutrophils with formyl peptide (f-Met-Leu-Phe) results in neutrophil activation concomitant with stimulation of PI 3-kinase activity as measured by production of PI-3,4,5-P_3 in [^{32}P]-orthophosphate labeled cells. Antiphosphotyrosine immunoprecipitates were assayed for PI 3-kinase activity; essentially no activity was present in lysates from either stimulated or unstimulated cells. The 85 kDa regulatory subunit of PI 3-kinase, which normally serves as a substrate for tyrosine kinases, was not detected by SDS-PAGE or Western blot analysis in antiphosphotyrosine immunoprecipitates. However, immunoprecipitates using polyclonal antibodies against PI 3-kinase showed high PI 3-kinase activity in neutrophil lysates and the 85 kDa subunit of PI 3-kinase was detected in Western blots; no differences in activity were observed in unstimulated or formyl peptide-stimulated neutrophils. In addition, the tyrosine kinase inhibitors genestein and staurosporine failed to prevent formation of PI-3,4,5-P_3 in formyl peptide-stimulated neutrophils, further demonstrating that PI 3-kinase activation is independent of tyrosine phosphorylation. These results suggest that, in contrast to polypeptide growth factor signal transduction systems, the activation of PI 3-kinase by formyl peptide does not require tyrosine phosphorylation, and further suggest a novel mechanism by which G-protein coupled receptors activate PI 3-kinase.

P 141 IN VIVO GENE INJECTION INTO MYOCARDIUM TO IDENTIFY REGULATORY ELEMENTS IMPORTANT FOR THE DEVELOPMENT OF CARDIAC HYPERTROPHY

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The mechanisms involved in the development of cardiac hypertrophy are barely understood. In part, this is attributable to a lack of a suitable model which would allow to determine a relationship between pathophysiological events and the regulatory mechanisms at the molecular level.

Therefore, we established a model where reporter gene constructs are injected into the left ventricle of canine myocardium in situ. Simultaneously, an acute cardiac pressure overload is induced by banding of the ascending aorta creating gradients of 60 - 100 mm HG in the conscious animal postoperatively. Animals are sacrificed 7 days thereafter. During that period, no increase of activity has been found for the hepatocyte-specific apolipoprotein A1 (-256apoA1) and the promiscuous mous sarcoma virus (MSV) promoter constructs. There was a slight increase of activity for the muscle-specific ß-myosin heavy chain (-667rß-MHC) promoter construct (2-fold). In contrast, there was a marked induction of the activity of the ANF (-3400rANF) promoter construct (6 - 10 fold). Moreoever, the deletion of a 550 bp fragment of the ANF-promoter completely abolished the hypertrophic response indicating the presence of a yet unidentified recognition sequence(s) responsible for the induction of the ANF-gene in cardiac hypertrophy. Currently, we are in the process of identifying the recognition sequence and its trans-activator. This may provide an important clue for the understanding of the cascade of activated pathways which eventually lead to hypertrophic growth in the mammalian myocardium.

In conclusion, the model of injection of reporter gene constructs into canine myocardium in situ has proven useful to identify regulatory gene sequences important in the regulation of pathophysiological events.

P 142 CARDIAC EXPRESSION OF POLYSIALYLATED NCAM IN THE CHICKEN EMBRYO CORRELATES WITH ZONES OF SLOW IMPULSE CONDUCTION,

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Measurement of conduction velocity in embryonic chicken hearts has revealed that the tubular heart is uniformly slow in impulse conduction while the septating heart has alternating regions of slow and fast conduction (de Jong *et al.*, 1992, *Circ. Res.* 71:240-50). It is our hypothesis that the mechanism by which heart cells regulate conduction at these early stages involves the up and down regulation of the polysialylated form (PSA) of the neural cell adhesion molecule NCAM. If PSA performs a regulatory function in cardiogenesis as it does in neurogenesis, the presence of PSA would indicate that cell-cell interactions are down-regulated which for myocardial tissue could mean that conduction of an impulse from cell to cell is slowed. Chicken embryos were fixed, embedded, and frozen sectioned. Serial sections were collected and immunofluorescently stained for polysialic acid using a monoclonal antibody. Embryos of stage 13 were analyzed to represent stages when the tubular heart is uniformly slow in impulse conduction. Stage 23 embryos were selected to represent stages when the heart has alternating regions of slow and fast conduction. The myocardium of hearts from stage 13 embryos were positive for PSA with some variation in staining intensity. In contrast, only selected regions of the myocardium of hearts from stage 23 embryos were positive for PSA. These regions included the myocardium lining the outflow tract, lumenal tips of the ventricular trabeculae, the atrio-ventricular junction, and a segment of the right atrial wall. Thus the staining pattern for PSA corresponded to regions previously determined by de Jong *et al.*, (1992) to have slow impulse conduction with the exception of the ventricular trabeculae. The pattern of PSA expression is consistent with its involvement in regulating cardiac impulse conduction before the emergence of the mature conduction system.

P 143 ISOLATION AND CHARACTERIZATION OF A CHICKEN ATRIAL-SPECIFIC MYOSIN HEAVY CHAIN cDNA,

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During chicken heart development, at least four myosin heavy chain genes are expressed in temporally and spatially distinct patterns. Elucidating these patterns is important for understanding cardiogenic commitment and differentiation events and the diversification of cardiac myocytes into atrial and ventricular lineages. In order to study myosin gene expression in the heart, specifically in the atrium, a cDNA library derived from chicken embryo day 14 atrium RNA was screened with a fast MHC cDNA at low stringency. As a result of these experiments, a novel >2.5 kb cDNA that corresponds to the 3' end of an atrially expressed myosin heavy chain gene (AMHC1) was isolated. Sequence analysis of the 3' untranslated region of the AMHC1 cDNA revealed that it is distinct from all other reported MHC genes. Previous studies utilizing monoclonal antibodies directed against atrial specific MHCs demonstrated the existence of a MHC expressed exclusively in atrial cells which may correspond to AMHC1 gene product. The expression of AMHC1 determined by northern analysis of RNA isolated from chicken embryos day 7, 11, 14, 21 revealed that AMHC1 is heart specific at these stages and is expressed exclusively in atrial cells after day 7. Unlike other MHCs expressed in the heart, it is not detected in skeletal muscle tissue at these stages. Studies are currently underway to determine the pattern of AMHC1 expression in very early heart development. In addition, the pattern of AMHC1 expression will be compared directly with the patterns of expression of other heart-specific genes such as VMHC1 and cardiac troponin I to study the coordination of gene expression during cardiac differentiation *in vivo*. The restriction of AMHC1 expression to the heart and specifically to the atrium make this gene important in studying the regulation of cardiac differentiation and diversification.

P 144 BRADYKININ SUPPRESSES RIBOSOMAL RNA SYNTHESIS IN VASCULAR SMOOTH MUSCLE CELLS, Peter Zahradka and Maury Pinsk, Department of Physiology, Division of Cardiovascular Sciences, St. Boniface General Hospital Research Centre, University of Manitoba, 351 Tache Avenue, Winnipeg, Manitoba, Canada R2H 2A6
Angiotensin converting enzyme (ACE) inhibitors have been successfully used to treat hypertension and atherosclerosis. Their ability to restrict *vascular smooth muscle cell* growth, a major contributing factor to these conditions, has generally been attributed to a reduction in angiotensin II levels. However, it has become apparent that the kininase activity of ACE also has a bearing on the physiological responses associated with this therapy. Thus, the accumulation of bradykinin that accompanies the use of ACE inhibitors accounts for some of the benefits derived with this treatment. Ribosomal RNA gene transcription is one of first processes activated when cell growth is stimulated. The incorporation of [^{3}H]-uridine into trichloroacetic acid insoluble material is a suitable method for measuring rRNA gene activity, due to the vast excess of this cellular component relative to mRNA. Using this assay, we have examined the effect of bradykinin on rRNA synthesis in both vascular (responsive) smooth muscle and non-vascular (unresponsive) hepatoma cell populations. Bradykinin did not influence the rate of uridine incorporation in cultures of proliferating H4IIE hepatoma or A10 smooth muscle cells. Quiescent hepatoma cells, obtained following a three day period in serum-free medium, also did not respond to bradykinin. In contrast, bradykinin reduced the level of RNA synthesis by 40% in A10 cells maintained for three days in a defined medium containing insulin, transferrin and selenium. Both indomethacin, an inhibitor of the cyclooxygenase responsible for prostaglandin synthesis, and des-Arg^9,[Leu^8]-bradykinin, a BK_1 receptor antagonist, prevented this effect of bradykinin on RNA synthesis. These results indicate that the action of bradykinin on rRNA gene activity is not only limited to specific tissues, it is also dependent on the differentiation status of the cell. Furthermore, the potential anti-proliferative influence of bradykinin on smooth muscle cells involves the BK_1 receptor and is mediated via the production of prostaglandins. Support for these studies was provided by NSERC Canada and the Manitoba Health Research Council.

P 145 *HEARTLESS*, AN INSERTIONAL MUTATION THAT CAUSES DEFECTS IN MOUSE CARDIOGENESIS. Maobin Zhang,[1] Maureen Gendron-Maguire,[2] Thomas Gridley,[2] and Joseph Grippo.[1] [1]Investigative Toxicology, Dept. Toxicology and Pathology, Hoffmann-La Roche and [2]Dept. Cell and Developmental Biology, Roche Institute of Molecular Biology, Nutley, NJ 07110

A line of transgenic mice that carries a recessive embryonic lethal mutation has been identified. This mutation was generated during a study characterizing the cis-acting regulatory elements for Hox 1.6 expression. Four lines of transgenic mice were generated by pronuclear injection of a transgene containing a *lacZ* coding sequence fused to the presumptive *Hox 1.6* promoter. While no detectable transgene expression could be found in three lines, the fourth line showed an unusual phenotype. In the hemizygous animals, *lacZ* expression is detected from 8.5 to 13.5 days p.c. and is prominently restricted to the conotruncus and right ventricle, the embryonic structures which give rise to the aorta, pulmonary trunk and right ventricle of the cardiovascular system. These mice exhibit normal morphology and breeding performance. When progenies of intercross matings between hemizygous mice were screened, no offsprings could be found homozygous for the transgene and the litter sizes appeared smaller, suggesting that the homozygous mice may not be able to survive to term. At 10.5 days p.c., homozygous embryos were found considerably smaller than their littermates. More interestingly, the conotruncus and right ventricle of the homozygous were not developed, which may account for the embryonic lethality. It appears that the transgene introduced served as an enhancer trap, reporting the expression of a gene active during cardiogenesis. The insertion of the transgene also disrupted this gene, causing a lethal defect in the formation of the embryonic cardiovascular system. Molecular analyses are in progress to clone the DNA sequence flanking the transgene insertion site to identify this heart-specific gene.

Growth Factors and Development; Transcriptional Control

P 200 INTERACTIONS OF THROMBOSPONDINS WITH SKELETAL MYOBLASTS. Jossephine C. Adams and Jack Lawler, Vascular Research Division, Dept. of Pathology, Brigham and Women's Hospital, Boston MA 02115.
Thrombospondins form a family of structurally-related, multi-domain glycoproteins. Thrombospondin-1 (TSP-1, platelet thrombospondin) and thrombospondin-4 (TSP-4) are both expressed in developing heart and skeletal muscle. In the adult, thrombospondin expression is upregulated in skeletal muscle after wounding. As a first step towards examining the biological roles of thrombospondins in this tissue, we are using skeletal myoblast cell lines for in vitro assays. Myoblasts adhere to TSP-1 coated-substrata and undergo extensive spreading. Adhesion cannot be blocked by heparin, or GRGDSP or VTCG peptides, alone or in combination, indicating that previously defined cell-binding sites are not the primary adhesive sites for myoblasts. Using anti-thrombospondin monoclonal antibodies we have mapped adhesive activity to the type 1 repeats and the carboxy-terminal domain and myoblasts adhere to a tryptic fragment of platelet thrombospondin which contains both these domains. To explore the roles of the two sites in more detail, the type 1 repeats and carboxy-terminus of human TSP-1 have been expressed as fusion proteins in the bacterial expression vector, pGEX. The type1 repeats are not adhesive, but the carboxy-terminus supports myoblast attachment and spreading at about 40% the level of the intact molecule. Deletion constructs are being used to define the myoblast binding site in more detail. Myotubes express lower levels of TSP-1 than myoblasts, and the fusion proteins are being used to examine the roles of TSP-1 in myoblast proliferation and terminal differentiation. While TSP-1 is expressed by many tissues, TSP-4 is principally expressed by cardiac and skeletal muscle. TSP-4 does not contain the type 1 repeats and its carboxy-terminal domain has about 65% amino acid identity to that of TSP-1. No functions are known for TSP-4, and we are using the carboxy-terminal domain expressed in pGEX to explore the interactions of this protein with myoblasts.

P 201 THE ROLE OF TGFβs IN MOUSE CARDIOGENESIS. Rosemary J. Akhurst, Marion C. Dickson, Elizabeth Duffie, Julie S. Martin, Department of Medical Genetics, Glasgow University, Yorkhill, Glasgow G3 8SJ, Scotland, UK.

Transforming growth factors β have pleiotropic effects on all cells of the cardiovascular system, including growth inhibition and capillary tube formation of endothelial cells, and stimulation or inhibition of myogenesis. Our detailed *in situ* hybridisation and immunolocalisation studies on early mouse embryos have suggested that TGFβ1 might be involved in endocardial tube formation and valve morphogenesis (1) and that TGFβ2 might regulate cardiomyogenesis and induction of cardiac cushion tissue formation (2,3,4). Two approaches are being taken to ratify these conclusions; a) *in situ* hybridisation studies to localise expression of the type II and type III TGFβ receptor genes during early cardiogenesis, and b) "knock out" experiments on a mouse cardiac culture system, using anti-sense oligonucleotides against the TGFβs and their receptors. Results of these experiments will be presented.

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P 202 *Abstract Withdrawn*

P 203 REGULATION OF MYOCARDIAL CELL DIFFERENTIATION IN THE AVIAN EMBRYO. Parker B. Antin, Department of Animal Sciences, University of Arizona, Tucson, AZ 85721
The vertebrate myocardium is formed by fusion of paired regions of lateral plate mesoderm. Experiments in lower vertebrates have shown that both the appearance of precardiac mesoderm during late gastrula stages and its subsequent differentiation into myocardium is regulated by positive and negative influencing signals from surrounding tissues. Soluble growth factors have been implicated as mediators of these signaling processes. In higher vertebrates, much less is known about the mechanisms controlling the appearance of myocardial cells in the early embryo. As a first step towards understanding how myocardial cell development is regulated in higher vertebrates, a series of experiments were performed in chick to define the role of surrounding cell layers in regulating the appearance of myocardium. Endodermal, mesodermal and ectodermal layers were isolated from the precardiac regions of embryos between stages 4+-7. Tissue fragments were cultured alone or in various combinations on a fibronectin substrate, either in defined medium (DMEM, 5ug/ml insulin, 5ug/ml transferrin, 1nM selenium) or in defined medium containing 10% fetal calf serum (FCS). Explants were examined after 48 hours for the presence of contractile cells, then fixed and scored by immunofluorescence for the presence of muscle-specific myosin heavy chain. Results are summarized as follows: 1) Of 5 mesodermal explants cultured in defined medium, none survived. Of 9 mesodermal explants cultured in defined medium containing FCS, all survived and 8 exhibited large numbers of myosin-positive cells after 48 hours. 2) Culturing of mesoderm with endoderm or with endoderm plus ectoderm resulted in myocardial cell differentiation at high frequency in both defined medium (19 of 21 cases and 6 of 8 cases, respectively) and in FCS-containing medium (13 of 14 cases and 6 of 7 cases, respectively). 3) Explants of mesoderm plus ectoderm showed no differentiation capacity in defined medium (0 of 11 cases), whereas in FCS-containing medium 14 of 16 mesoderm-ectoderm explants showed myosin-positive cells after 48 hours. 4) Explants of isolated endoderm or ectoderm only rarely contained one or a few myosin-positive cells after 48 hours of culture in either medium. These results indicate that although ectoderm and endoderm can support the survival of precardiac mesoderm, endoderm provides a signal(s) that is required for differentiation into myocardium. The finding that FCS contains signals for both survival and differentiation of precardiac mesoderm suggests that soluble factors may be involved in these signaling events. Experiments are presently underway to identify these factors.

P 204 REGULATION OF CARDIAC MYOCYTE GROWTH BY A SOLUBLE FACTOR RELEASED FROM THE PC12 SYMPATHETIC NEURONAL CELL LINE, Dianne L. Atkins, Kari L. Roletter, Penny A. Krumm and Steven H. Green, Departments of Pediatrics and Biology, University of Iowa, Iowa City, IA 52242
Sympathetic innervation of cardiac myocytes *in vitro* induces growth. To establish a model system which will allow purification and identification of the neuronal factor(s) responsible for mediating this regulation, we have initiated studies utilizing conditioned medium (CM) from the PC12 cell line. PC12 cells acquire a sympathetic neuronal phenotype when exposed to NGF. Neuronal PC12 cell CM induces growth in newborn rat cardiac myocytes as measured by surface area and rate of incorporation of [^{35}S]-methionine into protein. The myocyte response to CM is not detectable until after 24 h of exposure; maximal rate of protein synthesis is obtained within 48 h. The growth response is dependent on the proportion of CM added to the culture medium; it is detectable at 15% CM and maximal at 50% CM. The CM factor is heat stable and behaves upon filtration as possessing mass <10,000 Da, indicating that it is a small molecule, possibly a small peptide. We, therefore, examined the potential role of neurotransmitters and peptides known to be present in PC12 cells and sympathetic neurons. CM stimulation of growth could not be mimicked by neuropeptide Y, by somatostatin, nor by α- or β-adrenergic agonists. The effect of CM was not inhibited by α- and β-adrenergic antagonists nor by muscarinic antagonists. These data indicate that neuronal cells release a small soluble factor, different from neurotransmitter, which stimulates myocyte growth. They further identify the PC12 cell line as providing a convenient and abundant supply of this novel molecule, thus facilitating its further characterization.

P 205 THE RELATION BETWEEN THE ENERGY COST OF CROSSBRIDGE CYCLING AND TROPONIN ISOFORM IN FETAL RABBIT HEARTS, Edward M. Blanchard, *Lizhu Gao, *R. John Solaro. Departments of Physiology and Biophysics, University of Vermont, Burlington, VT 05405 and *University of Illinois, Chicago IL.
There are important instances when significant changes in the energy cost of crossbridge cycling occur in the absence of shifts in the myosin heavy chain isozyme profile, V_1/V_3, e.g. in failing human hearts due to either mitral regurgitation or idiopathic dilative cardiomyopathy. One idea advanced to account for some of the functional changes of the contractile apparatus of failing human hearts is that the disease represents a partial recapitulation of fetal troponin isoforms. We have made preliminary measurements of mechanical/thermal performance (30°C, 0.33 Hz) of papillary muscles and troponin isoform profile from samples of right ventricles of fetal rabbit hearts at gestation day 29. Controls are compared to a group exposed to indomethacin 48 hrs prior to sacrifice to close the ductus arteriosus and induce right ventricular pressure overload. The treatment produced significant right ventricular hypertrophy (~37%), a depression in mechanical/thermal output of isometric twitches (~40%), and a decrease in the energy cost of crossbridge cycling (~30%). These changes in papillary muscles due to the indomethacin treatment were associated with shifts in both the troponin T and troponin I isoform profile toward more mature forms, not toward less mature forms, along with no change in the myosin V_1/V_3 profile. Interestingly, the treatment caused the troponin isoform profile of left ventricular samples to shift in opposite directions, toward a more mature profile for TnT and a less mature profile for TnI. Additional myothermal data from left ventricular preparations should help identify which troponin isoform shift is correlated with changes in the energy cost of crossbridge cycling.

P 206 ANF AND TYPE B NATRIURETIC FACTOR GENE ACTIVATION DURING CARDIAC MYOGENESIS IN CELL-CULTURE MODELS. Poppo H. Boer (1), Jeanne Phipps (2) and Zahra Rassi (1), University of Ottawa Heart Institute (1), 40 Ruskin Street, Ottawa, Ontario, Canada K1Y 4E9 and National Research Council of Canada (2), M54, Montreal Road, Ottawa, Ontario, Canada K1A 0S2.

We have surveyed a number of multipotential mouse stem-cell lines that can be induced in vitro for cardiac myogenesis using RT-PCR (reverse transcriptase- mediated polymerase chain reactions) to monitor activities of induced genes, such as those encoding isoforms of cardiac muscle proteins and, as positive control, activity of the housekeeping gene pgk-1, in small culture samples taken at daily intervals. Differentiation was induced by aggregation, for P19 embryonal carcinoma (EC) cells for 5 days in the presence of 0.5% v/v DMSO, and for D3 embryonic stem cells (ES) after LIF growth factor withdrawal.

Developing muscle tissue with centers of spontaneous beating activity appeared after 8 - 10 days in both EC and ES cell-culture models and this was accompanied by a relatively high level of structural muscle gene activation such as the cardiac alpha-actin gene. In contrast, ANF and BNF transcripts were not detected in D3 cultures, and, in the P19 cultures they were induced but at lower steady state levels compared with accumulated alpha-actin gene transcripts. ANF transcripts were first seen at day six, whereas BNF transcripts appeared later in beating muscle structures. These results suggest that when comparing ANF and BNF genes, these are subject to different control mechanisms, and also, that ANF/BNF gene activation differs from that of structural cardiac muscle genes. By modifying the culture conditions we aim to increase the endogenous ANF/BNF gene activities and that of stably transfected promoter-reporter DNA constructs.

Supported by the Medical Research Council of Canada and the Heart and Stroke Foundation of Ontario.

P 207 DOMINANT NEGATIVE MUTANTS OF THE TYPE II TGFβ RECEPTOR AND p21 H-ras INHIBIT TGFβ DEPENDENT SKELETAL α-ACTIN TRANSCRIPTION IN CARDIAC MUSCLE CELLS, Thomas Brand, Maha M. Abdellatif and Michael D. Schneider, Molecular Cardiology Unit, Baylor College of Medicine, Houston, TX, 77030
Transforming growth factor β (TGFβ) and related peptides are important for mesoderm formation and early cardiogenesis. TGFβ stimulates heart muscle formation in embryonic stem cells and amphibian cardiogenic mesenchyme. In neonatal cardiac myocytes, the expression of at least 7 tissue-restricted genes including skeletal α-actin (SkA) is regulated by TGFβ1. In order to prove the functional role of TGFβ in cardiac development, specific inhibitors of TGFβ signal transduction are needed. The recently cloned type II TGFβ receptor (TβRII) has a serine/threonine kinase domain as its predicted signal transducing component and therefore differs from all classes of growth factor receptors with tyrosine kinase activity (RTK). Kinase-defective RTK mutants inhibit function of the respective wild type receptors, but whether a kinase deletion mutant of TβRII can function as a dominant inhibitor is not known. We constructed a truncated TβRII mutation (ΔTβRII) lacking cytoplasmic residues 280-560, by polymerase chain reaction. To further investigate signal transduction of TGFβ we studied the role of p21 H-ras. We used the N17 mutant of p21 H-ras, which has been well characterized as a dominant suppressor of RTK signal transduction. Both mutants were tested by transfecting the mutant or expression vector into Percoll-purified neonatal rat cardiac myocytes, together with firefly luciferase under the control of the chicken skeletal α-actin promoter (nt -394/+24) as the reporter gene, and CMV-lacZ to control for transfection efficiency. TGFβ stimulated SkA transcription ~6-fold. ΔTβRII decreased TGFβ-dependent stimulation of the SkA promoter to 1.9-fold, with little or no effect on basal expression. Similarly, N17 ras selectively inhibited TGFβ-dependent SkA transcription. Thus, a kinase-defective TβRII and N17 ras mutation can efficiently block TGFβ-dependent transcription. The dominant-negative TβRII provides a specific means to investigate the function of endogenous TGFβs during cardiac organogenesis.

P 208 NEURAL CREST CELLS PROTEOLYTICALLY ACTIVATE LATENT CARDIAC TGF-ß, Philip R. Brauer, Department of Biomedical Sciences, Creighton University, Omaha, NE 68178
Evidence suggests that cell-cell interactions between neural crest (NC) cells and cells of the outflow tract are required for normal cardiac development and may be mediated through TGF-ß. TGF-ß is usually secreted as a latent molecule that must be "activated" in order to interact with receptors on target cells and can be activated by cell-mediated proteolysis. The purpose of study was to determine whether 1) cultured outflow tract segments secrete TGF-ß in active or inactive form, 2) latent TGF-ß is activated by NC cell-associated plasminogen activator (PA) activity, and 3) active TGF-ß or outflow tract conditioned medium (CM) regulates NC cell PA activity. Using a TGF-ß bioassay and specific neutralizing TGF-ß antibodies, only latent TGF-ß3 (acid activatable) was detected in serum-free CM of cultured chick stage 13-14 and 18-19 outflow tract segments. To determine if cranial NC cells can activate latent cardiac TGF-ß, NC cell cultures were incubated in serum-free outflow tract CM in the presence or absence of plasminogen and the level of active versus latent TGF-ß in the medium was measured after 24 hr. Only in the presence of plasminogen was active TGF-ß3 detected in the medium. To determine if TGF-ß or outflow tract CM mediates NC cell-associated PA activity, NC cells were incubated in various concentrations of active TGF-ß or outflow tract CM and the NC cell PA activity was measured using an amidolytic microtiter assay. TGF-ß significantly decreased NC cell PA activity in a dose-dependent manner. At 1000 pg/ml TGF-ß, PA activity was reduced by 60% (45.5 $\pm$ 1.3 uU/ug protein/hr in untreated vs. 19.0$\pm$ 0.6 uU/ug protein/hr in TGF-ß treated, $P<0.0001$) and significantly decreased it within 6 hr. No increase in PA activity was observed in presence of fibrin fragments suggesting a lack of tissue-type PA component to the PA activity measured in NC cells. NC cells that were incubated in outflow tract CM also had a significant decrease in cell-associated PA activity (46.4 $\pm$5.4 uU/ug protein/hr in untreated vs. 8.5 $\pm$ 0.6 uU/ug protein/hr in outflow tract CM-treated; $P<0.0003$). This effect was neutralized by the addition of TGF-ß antibodies. These studies show that outflow tracts secrete latent TGF-ß and that NC cells can activate it by plasminogen-dependent mechanisms in vitro. In addition, NC cell PA activity is regulated by active TGF-ß and outflow tract-derived TGF-ß. From these observations, we hypothesize that upon entering the outflow tract, NC cells regulate key outflow tract developmental events by activating latent trophic factors through autocrine/paracrine-regulated proteolysis.

P 209 SV40-T ANTIGEN ASSOCIATED PROTEINS IN AT-2 CELLS DURING THE G1/S-PHASE TRANSITION. Amy S. Brooks*, Joseph B. Delcarpio*, Loren J. Field** and William C. Claycomb*, *Departments of Anatomy and Biochemistry and Molecular Biology, Louisiana State University Medical Center, New Orleans, LA 70112 and **Krannert Institute of Cardiology, Indiana University School of Medicine, Indianapolis, IN 46202
AT-2 cells represent an immortalized cell line derived from primary cultures of atrial cardiac muscle cells, originating from αMHC-SV40 T-antigen transgenic mice. AT-2 cells grow as a monolayer, exhibit a doubling time of 24 hours and have been passaged over 200 times. T-antigen is responsible for immortalizing AT-2 cells but the molecular details concerning how T-antigen controls passage through the G1 restriction point and entry into the S phase are not known. One step toward understanding AT-2 cell cycle regulation is to identify proteins that either associate with or dissociate from T-antigen during the G1/S transition. AT-2 cell growth can be blocked at the G1/S boundary using aphidicolin (7.5 μg/ml). Following release from an 18 hour exposure to aphidicolin, AT-2 cells show a peak incorporation of ^{3}H-thymidine within two hours. For immunoprecipitation of T-antigen complexes during the G1/S transition, three separate cultures, *untreated, aphidicolin blocked* (t = -4hr) and *aphidicolin released* (t = +2hr) were radiolabeled for four hours using either ^{32}P orthophosphate or ^{35}S methionine. Lysates were precleared using Sepharose A beads and total lysate protein concentrations were determined. Standard techniques of immunoprecipitation were followed using commercially available monoclonal antibodies to T-antigen (MAb 419), p53 (MAb 421) and cdc2 (PSTAIRE). Immunoprecipitated complexes were analyzed on 7.5% polyacrylamide gels and sample volumes loaded were normalized to results of the protein assay. Preliminary results show distinct changes in the association of proteins with T-antigen as the cells enter the S phase of the cell cycle.

P 210 MOLECULAR ANALYSIS OF CARDIAC-LIKE MYOSIN HEAVY CHAIN (MHC) EXPRESSION IN DIFFERENT SKELETAL MUSCLES UNDERGOING REGENERATION, Blanca Camoretti-Mercado, Evelyn Dizon, Kris Paterson, Smilja Jakovcic, and Radovan Zak, Department of Medicine, The University of Chicago, Chicago, IL 60637

Immunological studies from our laboratory have shown that ventricular (V) MHC is present in somites during early avian development, and in regenerating adult ALD muscle after cold injury. We have cloned both the adult V-MHC and a ventricular-like (primordial, P) MHC cDNAs expressed in ALD muscle at 3 days of regeneration. Comparison of partial nucleotide sequence revealed more than 97% homology in the C-terminal as well as in the 3' UT portion of these genes. The expression pattern in different muscle types following cold injury of 11-week old chicken was examined by RNase protection assay. Total RNA from ALD, PLD, gastrocnemius and pectoralis muscles were isolated at 0-1-2-3-4-6-9 and 14 days of regeneration as well as from the adult ventricle. Using either a probe specifying the coding region of V-MHC or the 3' UT portion of P-MHC, expected protected fragments were detected very early (1d) and up to 6d in ALD muscle. A lower and transient expression (3d) was observed in PLD muscle. However, no expected protected band was seen neither in pectoralis nor in gastrocnemius muscles. These results suggest that the primordial phenotype is restricted, at least in part, to specific muscles and it might reflect differences in the genetic reprogramming potential of adult myoblasts, their embryological origin and physiological roles.

P 211 CARDIAC FIBROBLASTS DIFFERENTIATE INTO ENDOTHELIAL CELLS WITH RETINOIC ACID TREATMENT, Lorraine E. Chalifour and Ala-Eddin Al Moustafa, Bloomfield Center for Research in Aging, Sir Mortimer B. Davis-Jewish General Hospital and Department of Microbiology and Immunology, McGill University, Montreal, Quebec, Canada H3T 1E2.

We isolated a single line of transgenic mice which develop a 3 to 5-fold enlarged heart due to the expression of the immortalizing gene, polyomavirus large T-antigen in cardiomyocytes (Chalifour et al 1990 Oncogene 5:1719-1726). Immortal cell lines were isolated from adult transgenic but not from non-transgenic hearts. All of the 20 individual clones tested to date expressed vimentin but not desmin or myosin heavy chain. We conclude the cell lines isolated are non-muscle, cardiac fibroblasts. All the cardiac fibroblast cell lines demonstrated profound morphological and biochemical effects when incubated with between 10^{-9}M to 10^{-6}M retinoic acid. Morphologically, all clones examined showed arrested cellular proliferation and the formation of lines and vesicles. Retinoic acid did not induce expression of desmin or myosin heavy chain. Treated cells were able to incorporate acetylated low density lipoprotein and expressed Factor VIII. Incubation with dimethyl sulfoxide or transforming growth factor-ß decreased cell proliferation with no effect on morphology, desmin or myosin heavy chain expression. We conclude that these cardiac fibroblasts differentiate to an endothelial lineage in the presence of retinoic acid.

P 212 **EXPRESSION OF HOMEOBOX GENES *Msx-1* (*Hox-7*) AND *Msx-2* (*Hox-8*) DURING CARDIAC DEVELOPMENT IN THE CHICK,** Penny S. Chan-Thomas, Robert P. Thompson[1], Benoît Robert[2], Magdi H. Yacoub and Paul J.R. Barton, Department of Cardiothoracic Surgery, National Heart and Lung Institute, London, U.K., [1]Department of Cell Biology and Anatomy, Medical University of South Carolina, Charleston, S.C., USA, [2]Département de Biologie Moléculaire, Institut Pasteur, Paris, France.

The vertebrate homeobox genes *Msx-1* and *Msx-2* are related to the Drosophila *msh* gene and are expressed in a variety of tissues during embryogenesis. We have examined their expression by *in situ* hybridisation during critical stages of cardiac development in the chick from stages 15+ to 37. *Msx-1* expression is apparent in a number of non-myocardial cell populations, including cells undergoing an epithelial to mesenchymal transformation in the atrioventricular junction and the outflow tract. These cells form endocardial cushions, which are integral to heart septation and valve formation. The pattern of expression of *Msx-1* suggests that it has a role during both the induction of the mesenchyme formation and the maturation of the developing cushions.

Msx-2 expression is restricted to a distinct subpopulation of myocardial cells that, in later stages, coincides morphologically with the cardiac conduction system. The timing of *Msx-2* expression suggests that it plays a role in conduction system tissue formation and that it identifies precursor cells of this specialised myocardium. The pattern of *Msx-2* expression has important implications for the understanding of the origins and development of the conduction system of higher vertebrates.

P 213 MYOCARDIAL ENLARGEMENT IN DEFECTIVE HEART DEVELOPMENT, Tony L. Creazzo and Jarrett Burch, Department of Cellular Biology and Anatomy, Medical College of Georgia, Augusta, GA 30912

Wet and dry weights of ventricles and whole embryos and the number and density of ventricular myocytes, were compared in sham-operated embryos and in embryos with a severe outflow tract defect known as persistent truncus arteriosus (PTA). The experiments were conducted at day 11 of incubation in the chick embryo. PTA was produced by bilateral ablation of the cardiac neural crest (neural crest extending from the mid-otic placode to the caudal region of somite 3) prior to migration. We found that the wet and dry weights of ventricles from hearts with PTA were not different from those of normal hearts from the sham-operated groups. However, the embryos with PTA weighed less than embryos with normal hearts (see Table). The ratios of the ventricle to embryo weight were 19 and 58% greater in embryos with PTA for wet and dry weights, respectively. This discrepancy between the wet and dry weight ratios indicated that the embryos with PTA were edematous. The number (~8.5×10^6/vent.) and density (~2.7×10^5 myocytes/mm^3) of ventricular myocytes, as determined by morphometric analysis, were not different when comparing sham-operated and experimental groups. The results indicated that, although the ventricles were enlarged with respect to body weight in embryos with PTA, there was no apparent change in the size or number of ventricular myocytes. When plotting embryo vs. ventricular dry weights the embryo weight increased linearly with respect to ventricle weight for both sham and PTA. However, the slopes (79 & 48, respectively) and y-intercepts (0.0 & 66.0, respectively) were different. These results suggest that a progressively larger heart was required to support a similar sized embryo when comparing PTA with sham-operated controls. Supported by NIH grants HL39039 and HL36059.

Group	Embryo		Ventricle	
	wet (g)	dry (mg)	wet (mg)	dry (mg)
sham	4.47±0.15	376±22	33.4±1.6	4.01±0.22
PTA	3.81±0.15	266±13	32.8±1.6	4.49±0.29
p (t-test)	0.006	<0.001	0.80	0.23

N = 12 to 23

P 214 CARDIOMYOCYTE TRANSFER INTO THE RAT AND MOUSE HEART, Joseph B. Delcarpio[1], R. Wayne Barbee[2], Bret D. Perry[2] and William C. Claycomb[3], Departments of [1]Anatomy and [3]Biochemistry and Molecular Biology, Louisiana State University Medical Center, New Orleans, LA 70112 and [2]Division of Research, Alton Ochsner Medical Institutions, New Orleans, LA 70121

Cardiomyocytes (AT-1 cells) isolated from a transplantable tumor lineage derived from transgenic mouse atrial cardiomyocytes that express the SV40 large T oncogene were transferred into the ventricles of immunosuppressed rats and syngeneic mice. To prevent rejection of injected AT-1 cells, adult female Sprague Dawley rats were administered cyclosporine (SandImmune) by gavage tube approximately two hours prior to surgery and then once daily thereafter. Using aseptic technique, a left parasternal thoracotomy was performed and the thoracic cavity was opened exposing the heart. Ten μl (approximately 10^4 AT-1 cells in Joklik's media) were injected into the ventricular wall. Some rats were injected with media containing basic fibroblast growth factor (bFGF). Similar technique were utilized for adult female C57BL/6J mice. Animals were examined immediately following surgery, and at various intervals post-injection using light and transmission electron microscopy. AT-1 cells were easily identifiable in rats and mice examined at each time point by their intense basophilia following hematoxylin and eosin staining. Use of Mason's trichrome indicated the presence of some connective tissue at the site of injection and demonstrated that the incorporated AT-1 cells stained similarly to the normal muscle cells present in the host myocardia. Electron microscopic examination of serial sections from injected regions in the same animals confirmed that AT-1 cells were present and clearly identifiable by their atrial specific granules and loosely organized myofibrils compared to the host's ventricular muscle cells. These results indicate potential therapeutic uses for myoblast transfer into the heart.

P 215 RNA AND PROTEIN LOCALISATION OF TGFβ2 IN THE EARLY MOUSE EMBRYO SUGGESTS AN INVOLVEMENT OF THIS MOLECULE IN CARDIOGENESIS Marion. C. Dickson, Elizabeth Duffie, Rosemary J. Akhurst. Duncan Guthrie Institute of Medical Genetics, University of Glasgow, Glasgow G3 8SJ UK.

Three mammalian transforming growth factor β molecules have been identified, and extensive descriptive analysis of the expression patterns and protein localisation of these molecules suggest they each have distinct biological functions (Akhurst et al. 1990a). We are interested in the role of these molecules during early morphogenesis of the heart (Akhurst et al. 1990b). We have performed a detailed analysis of the localisation of RNAs for TGFβ2 and TGFβ3, and of TGFβ2 protein in mouse embryos from 6.5 to 9.5 days *post-coitum*, (*p.c.*), using *in situ* hybridisation and immuno-histochemistry to serial sections, and whole mount *in situ* hybridisation to complete embryos (Dickson et al. submitted). TGFβ3 RNA was only detected at very low levels by whole mount *in situ* hybridisation around the outflow tract of the heart at 8.5 days *p.c.* TGFβ2 RNA was expressed at high levels, as early as 7.5 days *p.c*, in all cells with the potential to differentiate into cardiomyocytes. TGFβ2 RNA levels diminished within the myocytes as cardiomyogenesis proceeded, however, concomitantly there was an increase in staining for TGFβ2 protein in these cells. These results implicate the involvement of TGFβ2 in cardiomyogenesis. Additionally, an upregulation of TGFβ2 RNA in the myocardium of the outflow tract and atrio-ventricular canal between 8.5 and 9.5 days *p.c.*, suggests that TGFβ2 may be a good candidate for the myocardial induction signal necessary for formation of cushion tissue (Millan et al 1991).

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P 216 TEMPORAL PATTERN OF EXPRESSION OF MYOSIN HEAVY CHAIN, ANF AND BNF IN MONOCULTURE AND COCULTURE OF ADULT RAT VENTRICULAR MYOCYTES WITH EPICARDIAL MESOTHELIAL CELLS. Hoda Eid, Jia Hua Chen, Mercedes Kuroski de Bold and Adolfo J. de Bold, University of Ottawa Heart Institute, Research Centre, Ottawa, Ontario, Canada K1Y 4E9

We have recently isolated, purified, and characterized epicardial mesothelial cells (EMC) from adult rat heart. We demonstrated that these cells, when cocultured with adult rat ventricular myocytes (ARVM), affect myocytes phenotype in long term culture. ARVM in coculture had abundant thick and thin filaments in sarcomeric organization with synchronous large amplitude contractions. To further characterize this cell-cell interaction we studied the temporal pattern (7 and 14 days) of myocytes reorganization in monoculture and coculture. Using SDS-PAGE and radioimmunoassay techniques we studied the expression of myosin heavy chain (MHC) and the secretion of atrial natriuretic peptide and brain natriuretic peptide (ANF and BNF) respectively. As early as 7 days there was at least three times more MHC in cocultured ARVM. The total amount of MHC continued to increase until day 14, no change was observed in monocultured cells. α-MHC was present during the first week in both monocultured and cocultured ARVM. However, at day 14 a decrease of α-MHC of about 50% was seen in monocultured ARVM concomittantly with the reappearance of about 50% of ß-MHC. In contrast, cocultured cells show the same abundance of α-MHC from day 7 to 14 with the reexpression of ß-MHC. Previously, we have shown that pre-pro ANF mRNA reexpressed in monoculture was not affected after 14 days of coculture. ANF and BNF secreted in the cultured media at day 14 show an increase of 210% and 148% in monoculture and a decrease of 180% in coculture when compared to the levels obtained at day 7. These results show that EMC cocultured with dedifferentiated ARVM induce a rapid increase in total MHC content without affecting α-MHC expression. Interestingly there was a significant decrease of ANF and BNF released into the condition media of cocultured cells.

P 217 THE INFLUENCE OF THYROID HORMONE ON CARDIAC SARCOPLASMIC RETICULUM Ca^{2+} PUMP (SERCA2) EXPRESSION, David J. Fisher, Tim McQuinn, Sharon Phillips, Charlotte A. Tate, Orla M. Conneelly, and Robert Schwartz, Departments of Pediatrics and Cell Biology, Baylor College of Medicine, Houston TX, 77030, and Department of Pharmacology, University of Houston, Houston, Tx, 77024.

We have previously demonstrated developmental regulation of cardiac sarcoplasmic reticulum (SR) Ca^{2+} pump expression during perinatal maturation in the rabbit in vivo. In order to evaluate a potential responsible factor, we examined the role of thyroid hormone because it increases during perinatal gestation in a manner similar to cardiac SR Ca^{2+} pump expression. We examined the effects of T_3 on SERCA2 mRNA, SR mediated Ca^{2+} uptake, and immunodetectable SR Ca^{2+} pump protein in cultured chick embryo cardiomyocytes. We digested freshly minced hearts with collagenase A/trypsin, and isolated the cardiocytes with a Percoll gradient. The cells were maintained in MEM supplemented with 5% charcoal stripped HIHS, 10^{-6}M insulin, and $\pm 10^{-8}$M T_3 for 3 days. Northern analysis of isolated RNA with a human SERCA2 riboprobe demonstrated that treatment with T_3 produced a 3.2 fold increase in SERCA2 specific mRNA (n=6; $p<0.01$). T_3 also increased the oxalate sensitive (ie, SR Ca^{2+} pump specific) $^{45}Ca^{2+}$ uptake rate by 1.75 fold in digitonin permeabilized cultured cardiomyocytes (n=5; $p<0.01$). The T_3 induced change in $^{45}Ca^{2+}$ uptake was inhibited by submicromolar thapsigargin, a specific inhibitor of the SR Ca^{2+} pump. Preliminary data (n=1) by Western analysis suggests that T_3 stimulates a 2-3 fold increase in immunodetectable SR Ca^{2+} pump protein. These results demonstrate that T_3 is an important regulator of cardiac SR Ca^{2+} pump expression in the cardiomyocyte. The parallel augmentation of specific mRNA, Ca^{2+} uptake activity and protein suggest that the effect of thyroid hormone is mediated by transcriptional regulation of the cardiac SERCA2 gene.

P 218 CONTRACTILE ALTERATIONS DURING MYOCARDIAL DEVELOPMENT IN THE CHICK SUBSEQUENT TO ABLATION OF CARDIAC NEURAL CREST.

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In chick, neural crest ablation (at stage 9) has been used to produce heart defects (e.g., persistent truncus arteriosus, PTA) analogous to those in humans (Kirby & Waldo, *Circ. 82*:332-340, 1990). Previous studies using *in ovo* microcinephotography suggested that myocardial contractility was decreased in hearts prior to development of PTA (Leatherbury et al., *Circ. 81*:1047-1057, 1990). We examined isometric force generated by isolated preparations from chicks between embryonic days 3 (stage 18) and 15. At stage 18 twitch force of the whole heart (truncus arteriosus to atrium) was similar in normal and neural crest ablated animals. At days 7 and 15, however, twitch force of isolated trabeculae from left ventricle was reduced by 50 % in hearts showing PTA. At all ages, similar results were obtained when twitch force was maximized by elevation of extracellular [Ca^{2+}] to 10-20 mM. When the membranes of the trabeculae (days 7 and 15) were removed using Triton X-100 detergent, the maximum Ca^{2+}-activated force was similar to the maximal twitch before detergent treatment, suggesting that the reduction in twitch force was due to alterations in the contractile apparatus. Electron micrographs showed that sarcomere organization was unaffected by neural crest ablation. Western blot analysis showed that, at embryonic days 7 and 15, the amount of expression of myosin heavy chain and tropomyosin was the same in normal and experimental hearts. However, in the experimental hearts, there was an increased amount of α-actinin and desmin, and isoform switching of cardiac troponin T from the embryonic to adult form was precocious. It is not clear how these alterations in sarcomeric proteins could lead to diminished force production. (Support: NIH grants HL36059 and HL42266)

P 219 CYTOKINE EXPRESSION IN MYOCARDIAL AND CARDIAC MESENCHYMAL CELLS,

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Cytokines are critical signaling molecules for growth and differenciation of a variety of cell type. Little is known, however, what cytokines are produced by myocardial and cardiac mesenchymal cells. To adress this question, primary cultures of cardiac myocytes (MYO) and cardiac mesenchymal cells (MES) were prepared from the ventricles of 20-day fetal mice. The MES were collected by differencial adhesiveness, and the fourth subculture was used to deplete residual MYO. RNA was isolated, and RT-PCR was carried out with specific synthetic primers including interleukin (IL) 1α, 1β, 2, 3, 4, 5, 6, 7, 10, G-, M-, GM-CSF, interferon (IFN)γ, leukemia inhibitory factor (LIF) and tumor necrosis factor(TNF)β. The spleen and a fibrosarcoma cell line BMT11-Cl9 were used as a positive control. Amplified bands were identified by Southern blot using internal synthetic oligonucleotides. The results were summerized in the table. (nd:not determined)

	IL1α	1β	2	3	4	5	6	7	10	GCSF	M-	GM-	LIF	IFNγ	TNFβ
MYO	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-
MES	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
spleen	+	+	+	+	+	+	+	+	-	nd	nd	nd	+	-	+
BMT11	nd	nd	nd	nd	nd	nd	nd	nd	nd	+	+	+	nd	nd	nd

LIF and IL1β transcripts were detected in MYO. No cytokine mRNA was detected in MES. As a bioassay of LIF and IL1β activity, the lipoprotein lipase (LPL) activity of 3T3-L1 cells (for LIF and IL1β), and the colony formation activity of myeloid cell line Da-E7 (for LIF) were measured using the conditioned medium of MYO and MES. The MYO conditioned medium markedy inhibited the LPL activities, and had a strong colony formation activity, while that of MES did not. In conclusion, MYO produce functionaly active LIF and probably IL1β as well.

P 220 NEURAL CREST AND CORONARY ARTERIAL DEVELOPMENT,

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Study of development of the coronary vasculature has been followed in the quail embryonic heart using the quail-specific anti-endothelial antibody QH1. The origin of the coronary endothelial cells has been traced to be of extracardiac origin. The endothelial cells cover the surface of the heart following the formation of the epicardial covering. At the level of the future arterial orifices the endothelial precursors form two connected circles around the future aortic and pulmonary orifice. Special attention was paid to the highly stable site of the coronary arterial orifices in the two sinuses of the aorta facing the pulmonary orifice. From quail- chicken experiments it was obvious that the ingrowth of endothelial cell strands into the aortic wall was multiple; the pulmonary truncal wall was not invaded, the endothelial cells being more positioned within the upper myocardial cuff. Careful histological survey shows that the two actual coronary arteries that obtain a lumencontact with the aortic semilunar sinuses posses a media as well as a marked presence of ganglionic cells along their proximal coronary wall. The other parts of the aortic ring and ingrowth sites lack this development. It is postulated that the definitive position of the coronary arterial orifices and most probably their inherent proximal course are regulated by neural crest cells. Ablation experiments leading to common arterial trunk as well as cardiovascular pathology in the human such as common arterial trunk and bicuspid aortic valve in combination with aortic arch abnormalities show a high percentage (40-60%) of coronary arterial origin abnormalities, forming an indirect evidence for neural crest involvement in coronary arterial development.

P 221 EPIDERMAL GROWTH FACTOR INHIBITS DIFFERENTIATION OF HUMAN FETAL CARDIAC MYOCYTES IN VITRO,

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Previous work has shown that a change from high-mitogen medium (growth medium, GM) to serum-free medium containing insulin (SFMI) promotes differentiation of propagated human ventricular cardiac myocytes in vitro, and that neither basic fibroblast growth factor nor transforming growth factor β inhibits this process. In order to identify components of GM which inhibit cardiac differentiation, we used immunocytochemistry to study the effects of platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) on expression of sarcomeric proteins in cultured human fetal ventricular myocytes. Myocyte-enriched cultures prepared from a 10 week human fetal cardiac ventricle were expanded in GM and plated in multi-well dishes. One dish was fixed on day 0; medium in others was then changed to SFMI alone or SFMI plus PDGF (20 ng/ml) or EGF (20 ng/ml). Dishes were fixed after 9 days and the number of cells showing positive staining with monoclonal antibodies specific for sarcomeric actins (αsr-1) or sarcomeric myosin heavy chain (MF20) were counted. Results are as shown, expressed as mean ±SD:

Number of cells/mm^2	Day 0 GM	Day 9 SFMI	Day 9 SFMI + PDGF	Day 9 SFMI + EGF
Total	75 ± 12	73 ±2.3	77±4.9	107 ± 4.3
αsr-1 (+)	35 ±3.1	54 ± 1.3	50±4.6	18 ± 6.4
MF20 (+)	0 ±0	40 ± 2.0	31±5.3	10± 2.5

Serum withdrawal produced a significant increase in the number of cells expressing sarcomeric actins or myosin heavy chain ($p<.05$ by one-way ANOVA and Newman-Keuls testing) but did not increase total cell number. EGF sigificantly increased total cell number, but decreased the number of αsr-1- or MF20-stained cells in comparison with SFMI alone ($p<.05$). PDGF had neither effect. EGF may play an important regulatory role in human cardiac differentiation and development.

P 222 ISOLATION AND CHARACTERIZATION OF MOUSE *TWIST* HOMOLOGUES. Adam Greene and Seigo Izumo. Molecular Medicine and Cardiovascular Divisions, Harvard Medicial School, Boston MA 02215.

In *Drosophila* the*twist* gene is essential for the development of mesoderm and its gene product activates the expression of other mesodermal genes including *snail*. Xenopus and mouse homologs of *twist*, (xtwist and mtwist) have been cloned. In both xenopus and mouse embryos, twist is expressed in subsets of mesodermal non-myotomal cells and apparently *not in muscle primordia*.

We performed the polymerase chain reaction (PCR) using degenerate oligonucleotides corresponding to the peptide sequences of the basic and helix 2 regions of xtwist to amplify cDNA from a mouse 8.5 day embryo library. We isolated six different mtwist clones, each of which differed in nucleic acid and peptide sequence from the xtwist homolog. Some of our mtwist clones have basic-helix-loop-helix (bHLH) regions which are more closely related to the xtwist bHLH regions (94%/98% identical at the nucleotide/amino acid levels, respectively) than to the mtwist clone (79%/94% nucleotides/AA identical) described by Wolf et al. (Dev. Biol. 143:363-373).

Thus there appears to be a family of twist-related bHLH proteins expressed in early mouse embryos. Although previously the characterized mtwist mRNA is not expressed in developing heart there is the possibility that one or more of our new mtwist clones may be tissue specific and play a role in cardiac or skeletal muscle development. We are currently characterizing the developmental pattern of expression of our mtwist cDNAs.

P 223 COORDINATE GENE EXPRESSION DURING FETAL AND NEONATAL RAT HEART DEVELOPMENT.
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Mammalian heart development is characterized by rapid, near-terminal ventricular myocyte proliferation during fetal periods & initiation of cellular maturation and concomitant capillary angiogenesis and extracellular matrix (ECM) formation during the neonatal periods. Growth and maturation of cardiomyocytes has been reported to be under autocrine/paracrine mechanisms of action of locally produced growth factors (GFs). We present molecular (Northern/*in situ* hybridizations), biochemical (Western blots) and immunological (light & electron microscopy) data to clearly indicate that multiple GFs and/or GF-receptors (GFRs) are expressed at different relative levels in the fetal and neonatal myocardium and can be localized to specific ventricular cell types at distinct developmental time periods. The GF stimuli and ancillary GFRs associated with fetal expansion of the cardiomyocyte population are the FGFs & IGFs. The rapid "transition" to cardiomyocyte maturation and hyper-trophic ventricular growth in the neonate are influenced by ventric-ular TGF-ßs (both $ß_1$ & $ß_3$) as well as the rapid decline in cardio-myocyte GFR expression. A combination of both processes may be directly associated with the limited prolif-erative growth of the cardiomyocyte. In concert with cardiomyocyte-related changes in gene expression and proliferation, pronounced changes in the non-myocyte populations (endothelium & fibroblasts) of the ventricle occur in the neonate as well. These changes are associated with capillary neovascularization & ECM formation by the expanding non-myocyte populations. Cardio-myocyte-derived stimuli (FGFs, TGF-ßs & SPARC) operate in a true "paracrine" manner to possibly coordinate and or facilitate non-muscle cell growth and maturation in both capillary angiogenesis and ECM component formation (Collagen Types I, III, and IV). Together, these data indicate that heart development is reflective of a continua of changes in GFs and/or GFRs which appear to influence in a direct manner the growth and maturation of distinct ventricular cell types. Further analysis of direct effects specific GFs may play in cardiomyocyte development are ongoing.
Supported in part by HL-42218 (GLE).

P 224 **DIFFERENTIAL REGULATION OF CYCLINS AND CYCLIN DEPENDENT KINASES DURING TERMINAL DIFFERENTIATION OF C2C12 MUSCLE CELLS**
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During terminal differentiation of skeletal muscle cells a specific set of muscle specific genes is expressed and individual myocytes fuse into multinucleated myotubes, which withdraw from the cell cycle and go into a resting (G_0) state. Little is known about the expression of genes that drive the dividing myoblast through the cell cycle once the myocyte terminally differentiates. Using Northern blot analysis, histone H1 kinase assay and immunocytochemistry we investigated the expression pattern, biological activity and cellular localization of cyclins and associated kinases during terminal differentiation of mouse skeletal muscle derived C2C12 cells.

After induction of differentiation by low serum medium, cdc2 mRNA levels transiently increased (~2 fold) followed by a down regulation to barely detectable levels within 48 hours, while cdk2 mRNA stayed almost constant. Under these conditions cyclin A and cyclin B were down regulated within 24 hours to undetectable levels. CYL1, and CYL3, cyclins that seem to be important for G1/S transition, were regulated in different ways: While CYL1 is down regulated CYL3 mRNA levels remained constant. Re-stimulation with serum re-induced cdc2 as well as CYL1 RNA almost to the level observed in dividing myoblasts.

On protein level $p34^{cdc2}$ was detected in nuclei of proliferating myoblasts and nascent myotubes, but never in myotubes that were kept in low serum medium for more than 48 hrs. Re-stimulation induced $p34^{cdc2}$ protein in unfused myoblasts, but not in myotubes. Histone H1 kinase activity of $p33^{cdk2}$ was not altered during terminal differentiation, while $p34^{cdc2}$ kinase activity decreased during differentiation.

These findings suggest that terminal differentiation of skeletal muscle cells is associated with a differential regulation of cyclins and their associated kinases. C2C12 cells might be a useful model for investigating terminal differentiation and cell cycle regulating genes.

P 225 *IN VIVO* EFFECTS OF OVEREXPRESSION OF BASIC FIBROBLAST GROWTH FACTOR ON CARDIAC DEVELOPMENT
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Basic fibroblast growth factor (bFGF) is a heparin binding polypeptide with a wide range of biological effects. *In vitro* studies of the effect of bFGF on cardiac myocytes demonstrate increased proliferation, delayed differentiation, and induction of protein isoforms seen in cardiac hypertrophy. To determine whether increased endogenous production of bFGF in developing hearts would lead to increased myocyte proliferation, delayed or inhibited differentiation, and enhanced cardiac hypertrophy, we examined the *in vivo* effect of high level constitutive bFGF expression. This pattern of expression abolishes any regulation of bFGF production that would normally occur during development. A transgenic mouse strain was created using the 5' α-cardiac actin flanking sequence fused to bFGF. Northern analysis revealed that the transgene was highly expressed in the fetal heart, and that expression persisted in the adult heart. The content of functional FGF in the hearts of transgenic mice was fifty-fold greater than in control littermates. The overexpression of bFGF has no detectable effect on heart size, cardiac morphology, or expression of cardiac-specific protein isoforms. The results suggest that bFGF can be stored in cardiac tissues in a functionally inactive state, and that additional stimuli are necessary for this protein to display biological activity.

P 226 POSITIVE AND NEGATIVE REGULATORS OF VENTRICULAR SPECIFICATION IN TRANSGENIC MICE, K. Lee, R. Hickey, H. Zhu and K. Chien, AHA-Bugher Foundation Center for Molecular Biology, University of California, San Diego, La Jolla, CA 92093

Currently, little is known regarding mechanisms which govern the activation of regional specific programs of muscle gene expression during cardiogenesis. The cardiac MLC-2 gene has served as a model system to identify the molecular and positional cues which lead to ventricular specification. In the primitive heart tube, the endogenous MLC-2v gene is restricted in its expression to the ventricular segment, thereby providing a genetic marker for positional specification in the linear heart tube. A 250 bp MLC-2 promoter fragment can confer ventricular specific expression to a luciferase reporter gene during murine embryogenesis in independent mouse lines. To further address the pathways which lead to ventricular specification, we generated independent mouse lines which harbor point mutations in *cis* regulatory elements (HF-1a, HF-1b, HF-2, HF-3, E-box) that may mediate ventricular specificity. Transgenic mice which harbor mutations in the conserved HF-3 site display marked up-regulation (> 100 fold) in luciferase activity in muscle sub-types which do not express the endogenous gene, e.g. uterus. Luciferase activity in non-muscle tissues (kidney, liver, spleen) is not increased over background, and is not decreased in the ventricular chambers. Thus, HF-3 serves as a negative regulatory element to suppress the expression of cardiac genes in closely related muscle sub-types. Point mutations in the HF-1a element result in background levels of reporter activity in ventricular myocardium, and >100-fold increases in reporter activity in non-cardiac tissues (muscle and non-muscle (kidney, etc.)). Thus, depending upon cellular context, the HF-1a element may serve as both a positive and negative regulator of MLC-2 promoter activity. Mutations in the neighboring HF-1b site severely cripple ventricular specific expression, with little effect on luciferase activity in non-cardiac tissues, suggesting that HF-1b serves primarily as a positive regulatory element. Mutations in HF-2 or an E-box site have no detectable effect on MLC-2 luciferase transgene expression. These studies in transgenic mice suggest that regional specification of the MLC-2 gene may be due to a unique combination of regulatory pathways that include positive regulatory elements (HF-1b); lineage restricted negative regulatory elements (HF-3); and an element (HF-1a) which can serve as a positive and negative regulator, depending upon the cell context.

P 227 NONINVASIVE EVALUATION OF HEART DEFECTS IN EARLY TRISOMIC MOUSE EMBRYOS USING ECHOCARDIOGRAPHY: USE IN CELL AND MOLECULAR ANALYSES DURING DEVELOPMENT, Linask, K.K., Khowsathit, P., Huhta, J.C., and Y.H. Gui, Division of Cardiology, The Children's Hospital of Philadelphia, and Department of Pediatrics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

A genetic homology exists between regions of the human chromosome #21 and the mouse chromosome #16. Pathogenesis of features shared by mice with trisomy 16 (TS16) and Down Syndrome (TS 21) individuals, has been a basis for the TS 16 mouse being a model system for studies related to Down Syndrome. A major feature of the trisomy 16 mouse model is congenital heart disease (91.7%). About half of TS 16 mouse fetuses have endocardial cushion defects, specifically atrioventricular (AV) canal defects. Previously, there was no way to identify those trisomic mouse embryos that were developing a heart defect. Possible variability and severity of defects were difficult to ascertain. These drawbacks are overcome by using Doppler echocardiography to evaluate heart abnormalities as they are developing. The embryos identified as having defects, are karyotyped and further cellular, molecular, immunohistochemical studies can be carried out and compared to normal, control, litter mates. Physiological, cellular and molecular parameters of the developing heart can be thus obtained. It is suggested that this methodology may be useful in evaluating transgenic murine embryos where heart abnormalities may be suspected.

TS16 embryos were obtained on a C57 Bl/6J background using mice doubly heterozygous for the Robertsonian translocation. Normal embryonic blood velocities (peak diastolic and systolic filling velocities) from day 11 to term had been previously determined and no healthy embryos had Doppler evidence of AV valve regurgitation (AVVR), i.e. holosystolic, high velocity jet. Twenty-six embryos from 3 litters were examined with Doppler echocardiography daily starting at day 11 of gestation. Each embryonic heart was located and analyzed from multiple angles using pulsed Doppler to detect the blood velocity with a sample volume set to insonate the entire heart. On day 15, dams were sacrificed, embryos karyotyped, hearts were fixed and sectioned for immunohistochemical and *in situ* hybridization analyses. Results: 5 of the 26 embryos had AVVR and all were proven to have aneuploidy. 21 embryos had normal blood velocities and were euploid. FN localization has been determined in a spatiotemporal fashion in TS 16 mouse embryonic hearts after echocardiographic evaluation. Differences in FN localization are detected in TS 16 embryos in contrast to normal, euploid embryos.

P 228 INDUCTION OF CARDIOGENESIS BY ENDODERM: MEDIATION BY bFGF AND ACTIVIN?

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Previous work in this laboratory has shown that embryonic endoderm can induce cardiogenic differentiation of stage 5 precardiac mesoderm (*Ann. N. Y. Acad. Sci.* 588:421;'90). Subsequent analysis of endoderm cells and conditioned medium revealed the presence of bFGF (*Dev. Biol.* 146:139;'91) and activin (*Dev. Biol.* 146:242;'91), both of which induce mesoderm in *Xenopus* and chicken. While deletion of bFGF has indicated a critical autocrine role for this factor in the differentiation of precardiac mesoderm (*Dev. Biol.*;in press), endoderm-mediated paracrine roles for bFGF and activin in the cardiogenic process have yet to be established. To approach this problem, we devised a bioassay in which mesodermal explants containing approximately 10^4 stage 5 precardiac cells are grown on a fibronectin substrate in defined medium (M199). Without additives, these cells assumed a monolayer exhibiting no apparent growth or differentiation. By contrast, addition of endoderm or ITS (5 µg/ml insulin & transferrin; 5 ng/ml selenium) induced a proliferative, synchronously contractile cellular multilayer that expressed cardiac α-actin. When added individually, although up to 5 µg/ml transferrin (or BSA) had no effects, as little as 5 ng/ml insulin was cardiogenic. Activin or bFGF were also inductive, at respective concentrations as low as 0.5 and 5 ng/ml. To further establish that bFGF and activin induce cardiogenesis in paracrine fashion via endoderm, we are assessing whether their respective inhibitors, suramin and follistatin, can prevent endoderm-induced cardiogenesis. [Supported by NIH HL39829]

P 229 L6 SKELETAL MYOCYTES EXPRESS FGF RECEPTORS. Nicholas V. Matiuck, Kathleen B. Whitaker, Kimberly A. Scata, Ibrahim A. Tangoren, June M. Kaplow, Michael Jaye and Judith L. Swain. Rhone-Poulenc Rorer Central Research, Collegeville, PA 19426; University of Pennsylvania School of Medicine, Philadelphia, PA 19104.

The rat L6 skeletal myocyte cell line has been reported to lack endogenous fibroblast growth factor (FGF) receptors. We have, however, observed FGF receptor(s) at the RNA and protein levels in L6 myocytes. A 4.3 Kb transcript was detected by Northern analysis using probes derived from human and murine *flg* cDNA. Western blot analysis of L6 lysates with anti-*flg* antibody detected a single 150 KDa protein band. Chemical cross-linking of [^{125}I]aFGF to intact L6 myocytes indicates that FGF receptors reside on the cell surface. Treatment of L6 myoblast cultures with 30ng/ml FGF suppressed differentiation to myotubes by 50% compared to untreated control cultures as assessed by creatine kinase production. However, FGF treatment failed to produce a mitogenic response in L6 myoblast cultures. The results of this study indicate the presence of FGF receptors on the surface of L6 myocytes. In addition, the endogenous FGF receptor-effector pathway displays functional activity by suppressing differentiation of L6 myoblasts in the presence of exogenous FGF. Therefore, L6 skeletal myocytes cannot be used as a null cell line for FGF receptors.

P 230 OPIOID GROWTH FACTOR AND THE ZETA (ζ) OPIOID RECEPTOR IN THE DEVELOPING RAT HEART, Patricia J. McLaughlin, Dept. of Neuroscience and Anatomy, Pennsylvania State University College of Medicine, Hershey, PA 17033
Proliferation of cardiac cells is a critical event in heart development. The opioid growth factor (OGF), [Met^5]-enkephalin (MET), has been documented to tonically inhibit cell division in the nervous system by way of the zeta (ζ) opioid receptor. OGF action operates in a stereospecific and antagonist-reversable manner. This study examined the presence and relationship of OGF and ζ receptor during cardiac morphogenesis. Saturation binding isotherms of 1-day old rat heart with ^{125}I-MET demonstrated specific and saturable binding of radiolabeled OGF to the ζ receptor, with a binding affinity (K_d) of 2.5 nM and binding capacity (B_{max}) of 18 fmol/mg protein recorded. No specific and saturable binding of OGF was detected in the adult heart. Northern blot analysis of newborn rat heart revealed the presence of preproenkephalin A, the prohormone for [Met^5]-enkephalin. The role of OGF on cell proliferation of myocardial and epicardial cells from neonatal rat ventricles was examined using labeling indexes (-LI) ascertained from thymidine incorporation experiments. Eight hours following injection of 1 mg/kg OGF an LI of 16.7% was recorded for myocardial cells; this was a significant reduction from the values of sterile water controls (CO) (24.0%). Epicardial cells of the ventricles had an LI of 7.4% at 4 hours after injection of OGF; this was significantly different from the LI of CO (15.0%). These results are the first to indicate that OGF functions as a potent inhibitory growth factor in the developing mammalian heart. Moreover, receptors for OGF have been discovered in the neonatal but not adult heart, suggesting that the ζ receptor appears transiently in the heart and is only present during development. Finally, data showing the detection of preproenkephalin A mRNA in the neonatal heart would suggest that the prohormone for OGF is present in the developing heart and that OGF is produced in an autocrine and/or paracrine manner. Thus, these results serve to identify OGF as a modulator of cardiac myogenesis.
Supported by an Initial Investigatorship from the American Heart Association Pennsylvania Affiliate.

P 231 THE IDENTIFICATION OF MARKERS SPECIFIC FOR THE DEVELOPING CARDIAC CONDUCTION SYSTEM, F. Michiels, W.H. Lamers and A.F.M. Moorman, Dept of Anatomy and Embryology, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, the Netherlands.
We are searching for molecular markers specific for the developing conduction system in the rat heart. The method we are using is based on the observation that some antibodies, raised against neural antigens, cross-react with the conduction cells of the heart. This indicates that these cells, although muscular in phenotype, do express some neural genes. The identification and characterization of these genes will give us further insight into the functioning and development of cardiac conductive tissue. They will also enable us to study the regulation of their expression, which is crucial to understand the abnormalities in the conduction pattern of pathologic hearts.
The method we are currently employing is as follows: cDNA, prepared from 13 embryonic days (ED) rat hearts, is subtracted with liver cDNA and fibroblast cDNA. The remaining cDNA's, enriched for genes expressed in the heart and freed from ubiquitously expressed genes and genes that are expressed in blood cells and connective tissue, will be used to screen a cDNA library prepared from 15 ED rat neural tissue. In this way, we will select for cDNA's that are expressed both in the nervous system and in the heart.

P 232 **RETROVIRAL-MEDIATED OVEREXPRESSION OF TRUNCATED FGF-RECEPTOR 1 AND ITS ANTISENSE mRNA INHIBIT MYOCYTE PROLIFERATION IN DEVELOPING AVIAN HEARTS,** Tatsuo Mima*, Hikaru Ueno**, Donald A. Fischman*, Lewis T. Williams** and Takashi Mikawa*, *Department of Cell Biology and Anatomy, Cornell University Medical College, New York, NY, 10021, **Howard Hughes Medical Institute Research Laboratories, University of California School of Medicine, San Francisco, CA. 94143-0724

Although high levels of fibroblast growth factor (FGF) and its receptors have been demonstrated in embryonic and adult hearts, a role for FGF-signaling in myocardial formation has been uncertain. Replication-defective variants of avian spleen necrosis virus were engineered to encode two genes: bacterial ß-galactosidase (ß-gal), and a mutant form of FGFR-1 lacking the intracellular tyrosine kinase domains, in both sense and antisense orientations. The former was designed to overexpress a dominant negative mutant to block FGF-signaling of most FGFR isotypes, while the latter was designed to inhibit synthesis of FGFR1. Viruses were microinjected into developing hearts in ovo. After incubation for 3-10 days, daughter myocytes of the infected cells were examined by whole mount staining with X-gal. Overexpression of both truncated receptor and its antisense mRNA gave rise to much smaller colonies than wild-type myocytes. Electron microscopy of the transduced myocytes revealed well developed myofibrils, and normal cytodifferentiation. These results strongly suggest an important role for FGF-signaling in regulating myocyte proliferation during early stage of avian cardiac development.
Supported by: AHA (TM), HL-45458 (DAF), Aaron Diamond Foundation and HHMI.

P 233 *Abstract Withdrawn*

P 234 *SNOI*, A MUSCLE-SPECIFIC ALTERNATIVELY SPLICED ISOFORM OF *SKI* PROTO-ONCOGENE HOMOLOG *SNO*,
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The *ski* family genes, *ski* and *sno*, encode nuclear proteins that may act as transcription factors, but are not members of any of the major transcription factor families, including the bHLH or MyoD family. Two lines of evidence suggest that *ski* functions in muscle development: (1) Ed Stavnezer has shown that the chicken v-*ski* oncogene can convert quail embryo fibroblasts to the myogenic (muscle) pathway [1], activating the endogenous MyoD and myogenin genes [2], and (2) Steve Hughes' lab has shown that transgenic mice over-expressing *ski* in their skeletal muscles exhibit hypertrophy of certain types of fast myofibers [3]. Interestingly, mutant *ski* fails to convert quail embryo cells to muscle but retains its ability to activate MyoD and myogenin, suggesting that *ski* function is required for myogenesis [2]. We find that, in contrast to the MyoD family of genes, *ski* family genes are not expressed muscle-specifically in mice and humans, but are found to varying degrees in all tissues examined.

The other member of the *ski* family is called *sno*, for *ski*-related novel [4]. *Sno* shares extensive sequence similarity with *ski*, and is as similar to *ski* as members of the MyoD family are to each other. We have isolated a novel alternatively spliced cDNA for *sno* that accumulates specifically in skeletal muscle in humans. We are investigating the accumulation of *ski* and *sno* isoforms in mouse adult and embryonic tissues. We are testing the effects of overexpression of different *sno* isoforms in tissue culture cells and transgenic mice. We are making targeting constructs for homologous recombination gene knockout experiments in both *ski* and *sno* genes.

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P 235 NEURAL CREST CELLS DURING CARDIAC DEVELOPMENT,
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The development of the rhombencephalic neural crest contributing to the heart has been studied immunohistochemically in quail embryos. Antibodies were used that stain subpopulations of crest cells. HNK-1 detects migrating crest cells (among some other cell types), while antineurofilament antibodies stain with both undifferentiated crest cells and those that differentiate into neuronal cells.
In early developmental stages (i.e. before HH stage 16) crest cells are observed in the pharyngeal arches in the vicinity of the arch arteries. In stage 16 these cells are found encircling the aortic sac and in stage 19 both near the outflowtract and the sinus venosus. In older stages stained crest cells are found in the wall of the great arteries, in the semilunar valve leaflets and the outflow part of the interventricular septum. Furthermore, continuity is observed between cardiac ganglia located in this region, and the nodose ganglion that is part of the vagal nerve. Near the sinus venosus the crest cell area is continuous with both the vagal nerve and the sympathetic trunk. The latter derives from a more caudal level of the neural tube. The sinus venosus area develops into the sinus node, as can be deduced from a timed developmental series reaching up to stage 37.
In conclusion, this study of cell lineage and differentiation of a subpopulation of crest cells shows that the wall of the pharyngeal arch arteries and the aortic sac, the semilunar valve leaflets, the ventricular outflow septum, cardiac ganglia and the sinus node recruit cells from the neural crest.

P 236 CHRONIC ETHANOL TREATMENT *IN OVO* INDUCES CHANGES IN THE pH SENSITIVITY OF MYOFILAMENT CALCIUM ACTIVATION OF CHICKEN PECTORALIS BUT NOT CARDIAC MYOFILAMENTS,
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Compared to adults, embryonic and neonatal myofilament proteins of dog and rat hearts are resistant to a depression in Ca^{2+} activation with decreasing pH. This resistance to desensitization by acidosis is correlated with expression of embryonic isoforms of thin filament regulatory proteins, specifically troponin I (TnI) and troponin T (TnT). Myofilaments isolated from embryonic day 18 chicken cardiac and pectoralis muscles exhibit a similar resistance to desensitization of Ca^{2+}-activated ATPase activity at pH 6.5. Ethanol administration (100mg/100g egg; days 11 through 17 *in ovo*) did not modify this resistance to acidosis in the embryonic chick heart. However, similar to the adult pectoralis muscle, ATPase activity of ethanol treated pectoralis myofibrils was significantly reduced with acidosis at maximal activity and at all pCa's less than 6.125. Cooperativity, as judged by the steepness of the pCa-ATPase activity relation, and pCa_{50} were unchanged by ethanol treatment. K-EDTA ATPase activity of myofibrillar myosin was significantly diminished in the ethanol-treated pectoralis muscle whereas Ca-activated myofibrillar myosin ATPase activity was not altered. During the development of the chicken, multiple myosin, TnI and TnT isoforms have been observed in cardiac and skeletal muscles. Currently studies are underway to identify possible shifts in myosin, TnI and TnT isoforms induced by ethanol treatment which may contribute to the changes in Ca^{2+} sensitivity observed in pectoralis myofilaments with chronic ethanol administration.

P 237 EXPRESSION OF A UNIQUE MESSAGE PRIOR TO CARDIOVASCULAR DYSMORPHOGENESIS IN CHICK EMBRYOS.
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Cardiac neural crest cells migrate from the neural folds, through the pharyngeal arches and into the cardiac outflow tract. Removal of the cardiac neural crest disrupts normal cardiovascular development. Abdulla et al. (1993) have shown that the pharyngeal arches of stage 14 and 18 chick embryos with the cardiac neural crest removed express proteins which are not found in normal embryos. It was not possible to collect adequate protein for sequencing, and so an alternate method employing subtractive hybridization was devised. A normal stage 14 chick pharyngeal arch cDNA library was differentially screened for clones present in the embryos without cardiac neural crest. One clone, approximately 1.1 kb in length, was isolated. The cDNA was subcloned and sequenced. Preliminary sequence analysis indicates that the clone is 92% homologous at the amino acid level to a 28kd subunit of a *Drosophila* proteasome. In *Drosophila*, proteasomes may be involved in proteolytic events necessary for cell proliferation and morphogenesis during development (Klein et al., 1990). Expression of the proteasome mRNAs in the cardiac neural crest-deficient pharyngeal arches indicates that the cells of the pharyngeal arches may be proliferating to replace the missing mesenchyme normally produced by the cardiac neural crest.

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P 238 EXPRESSION OF THE FIBRONECTIN ISOFORMS IN RAT MYOCARDIUM DURING ONTOGENIC DEVELOPMENT

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Fibronectin (FN) is a protein or the basal membrane thought to play an important role during differentiation. The expression of the different FN isoforms that result from alternative splicing of a single transcript is developmentally regulated. Indeed the cellular forms, containing EIIIA and EIIIB domains are characteristic of most *embryonic* tissues and only rarely expressed in adult tissues. The expression of the various FN isoforms during cardiac development in mammals and their precise cardiac distribution is not known and is reported here. The accumulation of the FN mRNA isoforms in the myocardium was analyzed by in situ hybridization, using RNA probes complementary to EIIIA, EIIIB and a domain (5' terminus) common to all FN mRNAs. The relative quantitation of the EIIIA and EIIIB exons was determined by reverse transcription coupled to polymerase chain reaction (PCR).*In situ* hybridization reveals that the 3 mRNAs are generally coexpressed within the developing myocardium *in utero* but their distribution and relative accumulation are specific for each developmental stage. In the 11 day post coitum (dpc) fetus, all 3 isoforms are concentrated within the myocardium and the aorta. At 14 and 16 dpc, total hybridization decreases and the 3 mRNAs specifically accumulate as scattered dots in the trabecular endothelium (atrium and ventricle) and in the epicardium; they are almost absent from the ventricular wall. At 19 dpc, FN mRNAs are detected only in endothelial and smooth muscle cells of both atrium and ventricle. After birth, all forms decrease rapidly and are undetectable at 3 weeks of age. The evaluation of EIIIA+/EIIIA- or EIIIB+/EIIIB- mRNA ratios after PCR demonstrates the progressive decrease of both EIIIA and EIIIB expression in the heart from day 14 *in utero* (45%) to the perinatal period (20%). Both transcripts are completely absent in 13 d-old rat hearts but EIIIA may be reexpressed at a low level in aging adults. In conclusion the expression of FN mRNA and its isoforms which depends on the activity of noncardiomyocytes cells, appears to be tightly associated with the differentiation process.

P 239 STUDIES ON THE RELATIONSHIP BETWEEN THE MOUSE HOMEOBOX GENE HOX7.1 AND TGF-β1

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Hox7.1, a member of the *msh* class of mouse homeobox genes, is expressed in the endothelial cells of the atrioventricular (AV) canal just prior to their differentiation into mesenchymal cells. TGF-β1, a known effector of endothelial-mesenchymal transformation in the developing AV canal, also appears to be expressed in the endothelial cells of the AV canal at the same time as Hox7.1. The coincidence of Hox7.1 and TGF-β1 expression in the developing heart and elsewhere during mouse embryonic development prompted us to test the hypothesis that Hox7.1 interacts with the promotor or other regulatory sequences of the TGF-β1 gene to induce TGF-β1 expression. To test this hypothesis, we conducted cotransfection experiments in NIH3T3 cells using a chloramphenicol acetyltransferase (CAT) reporter gene under the control of the TGF-β1 promotor along with a plasmid, pCAGHox7.1, which overexpressed Hox7.1 mRNA as verified by RNAse protection assay. The reporter plasmid pTGF-β1cat was constructed by inserting a HindIII-BamHI fragment (position -1761 to +59) of the mouse TGF-β1 gene into the HindIII site of the promotorless CAT plasmid pSV00cat. These experiments showed no change in TGF-β1 promotor activity upon cotransfection with pCAGHox7.1, or upon transfection of the CAT construct into stable pCAGHox7.1 transfectants. Furthermore, we examined endogenous TGF-β1 mRNA levels in stable transfectants by RNAse protection assay. No difference was observed between NIH3T3 cells and stable pCAGHox7.1 transfectants. These results suggest that Hox7.1 does not regulate the TGF-β1 promotor in NIH3T3 cells. If Hox7.1 does regulate TGF-β1 expression in the developing AV canal, this functional relationship is not apparent in the NIH3T3 cell system.

P 240 VASCULAR ENDOTHELIAL GROWTH FACTOR EXPRESSION IN CARDIAC MYOCYTES IN VITRO AND ITS UPREGULATION BY TUMOR NECROSIS FACTOR-α,

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Vascular endothelial growth factor (VEGF), a heparin-binding glyco-peptide mitogen with target cell specificity to vascular endothelial cells has been shown to promote an array of responses in endothelium including hyper-permeability, endothelial cell proliferation and angiogenesis. Tumor necrosis factor-α (TNF-α), a cytokine predominantly secreted by activated macrophages has pleitropic effects on different cell types, however, the intracellular mediators of its action are not known. We examined the influence of TNF-α on the expression pattern of VEGF in cultured fetal mouse myocytes. Hearts from 18 day old fetal mice were removed and myocytes were isolated by trypsin digestion. Cells were grown in minimum essential medium containing 10% fetal calf serum and treated with recombinant TNF-α (1000 units/ml) for 1, 2, 4, 6, 8, 12 and 24 hrs. Total cellular RNA was isolated after each incubation and analysed by Northern hybridization using a VEGF specific cDNA probe. TNF-α induced arrhythmias and cessation of spontaneous contractions in a concentration dependent manner. We demonstrated a basal expression of VEGF mRNAs (3.9 and 3.7 kb) in fetal myocytes, however the expression was several fold induced by TNF-α being maximal at 4 hrs of stimulation and thereafter, the expression of VEGF declined. 24 hrs of TNF-α incubation resulted in the cell death. We conclude that the enhanced expression of VEGF in cardiac myocytes by TNF-α verifies the known indirect angiogenic functions of this cytokine which could be mediated by VEGF.

P 241 SMOOTH MUSCLE CELL (SMC) LINEAGE DIVERSITY IN THE CHICK EMBRYO: TWO TYPES OF AORTIC SMC DIFFER IN MITOGENIC RESPONSES TO SERUM AND TRANSFORMING GROWTH FACTOR-β1

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In vertebrates, SMCs that populate most blood vessel walls derive from locally recruited mesenchyme (MES). In contrast, the major outflow vessels from the heart also contain a population of SMC derived from ectomesenchyme (ECT) of neural crest origin. In the chick embryo, the 4th aortic arch is primarily composed of ECT, while the abdominal aorta contains only MES. In this study, we asked if the different origins of ECT and MES cells *in vivo* confer intrinsic differences in growth properties that are detectable *in vitro*. Based on the above rigid anatomical distribution, we isolated ECT and MES cells from 14-day chick embryo aorta and compared their responses to fetal calf serum (FCS) and defined growth factors at serial passages up to P5. Both populations expressed similar levels of desmin and α-smooth muscle actin thus confirming their SMC identity. Primary ECT cells were polygonal in shape, smaller than MES and formed a monolayer at confluence. In contrast, primary MES cells were elongated and grew in a multilayered "hill and valley" pattern. Upon serial passage ECT morphology came to resemble that of MES by P3 to P5. Irrespective of passage number, both ECT and MES (plated at $5*10^3$ cells/cm^2) required serum concentrations ≥ 2% to support growth, indicating a lack of autocrine growth potential under these conditions. In three separate isolations, primary or P1 ECT in 10% FCS had shorter cell cycle times and achieved confluence much earlier than paired MES. Responses of confluent ECT and MES (P1) to purified growth factors (PDGF-AA, PDGF-BB, basic FGF, EGF) (20 ng/ml) and TGF-β1 (0.01-10 ng/ml) were determined in a defined, serum-free medium. TGF-β1 produced peak increases in DNA synthesis (4-fold, 24h) in ECT (P1 to P3), while it had no effect on matched MES. ECT and MES did not differ in responses to the other growth factors tested. We conclude that ECT and MES SMCs share common properties related to their smooth muscle identity. However, they exhibit clear differences in cell shape, growth rate and ability to respond mitogenically to TGF-β1. Thus two distinct lineages in the chick embryo may produce vascular SMCs that differ in growth properties *in vivo*. These results may have important implications for large artery morphogenesis and disease. Supported by HL-47655.

P 242 HUMAN ADULT ATRIAL APPENDAGE MYOCYTES DEVELOP in vitro FROM A HUMAN ADULT ATRIAL APPENDAGE FIBROBLAST-STEM CELL, Laurace E. Townsend, Marilyn L. Seymour, Darlene M. Reitz-Vick and John L. Glover, Department of Surgery, Wm. Beaumont Hospital, Royal Oak, MI 48073-6769.
An explant culture system for the growth of human adult myocardial cells was developed in our laboratory. This method involves the initial outgrowth of cells from an explant of human atrial appendage. The cells that migrate out of the explant undergo two cell doublings over a two week period and develop into myocardial cells. Morphology is dependent upon growth factors present. These cells stain with antibody against striated muscle cell myoglobin, atrial natriuretic peptide, propyl 4-hydroxylase (anti-fibroblast) and variably against smooth muscle cell actin. This data supports the hypothesis that development of myocardial cells can occur from fibroblasts present in the atrial appendage of adults.

P 243 CHARACTERIZATION OF THE RNA FROM CONDITIONED MEDIUM WHICH RESCUES CARDIAC MUTANT AXOLOTL HEARTS, N. Erginel-Unaltuna, D.K. Dube, S. M. LaFrance and L.F. Lemanski. Department of Anatomy and Cell Biology, SUNY, Health Science Center, Syracuse, NY 13210.
Homozygous mutant (c/c) axolotl embryos lack sarcomeric myofibrils and their hearts fail to beat. This defect can be corrected by culturing mutant hearts with normal tissue from the anterior endoderm region or in a medium which is conditioned by this tissue. Preliminary studies suggest that the active material is RNA from the anterior endoderm region. A cDNA library has been constructed using total RNA from conditioned medium which showed positive induction activity utilizing random priming. The transfected clones are amplified and sequenced employing the dideoxy chain terminator method. The sequences are compared to other known sequences in all available databases utilizing the Genetic Computer Group DNA analysis package. Six out of twelve clones are found to have homology with 28S rRNA from a variety of eukaryotic species. Two are homologous to 18S rRNA. No significant homology is detected with the remaining four clones suggesting the possibility that they may be products of distinct coding genes. The four unique clones contain open reading frames of 261, 186, 186 and 132 bases respectively and show no overlapping regions among themselves. RTPCR analysis with primers from the sequence with the largest open reading frame showed that this gene is expressed in brain, skeletal muscle, heart and lungs, but the expression is not detectable in liver. The most antigenic region of the peptide deduced from the nucleotide sequence was used to raise polyclonal antibodies. Preliminary Western blot analysis indicates that the antibody recognizes of polypeptide of approximately 70 and 150kD. Affinity purified antibodies will be used to further characterize the protein.

P 244 HF-1b: A NOVEL, TISSUE-RESTRICTED ZINC FINGER GENE MEDIATES AN E-BOX INDEPENDENT PATHWAY FOR CARDIAC MUSCLE GENE EXPRESSION, Hong Zhu, Van Nguyen, Anne Brown, Atossa Pourhusseini, Arnold Garcia, Marc Van Bilsen, and Kenneth R. Chien, Department of Medicine, Center for Molecular Genetics, and the American Heart Association-Bugher Foundation Center for Molecular Biology, University of California, San Diego, School of Medicine, La Jolla, California 92093
Utilizing southwestern screening of a rat cardiac cDNA library, the present study reports the isolation of a novel Sp-1 related zinc finger gene (HF-1b) that activates the expression of the rat cardiac myosin light chain-2 gene via an E-box independent pathway. The deduced amino acid sequence of the HF-1b cDNA reveals three C2H2 zinc finger domains with a high degree of homology (89%) to the three Sp-1 zinc fingers, as well as amino terminal glutamine and serine/threonine rich domains that have been shown to be required for transcriptional activation by Sp-1. Northern blotting and RNase protection analyses reveal a tissue restricted pattern of expression in terminally differentiated tissues (cardiac, skeletal, and brain), with a single predominant mRNA species. A trpE-HF-1b fusion protein, which contains the three zinc fingers, binds specifically to the A-T rich HF-1B site in the MLC-2 promoter, as assessed by a combination of gel shift assays with duplex oligonucleotide competitors and DEPC interference analyses. Pre-incubation with antibodies directed against the fusion protein removes a major component of the endogenous HF-1b binding activity in cardiac extracts. Co-transfection of an HF-1b expression vector with either the native 250 bp MLC-2 promoter-luciferase fusion gene or a HF-1 TK-luciferase heterologous reporter gene documents the transactivation of the reporter gene that is dependent upon an intact HF-1b binding site. Thus, three independent criteria (binding site specificity, removal of the endogenous HF-1b binding activity with fusion protein antibodies, and site-specific transcriptional activation indicate that this novel, tissue restricted zinc finger protein mediates an E-box independent pathway for cardiac muscle specific expression of the MLC-2 gene. The restricted expression of HF-1b to other terminally differentiated cell types (skeletal and brain) suggests the possibility that this zinc finger gene might play a more generalized role in the control of the myogenic and neural gene program.

Genetics and Cardiovascular Disease: Ion Channels

P 300 SODIUM CURRENT IN CARDIAC- AND NEURON-LIKE CELLS DERIVED FROM AN EMBRYONAL CARCINOMA CELL LINE BY *IN VITRO* DIFFERENTIATION,

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Pluripotent murine embryonal carcinoma cells (P19) were differentiated into cardiac- and neuron-like cells with dimethyl sulfoxide (0.5%) and retinoic acid (0.5 μM), respectively. The *cardiac-like* cells (20-30 μm) beat at about 1 Hz (room temperature). Neuron-like cells are smaller (10-15 μm) and express extensive neurites. Voltage-gated sodium currents recorded with whole cell voltage clamp have a maximum amplitude of -3.07±0.55 nA and -1.22±0.25 nA in cardiac- and neuron-like cells, respectively. Control undifferentiated cells have a very small inward current with a maximum amplitude of -0.15±0.02 nA. The voltage dependence of the peak sodium current in both cell types was similar. Significant inward current was recorded at -40 mV and was maximum around -10 to 0 mV of membrane potential. Cardiac-like sodium channels were 50% inactivated at -80.6±3.7 mV with a slope factor of 12.1±0.9 mV. In contrast, these values for neuron-like sodium channels were -45.9±0.8 mV and 7.0±0.5 mV. The time course and current-voltage (I-V) relationship of sodium currents recorded from the cardiac-like cells were quite similar to those from guinea pig ventricular myocytes. Tetrodotoxin (TTX) blocked cardiac- and neuron-like sodium channels in a dose-dependent manner with K_d's of 650 nM and 8.6 nM, respectively. The maximum current through cardiac-like sodium channels was inhibited 23% and 42% by 15 μM dibutyryl cyclic-AMP and 50 nM phorbol 12,13-dibutyrate, respectively. It is concluded that *in vitro* generated cardiac- and neuron-like cells express sodium channels with different TTX sensitivities. These cardiac-like sodium channels are targets of regulatory processes. Finally, P19 is a cell line that may be a suitable model to study *in vitro* the electrophysiological changes during the cellular differentiation process toward an excitable cell.

P 301 AN INDUCIBLE LINEAGE SYSTEM FOR DETERMINATION AND DIFFERENTIATION OF SMOOTH MUSCLE,

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Despite intense interest in understanding differentiation of vascular smooth muscle, very little is known regarding the cellular and molecular mechanisms that control differentiation of this cell type. The field has been greatly hampered by the lack of an inducible in vitro differentiation system for studying the early steps of smooth muscle differentiation. The present study provides evidence for an inducible smooth muscle lineage system in which multipotential mouse P19 embryonal carcinoma cells (P19s) are induced to form smooth muscle lineages. Treatment of P19s with retinoic acid was associated with profound changes in cell morphology and with appearance of a high frequency of smooth muscle alpha actin positive cells which were absent or present at an extremely low frequency in parental P19s. A clonal line derived from retinoic acid treated P19s (designated 9E11G) expressed multiple characteristics of smooth muscle, including SM alpha actin and SM myosin heavy chain as well as functional responses to the contractile agonists phenylephrine, angiotensin II, ATP, bradykinin, histamine, PDGF-AA, and PDGF-BB. Additionally, 9E11G cells expressed transcripts encoding Mhox, a muscle homeobox gene expressed in smooth, cardiac, and skeletal muscles, but not the skeletal muscle-specific regulatory factors, MyoD and myogenin. Our results indicate that retinoic acid treatment of P19s is associated with induction of smooth muscle lineages. Retinoic acid induced smooth muscle clonal lines should be of utility in identifying genetic elements involved in smooth muscle differentiation control and/or lineage determination.

P 302 THE *tinman* GENE SPECIFIES HEART AND VISCERAL MUSCLE FORMATION IN *DROSOPHILA*,

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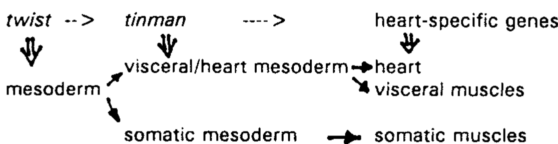
The homeobox-containing gene, *tinman*, is expressed at blastoderm in the mesodermal primordia. This expression is dependent on the zygotic mesoderm determinant *twist*, which suggests that *tinman* may be involved in subsequent steps of mesoderm development. When the mesoderm subdivides into primordia of different fates *tinman* expression becomes restricted to the portion of the mesoderm that is destined to develop into heart and visceral muscles. Later, expression becomes exclusive to the developing heart. Mutant embryos that lack a functional *tinman* gene do not form heart or visceral muscles. There is also no evidence for the appearance of precursor cells for cardiac or visceral muscles. Formation of somatic muscles, on the other hand, is not severely perturbed. It is concluded that *tinman* is involved in the determination of a major subdivision of the mesoderm, namely the heart and visceral mesoderm as opposed to the somatic mesoderm, and that *tinman* is required in these primordial cells to specify heart and visceral muscle development. Function and expression of *tinman* in the heart suggest that *tinman* may be the first determinant of heart development.



P 303 DEVELOPMENTAL REGULATION OF THE SKELETAL MUSCLE DIHYDROPYRIDINE RECEPTOR SUBUNITS

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Transduction of a sarcolemmal action potential to sarcoplasmic calcium release during excitation-contraction (E-C) coupling in striated muscle involves activation of the dihydropyridine receptor (DHPR), a voltage-sensitive L-type calcium channel, and the ryanodine receptor (RYR), the calcium release channel of the sarcoplasmic reticulum. The DHPR is composed of 5 heterologous subunits: α1, α2, β, δ and γ. Although the α1-subunit alone has been shown to possess slow calcium channel properties, its kinetic properties are significantly modulated by the remaining subunits. We have previously reported developmental regulation of α1 subunit expression in skeletal muscle. In order to determine the relative expression of the α1, α2, β and γ-subunits during skeletal muscle development, Northern blot analyses of 26 day fetal, 0, 1, 3, 5, 7, and 28 day neonatal and adult rabbit skeletal muscle were performed. cDNAs encoding each of the DHPR subunits were isolated by and sequenced using polymerase chain reaction amplification of reverse-transcribed. Amplification was performed with primers designed from published cDNA sequences. Densitometric analyses of mRNA abundance showed a 2-fold increase in α1-subunit mRNA on the day of birth and final adult levels were increased by ~3.5-fold compared to fetal levels. The α2- and β-subunit mRNA levels increased ~1.5-fold on the day of birth and continued to increase, reaching adult levels of ~3.5-fold greater than fetal levels. In contrast, γ-subunit mRNA increased ~1.5-fold on the day of birth but did not significantly change during postnatal development and adulthood. These data indicate that expression of the DHPR subunits is differentially regulated. Differential expression of the DHPR subunits may regulate slow calcium channel activity during skeletal muscle development.

P 304 EVIDENCE FOR CHANGES IN ACIDIC FIBROBLAST GROWTH FACTOR PEPTIDES IN THE HEART, Peter Cummins, Derrick C. Chilton, Sarah Beestone, Molecular Cardiology Research Group, Department of Physiology, The Medical School, University of Birmingham, Birmingham, B15 2TJ, UK.
There is clear evidence from a number of tissues for the presence of at least three forms of basic fibroblast growth factor (bFGF) which are N-terminally extended versions of the 'mature' 18kDa peptide arising from transcription initiation at different start sites. The various forms of bFGF are thought to be differentially located both intra- and extra-cellularly suggesting functional differentiation and may be developmentally regulated. It is not clear however whether acidic fibroblast growth factor (aFGF) also exhibits similar diversity of expression. We have investigated aFGF expression in the developing rat and bovine heart by gel electrophoresis and immunoblotting of heparin-Sepharose extracts from atria and ventricles. Purified polyclonal rabbit antibodies to recombinant bovine aFGF were used to detect <1 ng aFGF with an enhanced chemiluminescent detection system. Three aFGF peptides were detected in the rat ventricle with apparent kDa's of 16.0, 16.5 and 18. Recombinant standard bovine aFGF co-migrated with the 16kDa form. Prior to birth, in the 18 day fetal ventricle, only the 18kDa peptide was present. However, by two days after birth the 16.5kDa form appeared and by 14 days the 18 and 16.5 kDa forms were present in equal amounts. From 18 days after birth, the 18 kDa form decreased and the 16kDa form started to appear. In the adult, there were equal amounts of both the 16 and 16.5kDa forms but less than 20% of the 18kDa form. Throughout the developmental period studied there was an overall increase in total aFGF from about 50ng/g heart in the 18 day fetus to 200 ng/g in the adult. In contrast to the rat, the bovine heart demonstrated no diversity of aFGF forms. A single aFGF form of 17kDa was present in both atria and ventricles from the 24 week fetus upto gestation at 40 weeks, in the 3-4 week neonate and in the adult. As in the rat, the total levels of aFGF increased significantly with development in the bovine heart. These results suggest that as with bFGF, there are also different isoforms of aFGF that are developmentally regulated and that these in turn could have different functional roles. This could have important implications for growth of the heart in the early neonatal preriod.

P 305 BIOCHEMICAL PROPERTIES OF THE RABBIT CARDIAC L-TYPE CALCIUM CHANNEL α1 SUBUNIT,
Karen S. De Jongh, Anita A. Colvin, Masami Takahashi* and William A. Catterall, Department of Pharmacology, University of Washington, Seattle, WA 98195 and *Mitsubishi Kasei Institute of Life Sciences, Machida, Tokyo 194, Japan.
L-type calcium channels mediate entry of calcium into cells in response to membrane depolarisation and are modulated by cAMP-dependent phosphorylation events. In previous work we have identified two forms of the principal α1 subunit of the skeletal muscle L-type calcium channel which we hypothesised to have separate functional roles as the voltage sensor for excitation-contraction coupling and in mediating ion flux respectively. We demonstrated differential phosphorylation of the two forms by cAMP-dependent protein kinase suggesting that they have distinct physiological functions in cAMP-modulated ion conductance. Although the skeletal muscle L-type calcium channel has been well characterized biochemically, little is known about the channel from other tissues. Attempts to purify the channel from heart have proven difficult and phosphorylation of the purified channel has not been demonstrated even though the flux through the channel has been shown to be modulated by phosphorylation in electrophysiological experiments. In the present study we have partially purified the rabbit cardiac L-type calcium channel and examined the properties of the α1 subunit. We have identified two forms of this subunit, one of which is truncated at its C-terminus. The full length form is phosphorylated by cAMP-dependent protein kinase. Our results are consistent with previous electrophysiological observations that L-type calcium channels mediate the stimulatory effects of β-adrenergic receptor agonists on cardiac contraction.

P 306 TRANSFORMING GROWTH FACTOR $BETA_1$ INCREASES EXPRESSION OF SARCOMERIC ACTIN mRNA IN MOUSE VENTRICULAR MYOCARDIUM, Mahboubeh Eghbali, Department of Anesthesiology, Yale University School Of Medicine, New Haven, CT 06510
The effects of transforming growth factor $beta_1$ (TGF-β_1) on cardiac fibroblast transformation have been shown in our previous studies. Based on several evidence such as induction of sarcomeric actin mRNA expression of sarcomeric actin filaments, loss of intermediate filament vimentin and appearance of myocyte-specific morphological characteristics in TGF-β_1-treated cardiac fibroblasts, we proposed that cardiac fibroblasts are predisposed to convert into myocyte phenotype and that TGF-β_1 is the inducer of cardiac fibroblast transformation in vitro. To investigate the biological relevance of those findings, we have examined the in vivo effect of TGF-β_1 in the mice ventricular tissue. Swiss albino male mice (3-4 months, 28-32 gr body weight) received daily injections of 100 ng TGF-β_1 intravenously. Mice were sacrificed at 24, 48 and 96 hour intervals. Northern hybridization analysis of RNA extracted from total ventricular tissue showed that at 24 hours the ratio of sarcomeric actin to cytoskeletal actin mRNA in TGF-β_1-treated mice heart compared to that in control mice heart was increased by 73% and remained elevated (48%) at 48 hours following treatment. At 96 hours following treatment this ratio returned to levels comparable to those in normal untreated mice. Immunofluorescence microscopy of frozen sections of TGF-β_1-treated hearts showed decreased staining with antibody to vimentin. Up-regulation of sarcomeric actin gene expression and decreased expression of vimentin in the myocardium may be considered indirect evidence of in vivo transformation of cardiac fibroblasts phenotype by TGF-β_1.

P 307 INDUCTION OF MUSCLE FIBER-SPECIFIC REGULATORY ELEMENTS BY THE SKI ONCOGENE
James C. Engert, Sabah Servaes, and Nadia Rosenthal. Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118.
The *ski* oncogene has been characterized in several avian and mammalian species, including man. First isolated from an avian transforming retrovirus, it has the ability to transform quail embryonic cells (QECs) and has also been shown to be involved in myogenesis. The Ski protein product, a nuclear localized protein, can convert QECs to fully differentiated myoblasts and has the ability to stimulate the massive hypertrophy of specific fiber types when expressed in a transgenic mouse. Though *ski* is expressed in all skeletal muscle tissues of the transgenic mice, the hypertrophic effect of *ski* appears to be limited to fast glycolytic fibers (IIb and IIx). Thus, the effect occurs in the same fiber types where the myosin light chain 1/3 gene (MLC1/3) is expressed. Using the regulatory regions of the MLC1/3 locus, we have recently shown that Ski can transactivate this fiber-specific regulatory region in the C2C12 muscle cell line. Conversely, this transactivation potential does not exist in NIH3T3 cells, and in fact Ski downregulates the ability of MyoD to transactivate the MLC enhancer in such a system. Northern analysis of the adult *ski* transgenic mouse muscle shows the MLC1/3 message to be only slightly increased. We are currently investigating the effect of the cellular background on the activity of *ski* as well as the nature of the negative effect on MyoD's transactivating potential with respect to the MLC enhancer. In addition, we are investigating if the significant positive effect seen in myoblasts is a result of the direct action of Ski on the MLC1/3 enhancer or a result of an indirect effect, possibly through one of the four myogenic factors. Preliminary evidence from our lab indicates that *c-ski* may also be involved in early events accompanying muscle regeneration.

P 308 MOLECULAR CLONING AND CHARACTERIZATION OF THE INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR FROM A HUMAN MEGAKARYOCYTIC CELL LINE. Loewe Go, Tania Nanevicz, Anne-Marie Brillantes, T. Jayaraman, and Andrew R. Marks. Molecular Medicine Program, Dept. of Medicine, Mount Sinai School of Medicine, New York, NY 10029

Platelet stimulation by thrombin generates inositol 1,4,5-trisphosphate (IP3) via G-protein coupled phosphoinositide hydrolysis. IP3 acts as a second messenger for mobilization of intracellular calcium during platelet activation. The molecular structure of the IP3-receptor (IP3R) in smooth muscle and brain has been described. The IP3R is a calcium release channel localized on the endoplasmic reticulum. To elucidate IP3R expression and regulation in platelet precursors, we utilized the Dami human megakaryocytic cell line. Dami cells express a functional thrombin receptor and express a platelet phenotype upon activation by phorbol myristate acetate (PMA). A 500 bp Dami IP3R cDNA clone was synthesized using reverse transcriptase followed by polymerase chain reaction with primers based on the rat aortic smooth muscle IP3R. Screening a Dami λGT11 cDNA library (a gift from R. Handin) yielded multiple overlapping cDNA clones with ~80% homology to the rat brain IP3R. Northern blot analysis of Dami RNA demonstrated a ~10 kb IP3R mRNA. Immunoblot analysis of Dami membrane preparations using rabbit polyclonal antibodies directed against an synthetic IP3R peptide identified a ~300 kD protein. These results are similar to prior molecular descriptions of the IP3R. Total RNA was then prepared from control, thrombin (1μ/ml), and PMA (20ng/ml) treated Dami cells at 2, 5, 24, and 48 hours after initiation of subculture. Northern blot analysis revealed that IP3R is constitutively expressed in Dami cells and that neither thrombin nor PMA regulates its expression. Cloning of the Dami cell IP3R will facilitate future studies of its role in platelet activation and megakaryocyte differentiation.

P 309 A NOVEL MEMBER OF THE BRAHMA FAMILY ENCODES AN E BOX-SPECIFIC DNA BINDING PROTEIN, Xiaohua Gong, Uta Grieshammer, Nadia Rosenthal, Department of Biochemistry, Boston University School of Medicine, 80 East Concord Street, Boston, MA 02118

The 173 bp comprising the downstream enhancer in the myosin light chain locus contains four E-box sites, one CArG box and one MEF2 binding site. One of the E-boxes(BMW; CATGTG) which is located in the 5' region of the enhancer does not bind myogenic factors, but is a binding site for a protein complex found in muscle and non-muscle cell lines. Using a DNA oligonucleotide probe which contains the BMW E-box, we screened a Hela lambda gt11 expression library and identified positive clones. The binding between the protein product of the clone and the BMW oligo was sequence specific as demonstrated by competition expriments. The amino acid sequence of the protein does not reveal the expected DNA binding domains, such as basic-helix-loop-helix domains which are typical of E-box binding proteins. Rather, a small helicase domain that is homologous to the Drosophila brahma and yeast SNF2/SWI2 genes (a class of transcriptional regulatory factors potentially involved in chromatin assembly). Northern blotting analysis showed that there are two positive bands (5kb and 7kb) in Hela and Jurkat cell lines. Using low stringency PCR, we isolated a corresponding DNA fragment from a mouse cell line which shows 90% homology to the human sequence.We are currently cloning the full length cDNA of this gene and are investigating tissue specific expression pattern in the mouse by *in situ* hybridization.

P 310 EXPRESSION OF THE INOSITOL 1,4,5-TRISPHOSPHATE AND RYANODINE RECEPTORS IN A RAT MODEL OF HEART FAILURE. Kushal K. Handa, Maria C. Moschella, and Andrew R. Marks. Molecular Medicine Program, Brookdale center for Molecular Biology, Mount School of Medicine, New York, NY 10029.

Intracellular calcium release in cardiac muscle occurs primarily via the caffeine-sensitive ryanodine receptor (RYR). The RYR is an intracellular calcium release channel on the sarcoplasmic reticulum. In cardiac muscle, during excitation-contraction coupling, the RYR is activated by calcium entering through the voltage-dependent calcium channel on the plasma membrane. The inositol 1,4,5-tripshosphate receptor (IP3R) is a related intracellular calcium release channel expressed on the endoplasmic reticulum of most tissues including the cerebellum and vascular smooth muscle. We have recently demonstrated that cardiac myocytes express IP3R as well. The significance if IP3R mediated intracellular calcium release in the heart as well as IP3R interaction with the cardiac RYR has yet to be elucidated. We have previously shown that RYR expression is down regulated in the ventricles of patients with end-stage heart failure. To understand the molecular basis underlying gene regulation during heart failure, we established a rat model. Adult rats were injected with adriamycin in a single dose of 8mgkg^{-1} intraperitoneally. Animals were sacrificed on days 0, 3, 6, 9 and 18 after injection of adriamycin as well as age and sex matched controls. Clinical correlations of heart failure included failure to thrive, marked ascites, pulmonary edema, increased heart weight, and pleural effusions in the adriamycin treated rats. Northern blot analyses were performed on total RNA extracted from the hearts of control and injected animals. Rat aortic smooth muscle IP3R and rabbit cardiac RYR cDNA probes were used. RYR mRNA expression was decreased three fold in adriamycin treated rats at 3 days comapred to controls and remained decreased. In contrast, IP3R mRNA levels were elevated five fold compared to controls at 6 and 9 days after injection. The reciprocal changes observed in the expression of the RYR and IP3R genes suggest that regulation of intracellular calcium release channels may play a role in the development of heart failure associated with adriamycin induced cardiomyopathy.

P 311 MOLECULAR ANALYSIS OF THE MURINE PULMONARY MYOCARDIUM W. Keith Jones, Arun Subramanium and Jeffrey Robbins, Department of Pharmacology and Cell Biophysics University of Cincinnati, Cincinnati, Ohio

Transcript analysis by RNA dot blot and PCR revealed that the cardiac α-myosin heavy chain (α-MHC) gene is expressed in the murine lung. *In situ* hybridization experiments localized α-MHC gene expression to a thick layer of striated muscle within the walls of the pulmonary and caval veins. This tissue was found to extend far into the pulmonary venous bed of the lung. The observation of α-MHC transcription is the first molecular evidence for what was previously designated the pulmonary myocardium on histological and morphological criteria.

In situ hybridization revealed that α-MHC gene expression occurs in the lung at 13.4 days post coitum (p.c.), much earlier than previously reported. By 14.3 days p.c., α-MHC gene expression is more extensive, but clearly lags behind the extension of the pulmonary veins. By 16 days p.c., many small veins in the lung contain the α-MHC transcript. These data are consistent with either the migration of cardiac myoblasts from the atria, or the differentiation of cardiac muscle from precursors in the pulmonary veins.

In the mouse, the β-MHC gene is transcribed in the embryonic ventricle until birth. The α-MHC gene is expressed in the atrium throughout development and in the ventricle after birth. We have found that the α-MHC gene is constitutively expressed in the pulmonary myocardium throughout development. Analyses of tissues from hypothyroid mice revealed α-MHC gene expression in the atria and pulmonary myocardium to be unaffected by thyroxin. This is not the case in the ventricle. By the criteria of MHC gene expression, the pulmonary myocardium is indistinguishable from atrial cardiac muscle. Hypotheses concerning the possible physiological relevance of the pulmonary myocardium and involvement in human disease are discussed.

P 312 COOPERATIVE INTERACTIONS OF MEF2 WITH OTHER TRANSCRIPTIONAL FACTORS IN SKELETAL AND HEART MUSCLE.
S.Kaushal, B. Nadal-Ginard, V.Mahdavi, Department of Cardiology, Children's Hospital and Harvard Medical School, Boston, MA 02115.
Expression of many muscle-specific genes is regulated by the basic helix-loop-helix transcription factors of the MyoD family and the recently cloned MEF2 transactivators, members of the MADS family. The MyoD family plays a pivital role in inducing and maintaining the skeletal muscle phenotype, however these factors are undetectable in the heart. In contrast, the MEF2 factors have specific DNA-binding and trans-activitating activities detected exclusively in skeletal, cardiac, and smooth muscles. Since many skeletal muscle specific enhancers include both MEF2 and E-box binding sites, we examined the functional interactions of MyoD and MEF2. When the E-box and the MEF2 elements of the MLC 1/3 were linked to a CAT gene construct, cooperative activation of the reporter was observed. This interaction appears to be spacially independent. We next demonstrated that MyoD and MEF2 forms a specific complex in vivo by performing immunoprecipitation of C2 myotube extracts. When gel shifts were performed using the single E-box probe, no supershift were seen with the MEF2 antibody. The same result was also seen when performing the reverse experiment with the MEF2 probe. This suggests that MEF2 and MyoD do not interact when one of them binds to its cis-element, but may form stable complexes when the two respective DNA binding sites are in close proximity. Finally, the exact domain of MyoD and MEF2 necessary for the interaction has been mapped in vitro. Since MEF2 interacts with the MyoD family, we hypothesize that MEF2 may interact with specific cardiac transcriptional factors. Currently, we are investigating the partners of MEF2 in the heart. Determining the functional partners of MEF2 will advance our understanding of cardiac gene regulation.

P 313 ALTERED PROLIFERATIVE ACTIVITIES IN CELLS OF Ca-DEFICIENT CHICK EMBRYOS,
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Chick embryos rendered Ca-deficient by long-term shell-less (SL) culture *ex ovo* develop hypertension and dyslipidemia. To study the cellular basis of these systemic developmental abnormalities, cardiomyocytes and hepatocytes isolated from day 15 normal (NL) and SL embryos were cultured in serum-free media, and were compared with respect to cell proliferative and biosynthetic functions, including growth in cell number, 2-bromo, 5-deoxyuridine (BrdU) incorporation, total RNA, the level of cell cycle specific proteins, myosin content in cardiomyocytes, and cholesterol biosynthesis by hepatocytes. In SL, the increase in cell number and BrdU incorporation were larger in both cell types studied. The BrdU labeled cells of SL entered into mitosis earlier than those of NL. Expression of the cell cycle G2 specific proteins, cyclin B1 and PSTAIRE, was higher in SL. Both of the cell number increase and BrdU incorporation were inhibited by EDTA treatment. These results suggest that, in SL cells, entry into S phase and mitotic activities at G2/M phase were both enhanced in a Ca-dependent manner. Cellular RNA content as stained with methyl green / pironin was stronger in SL cells. However, myocardial myosin and hepatic cholesterol biosynthesis were not greater in SL, consistent with their lower *in vivo* content in heart and liver. In view of our previous findings of up-regulated Ca handling in SL erythrocytes and cardiomyocytes, the Ca-dependent, accelerated cell proliferative functions of SL cells observed here may be a consequence of changes in cellular Ca homeostasis, and may be the cellular basis for the cardiac hypertrophy and other systemic developmental abnormalities in the SL, hypertensive chick embryos.

P 314 EXPRESSION PATTERN OF MEF2 TRANSCRIPTS DURING EARLY EMBRYO DEVELOPMENT IN MOUSE
D.A Laheru, V Mahdavi & B Nadal-Ginard, Department of Cardiology, Children's Hospital and Harvard Medical School,Boston, MA 02115
The identification of skeletal muscle specific proteins such as the basic helix-loop-helix (bHLH) family that can convert nonmyogenic cell lines into differentiated skeletal muscle has provided invaluable clues into events governing myogenesis. However,the bHLH protein family is not inclusive since its equivalent has not been identified in either smooth muscle or cardiac cell lines.

A family of factors that regulate tissue specific expression of a number of genes expressed in cardiac as well as skeletal muscle known as myocyte specific enhancer binding factor 2 (MEF2) has been isolated in our laboratory. These nuclear factors accumulate preferentially in skeletal muscle, heart, and also in smooth muscle and brain. MEF2 share homology to the recently identified MADS gene family. A minimum of 12 variants of MEF2 arise by alternative splicing of the transcripts of four different but related genes. The tissue specific expression pattern implicates MEF2 as participants in cell specific differentiation and defines the need to understand the functional differences between the MEF2 isoforms.

We report the identification of MEF2 mRNA transcripts and proteins in tissue sections and whole mounts of mouse embryos at different developmental stages in vivo. Isoform specific cDNA probes were used for in-situ hybridization and isoform specific antibodies were raised for immunohistochemistry. These results provide important clues into the regulation of MEF2 activity in skeletal muscle in relation to the expression of bHLH protein family members such as MyoD and myogenin. In addition, these results provide an initial understanding into the regulation of cardiac cell differentiation in the absence of the bHLH family. They also underscore the importance of the different MEF2 isoforms at different stages of development and in different tissues.

P 315 VERSICAN EXPRESSION IN ADULT BUT NOT PUP VASCULAR SMOOTH MUSCLE CULTURES,
Joan M. Lemire, Susan D. Perigo, Thomas N. Wight, and Stephen M. Schwartz, Department of Pathology, University of Washington, Seattle, WA 98195
Versican is a large chondroitin sulfate proteoglycan that has been isolated from cultured human fibroblasts, monkey vascular smooth muscle cells and bovine and human artery wall. We show here that versican proteoglycan is found in conditioned medium and extracted cell layers of smooth muscle cultures derived from adult rat aortas (adult SMC), but lacking, or expressed only at low levels, in SMC cultures derived from aortas of 12 day old rats (pup SMC). Versican mRNA could also be detected in RNA isolated from adult SMC but not pup SMC and versican mRNA was present in all 10 clonal lines of adult SMC by Northern analysis. The pup vs. adult difference in versican expression did not reflect differences in growth state as versican is expressed by adult SMC at different states of confluence and by serum-starved and serum-fed adult cells whereas pup SMC failed to express this mRNA in any of these conditions. The mRNA for another proteoglycan, perlecan, was expressed by both pup and adult SMC. These data suggest that versican expression is controlled as part of the development of vessel wall cells.

P 316 TRANSFECTION OF MYOGENESIS-IMPAIRED RAT MYOBLASTS WITH MYOGENIC FACTORS,

Theodore C.Y. Lo, and Xiao Y. Chen Department of Biochemistry, University of Western Ontario, London, Ontario, Canada N6A 5C1.

We have previously shown that a 112 kDa protein present on the cell surface of rat L6 myoblast was specifically phosphorylated by an ecto-protein kinase located on the same surface. Inactivation of either one of these two proteins by chemical reagents or by genetic manipulations resulted in the abolishment of myogenic differentiation. These myogenesis-impaired cells exhibited much reduced levels of the Myf-4, NCAM, MLC, MHC, and TnT transcripts; however, their Myf-5 transcript levels remained similar to those of the myogenesis-competent cells. The present investigation examined the sequence of events involved in myogenesis by transfecting mutants which lacked the phosphorylated 112 kDa protein (p112) with the Myf-4 cDNA. Transfectants harbouring low and high levels of the Myf-4 cDNA were isolated. While the Myf-4 mRNA was present in negligible levels in transfectants containing only the vector (T_C) or low level of the Myf-4 cDNA (T_L), it was present in significant level in the high level transfectants (T_H). The transcripts for the muscle-specific proteins, viz. the MLC, MHC, and TnT, were also substantially higher in T_H . More importantly, multinucleated myotubes could be observed in day-6 cultures of T_H, but not in T_C or T_L. In other words, T_H regained the ability to undergo myogenic differentiation. Since the p112 level, and the Myf-5 and NCAM transcript levels in T_H remained similar to those of T_C and T_L, it seemed likely that the Myf-4 might be involved in sites downstream from these components in the myogenic pathway. Since the introduction of Myf-4 could increase the transcript levels of the muscle-specific proteins, Myf-4 must functioning upstream from these components. (Supported by an operating grant from the Medical Research Council of Canada).

P 317 ECTOPIC MYOD EXPRESSION IN TRANSGENIC MICE INDUCES SKELETAL MUSCLE GENE EXPRESSION IN EMBRYONIC HEARTS. Gary E. Lyons, Department of Anatomy, University of Wisconsin, Madison, WI 53706, Jeffrey Miner and Barbara Wold, Department of Biology, California Institute of Technology, Pasadena, CA 91125.

We examined two independent lines of transgenic mice which express MyoD ectopically in cardiac muscle during embryogenesis. Transcripts of the transgene, which contains the muscle creatine kinase (MCK) promoter and enhancer linked to a MyoD cDNA, are first detected by *in situ* hybridzation in cardiac myocytes around 8 days *post coitum* (*p.c.*) in one transgenic line and at 12 days *p.c.* in the other. The temporal and spatial patterns of accumulation of transgene mRNAs in the two lines are also different. MyoD activates myogenin and embryonic myosin heavy chain gene expression predominantly in ventricular myocytes around 12.5 days *p.c.* in both transgenic lines, but the endogenous MyoD gene and the two other myogenic regulators, myf-5 and MRF4, are not detected in cardiac muscle at any stage of development. In areas of skeletal muscle specific gene expression, the cardiac myosin heavy chain α appears to be decreased. Beginning at 14.5 days *p.c.*, malformations of the ventricular myocardium become evident. By 16.5 days *p.c.*, the ventricular walls are thinner and grossly malformed and the atria appear hypertrophied compared to control littermates. Transgenic embryos die around 17 days *p.c.* These results indicate that MyoD can induce at least a partial skeletal muscle phenotype during mouse embryogenesis even in the presence of the cardiac muscle differentiation program. MyoD gene transcripts were also detected in a few nonmuscle tissues such as the epithelium of the gut and the notochord in one transgenic line. MyoD failed to activate the skeletal muscle phenotype in these nonmuscle tissues. This work was supported by a grant from the American Heart Association of Wisconsin.

P 318 MUSCLE-SPECIFIC ACTIVATION OF THE GLYCEROPHOSPHATE DEHYDROGENASE GENE IS REGULATED BY AN INTRAGENIC ENHANCER ELEMENT, Helena M. Madden and Deborah E. Dobson, Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118

Initiation of a differentiation program in skeletal muscle is accompanied by transcriptional activation of the adult isoform of glycerophosphate dehydrogenase (GPD). To localize cis-acting elements involved in the differentiation-dependent activation of GPD, sequences in and surrounding the GPD gene were tested for their ability to regulate expression of a reporter gene, chloramphenicol acetyltransferase (CAT), in differentiating mouse C2C12 muscle cells. Using this approach we have identified a 1 kb region of the GPD gene which confers correct developmental expression from the GPD promoter in a position and orientation-independent manner. Further delimitation of these regulatory sequences suggests that a muscle-specific enhancer is located within the first intron of the mouse and human GPD locus.

P 319 DIFFERENTIAL METHYLATION OF THE MYOSIN 1/3 LOCUS IN ADULT SKELETAL MUSCLES AND DURING MOUSE DEVELOPMENT. Michael McGrew, Uta Grieshammer, and Nadia Rosenthal. Boston University School of Medicine, Boston, MA 02118.

Transgenic mice containing a chloramphenicol acetyl transferase (CAT) reporter construct under the control of a 1.2 kb myosin light chain (MLC) 1 promoter and a 900 bp MLC 1/3 enhancer display varying levels of CAT mRNA and CAT activity in different skeletal muscles, in contrast to the endogenous MLC 1 gene which is expressed evenly in all fast fibers. To investigate the possible mechanisms leading to the differential transgene expression we have examined the methylation status of the transgenic and the endogenous rat MLC1 promoter and MLC 1/3 enhancer. Using methylation sensitive restriction endonucleases in conjunction with ligation mediated PCR and Southern analysis, we have determined that the endogenous enhancer is methylated to a different extent in several muscles. The transgenic enhancer was demethylated during limb development concomitantly with the initial transcriptional activation of the CAT gene and remained demethylated in all adult muscles assayed. On the other hand, differential expression of the transgene in various muscles correlated directly with the degree of methylation of the transgenic promoter. It appears that the MLC 1/3 enhancer is first demethylated to activate muscle-specific transcription during development whereas the extent of methylation in the MLC 1 promoter is involved in actively determining relative transgene expression levels in different muscles. We are currently investigating the functional importance of the methylation pattern of the MLC 1/3 locus.

P 320 MOLECULAR CLONING AND CHARACTERIZATION OF FK-506 BINDING PROTEIN FROM HUMAN HEART

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Calcium release from sarcoplasmic reticulum during excitation-contraction coupling in cardiac and skeletal muscle is mediated by the ryanodine receptor (RYR). We have previously shown that the FK-506 binding protein (FKBP-12) is associated with the RYR of rabbit skeletal muscle sarcoplasmic reticulum (J. Biol. Chem. 267, 9474, 1992.). Northern blot analysis of FKBP using the full-length skeletal muscle cDNA as a probe showed that the 1.8 kb mRNA is expressed in skeletal and cardiac muscle. Western blot analysis using antiserum against the N-terminal amino acid sequence of FKBP also indicates the presence of this protein in both sarcoplasmic reticulum and partially purified ryanodine receptor proteins from both skeletal and cardiac muscle. To characterize FKBP in cardiac muscle we have cloned the full length human cardiac FKBP cDNA. A human ventricular λGT10 cDNA library (gift from M. Tamkun) was screened with the full-length rabbit skeletal muscle FKBP cDNA and multiple overlapping cDNA clones were isolated. Northern blot analysis of total RNA purified from human heart with the full-length cardiac FKBP cDNA recognizes a single 1.8 kb mRNA. Isolation of the full-length cardiac FKBP cDNA will allow further investigations into the nature of its association with the intracellular calcium release channel.

P 321 IN VITRO DEVELOPMENT OF THE CARDIAC CONTRACTILE APPARATUS IN DIFFERENTIATING MOUSE EMBRYONIC STEM CELLS.

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Mouse embryonic stem cells (ES) are pluripotent cells that have the capability of differentiating *in vitro* into a variety of cell types, including cells that display spontaneous contractile activity, the characteristic phenotype of cardiac muscle. Previous work has shown that these cells express cardiac β- and α-myosin heavy chain (MHC) mRNAs (Robbins, JBC 265: 11905, 1990) providing evidence that this *in vitro* system may represent a model for examining the early molecular and cellular events underlying embryogenesis of mammalian cardiac contractile apparatus. In the present study we established differentiation cultures by removing ES-D3 cells from a feeder layer of embryonic fibroblasts. Cell aggregates were formed in hanging drop cultures and plated onto gelatin coated glass cover slips. After 9-12 days in differentiation culture, spontaneous contractile activity became apparent and was maintained for at least 30 days. Our initial studies were directed at determining whether myosin protein expression was localized to regions that were spontaneously contracting. Immunofluorescence microscopy with a polyclonal anti-myosin antibody (Sigma, M7648) showed that myosin protein expression was discretely localized to the contracting cells. SDS-PAGE electrophoresis on micro-dissected samples of the contracting cells demonstrated the presence of cardiac MHC. Functional studies on contracting cells were also conducted. Force production was determined by using glass microelectrodes to attach micro-dissected contracting cells to an ultrasensitive force transducer. Measurements of force produced by spontaneous contractions were obtained in these intact preparations. Following these force measurements the preparation was chemically permeabilized and contractile force was initiated by the direct application of free Ca^{2+}. Results showed that the Ca^{2+} sensitivity of contraction in these cells was markedly increased with respect to that of adult single cardiac myocytes. These biochemical and functional findings parallel those obtained in developing heart muscle and further establish the feasibility of using this differentiation culture system to study embryogenesis of the cardiac contractile apparatus.

P 322 ROLE OF FGF IN REGULATING MYOGENIN EXPRESSION AND FUNCTION DURING THE ACTIVATION OF MUSCLE PRECURSOR CELLS.

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We are currently examining an *in vitro* model of satellite cell activation in which we are able to reversibly growth arrest undifferentiatied muscle cells, myoblasts, in a G_0 quiescent state strongly resembling that of muscle precursor cells *in vivo*. C2C12 mouse myoblasts are maintained as a G_0 population by suspending single cells in methocel-containing media and because these cells are anchorage-dependent, they will not proliferate in the absence of adhesion. Proliferation of suspended myoblasts can be activated by removing cells from suspension and attaching them to a substratum in defined medium. It is possible to control the fate of the activated cells by varying the media composition. Cells reattached in high serum (20% fetal bovine serum) or 10ng/ml bFGF synchronously progress through the cell cycle exhibiting a peak of DNA synthesis at 24 hours after replating. There is a concominant phosphorylation of the Rb gene product and myogenin protein is undetectable at this time. In the presence of low serum (2% calf serum), cells progress down a differentiation pathway directly from G_0, do not phosphorylate Rb and express high levels of myogenin protein. Interestingly, G_0 myoblasts activated into the cell cycle in FGF are eventually arrested in a reversible post-mitotic state. The observation that these FGF treated cells are unable to phosphorylate Rb after the first round of the cell cycle indicates that they are arrested in early G_1. FGF also inhibits myogenin protein expression and these cells are unable to differentiate. We are presently studying how FGF and adhesion regulate transcription and translation of the myogenin gene, and are analyzing the mechanisms by which myogenin transactivates muscle-differentiation genes (MLC) during muscle precursor cell activation in vitro.

P 323 CHANGING PATTERNS OF EXPRESSION OF α-CARDIAC AND α-SKELETAL ACTIN mRNA IN THE DEVELOPING HUMAN HEART

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In the human heart two different sarcomeric isoforms of actin, α-cardiac and α-skeletal, are expressed. These isoforms are encoded by two different genes that have a highly conserved nucleic acid sequence. As the mRNAs encoding these two isoactins differ in their 3'- and 5'-untranslated regions they can easily be distinguished (Boheler et al., J. Clin. Invest., 88:323, 1991). During development the skeletal isoform becomes the major isoform and its level of expression does not change significantly in hypertrophied and failing hearts. However, the interesting question of how the distinct isoforms are distributed in the adult (normal and failing) and in the developing heart has not been addressed yet. To this end we have investigated the spatial distribution of the respective mRNAs in the developing human heart between the 6th and 12th week of development and in a 3 month-old neonatal heart by in situ hybridocytochemistry. A high homogeneous expression of α-cardiac actin mRNA was found in all myocardial structures in each heart examined. α-Skeletal actin mRNA could not be detected in hearts of 6 weeks of development. Interestingly, in subsequent stages of development, α-skeletal actin mRNA starts to accumulate specifically in the developing papillary muscles, whereas no expression of α-skeletal actin mRNA is observed in the rest of the ventricular myocardium or in the atrial myocardium up to 12 weeks of development. In the ventricle of the 3 month-old neonatal heart an intricate expression pattern of α-skeletal actin mRNA has become established, whereas in the atrium this isoform is still not expressed. Whether these changes in expression reflect a functional adaptation cannot be answered yet. Crucial in our understanding will be the analysis of the normal and failing adult hearts, that is currently carried out.

P 324 MUSCLE-SPECIFIC ALTERNATIVE SPLICING: A MECHANISM FOR PHENOTYPIC RESCUE OF AN INHERITED DEFECT OF AMPD1 IN MYOCYTE, Hiroko Morisaki, Takayuki Morisaki, L. Kristin Newby and Edward W. Holmes, Department of Medicine and Human Genetics, University of Pennsylvania, Philadelphia, PA 19104.
AMP deaminase (AMPD) plays a central role in energy metabolism in all eukaryotic cells, especially in skeletal muscle of vertebrates where the AMPD1 gene is expressed at high levels. Approximately 2 % of Caucasians and African-Americans are homozygous for a nonsense mutation in exon 2 of the AMPD1 gene. Prior studies in rat have shown that exon 2 is variably removed during muscle differentiation. If alternative splicing of exon 2 were to occur in the human AMPD1 transcript, the alternatively spliced transcript would encode a functional AMPD peptide. We have made the following observations on splicing of exon 2 in human AMPD1. Alternative splicing eliminates exon 2, the site of the nonsense mutation, in 0.6 to 2 % of AMPD1 mRNA transcripts in adult skeletal muscle. A much higher percentage of alternatively spliced transcripts are found during differentiation of human myocytes *in vitro*. Transfection studies with human minigene constructs demonstrate that alternative splicing of the primary transcript of human AMPD1 is controlled by tissue-specific and stage-specific signals. Alternative splicing of exon 2 in individuals who have inherited this defect provides a mechanism for a phenotypic rescue and variations in splicing patterns may contribute to the variability in clinical symptoms observed in these individuals. This alternative splicing of AMPD1 will also provide a good tool for studying the mechanisms of tissue-specific and stage-specific alternative splicing in myocyte.

P 325 DEVELOPMENTAL CHANGES IN CALCIUM UPTAKE IN SKINNED VENTRICULAR TABECULAE OF THE CHICK, Thomas M. Nosek, R.T.H. Fogaça, Paula Allee, and Robert E. Godt, Department of Physiology & Endocrinology, Medical College of Georgia, Augusta, GA 30912-3000
The role of the sarcoplasmic reticulum (SR) in the excitation-contraction coupling process of chick heart is believed to change with development (Vetter & Will, *J. Mol. Cell. Cardiol.*, *18*:1267, 1986). Alterations in calcium uptake and/or release underlie these changes. We used the protocol described by Zhu and Nosek (*Pflugers Archiv. 418*:1, 1991) to determine whether the calcium uptake properties of the SR of chick ventricular trabeculae made hyperpermeable (skinned) with saponin are modified during development. **Loading Time**: The time to 50% loading of the SR in the presence of 1 μM calcium at 22°C was approximately 60 sec. for trabeculae taken from mature (3 weeks post-hatching) as well as from 19 day embryonic chicks. Trabeculae from 7 day embryos exhibited more rapid loading of the SR; 50% of maximal loading was achieved in less than 30 seconds. **Calcium Sensitivity of Loading**: Calcium uptake by mature chick trabeculae increased with increasing calcium to a maximum at 1.78 μM (Ca_{50} = 0.56 μM). Loading decreased at higher calcium due to calcium induced calcium release (CICR). Embryonic muscle exhibited significantly greater sensitivity to calcium; Ca_{50} = 0.04 and 0.13 μM in 7 and 19 day embryonic muscle, respectively. CICR was evident at calcium concentrations greater than 1.78 μM calcium at all stages of development. Changes in the calcium-ATPase or calsequestrin are apparently not involved in these changes since there is no evidence for isoform switching of these molecules during development of the mammalian heart (Arai et al., *Am. J. Physiol. 262*:C614, 1992). However, phospholamban increases with development in the chick heart (Will et al., *FEBS Lett. 155*:326, 1983) and, in its unphosphorylated state, inhibits the SR calcium pump and decreases its sensitivity to calcium (Kirchberger et al., *Biochem. 25*:5484, 1986; studies on mammalian heart). These results suggest that the observed decrease in calcium sensitivity with development may be related to the changing concentration of phospholamban (Support: NIH HL 36059)

P 326 NEURAL INFLUENCE ON MYOGENIC REGULATORY GENE EXPRESSION DURING QUAIL EMBRYOGENESIS, M.E.Pownall and C.P.Emerson, Jr. Department of Biology, Gilmer Hall, University of Virginia, Charlottesville, VA 22901 and Institute for Cancer Research, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111.

We are examining the role of the neural tube in regulating the expression of the quail bHLH myogenic genes during somite development. The expression pattern of the qmfs during somite morphogenesis is tightly regulated both temporally and spatially. Activation of *qmf1* (the quail MyoD homologue) expression is detected earliest, in the immature somite, followed by *qmf3*. *qmf2* and contractile protein gene expression is not detected until the somite has matured. Expression of the *qmfs* and other muscle specific genes is restricted to the myotomal compartment of the mature somite. In the earlier, immature somite, *qmf1* and *qmf3* are expressed in the medial cells proximal to the neural tube. The activation of *qmf1* expression in the somite cells proximal to the neural tube supports the hypothesis that neural tube influences may regulate some initial events of myogenesis. Using whole mount digoxygenin *in situ* hybridization subsequent to experimental manipulation of the quail embryo, we are examining the capacity of the neural tube to induce ectopic *qmf1* expression in somites. We are also testing whether the neural tube is necessary for the maintenance of *qmf* expression in the myotome. Preliminary results indicate that the neural tube is necessary for both the activation and maintenance of qmf expression in somites. We are also generating antibodies specific to the different quail myogenic factors in order to establish the expression pattern of the proteins during quail embryogenesis as well as in response to experimental manipulation of the neural tube and somites.

P 327 **EMBRYOID BODIES RECAPITULATE *IN VITRO* CERTAIN ASPECTS OF MAMMALIAN, PRE-SOMITIC EMBRYOGENESIS,** Alejandro Sánchez, W. Keith Jones, Jeffrey Robbins, Department of Pharmacology and Cell Biophysics University of Cincinnati College of Medicine, Cincinnati, OH 45267

In the absence of inhibitory factors, pluripotent, murine embryonic stem (ES) cells spontaneously differentiate *in vitro* to form cystic conglomerations of cells known as embryoid bodies (EBs). After 8 days of culture 40-60% of the resulting EBs exhibit foci of rhythmical and spontaneous contractions. We have shown in previous work that EBs mimic the temporal and spatial regulation of the α-, β-cardiac and embryonic skeletal myosin heavy chain (MHC) genes. The early expression of the β-cardiac MHC (day 4 in EBs and 5 day p.c. in embryos) led us to further define the embryological context in which cardiogenesis was taking place. The expression patterns of the mesodermal marker T-Brachyury and the myogenic factors MyoD and Myogenin were investigated in both EBs and embryos. The data show that, similarly to the β-cardiac MHC gene, these factors are expressed at developmental stages where only ectoderm and endoderm are known to exist. PCR and *in situ* analyses also revealed that the myogenin RNA present during early embryogenesis is mostly unspliced. Unspliced myogenin transcripts may provide an explanation for the relatively late appearance of myogenin protein in murine embryos. The precocious expression of the *T*, *Myo D*, *Myogenin*, and β-cardiac MHC genes in EBs, and its corroboration in pre-somitic murine embryos testify to the usefulness of EBs as an *in vitro* system in which to study some of the molecular aspects involved in the early events of mammalian embryogenesis.

P 328 A Major MCK Enhancer Element Binds Skeletal and Cardiac Factors, and is Suppressed by AP2(A). **Edward A. Sternberg, Lifen Ren and Mark Heitman, Dept. of Physiology, Medical College of Wisconsin, Milwaukee, WI 53226**

The muscle creatine kinase gene (MCK), which is tightly differentially regulated in heart and skeletal muscle, requires an intact TC-II motif for full skeletal activity; yet AP2, which binds to the TC-II motif, has not been shown to be present in muscle. We have therefore sought to determine whether the factors in muscle that bind to this motif are or contain AP2, and whether AP2 can modulate the activity of the MCK gene. We report here that pure AP2 can bind to the MCK TC-II motif, as do a series of five skeletal and cardiac factors that bind this MCK region. However, the cardiac and skeletal factors do not contain AP2 as determined by mobility shift, competitive bandshifting and supershift analysis. MS2, the most important skeletal muscle factor that binds this motif, has been isolated. In C2 myotubes and mouse skeletal muscle, MS2 is composed of a 40 KDa protein monomer; mouse skeletal muscle also yields a 21 KDa protein. The competitive bandshift, supershift, footprint, and SDS gel mobility data concerning these factors rules out the possiblity that these skeletal and cardiac factors are AP2. Nonetheless, AP2 is able to modulate MCK activity: transient cotransfection of an AP2(A) expression plasmid with MCK-CAT marker vectors results in complete suppression of the MCK upstream sequences. This response is so strong that it must be accounted for in the mechanisms that regulate tissue-specific MCK expression. Though the mechanism of action may be simple or complex, these studies establish that differential regulation of MCK may be negative as well as positive.

P 329 E1A-MEDIATED INHIBITION OF MYOGENESIS: A DIRECT PHYSICAL INTERACTION OF E1A$_{12S}$ AND bHLH PROTEINS, Doris A. Taylor, Virginia B. Kraus, John J. Schwarz, Eric N. Olson and William E. Kraus. Departments of Medicine and Cell Biology, Duke University Medical Center, Durham, NC 27710 and Department of Biochemistry, MD Anderson Cancer Center, Houston, TX 77030.

The adenovirus 5 E1A gene product has been implicated in the control of cellular differentiation and proliferation. The observation, several years ago, that both E1A$_{12S}$ and E1A$_{13S}$ can inhibit differentiation of muscle cells and that a class of basic-helix-loop-helix (bHLH) proteins are master regulators of muscle differentiation, suggested to us that E1A may prevent differentiation of skeletal myocytes by interfering with the activity of myogenic bHLH proteins. Therefore, we examined the role of E1A in mediating repression of the muscle-specific muscle creatine kinase (MCK) enhancer in a human rhabdomyosarcoma (RD) cell line. In transfections, both E1A$_{12S}$ and E1A$_{13S}$ repressed MCK expression in a dose-dependent fashion. However, amino-terminal deletions (d2-36 and d15-35) of E1A$_{12S}$ were defective for repression. E1A$_{12S}$ also repressed expression of minimal promoters, containing multimers of the high affinity E-boxes of the MCK and IgH promoters. In these studies, activity of the promoters were dependent on co-expression of either a myogenin bHLH-VP16 fusion protein (MCK promoter) or E2.5 (IgH promoter), which were therefore determined to be the targets for E1A-mediated repression. To explore whether E1A$_{12S}$ may be interacting directly with myogenic bHLH proteins to repress muscle-specific gene transcription, we performed a series of co-precipitation experiments with GST-fusion and *in vitro* translated proteins. E1A$_{12S}$ wt, but not amino-terminal deletion mutants, bind to wild type myogenin and E12, and to deletion mutants of myogenin that spare the bHLH domains. Thus, domains of E1A that mediate repression of muscle-specific gene transcription *in vivo* also mediate binding to bHLH proteins *in vitro*. We conclude that E1A mediates repression of muscle-specific gene transcription through its amino-terminal domain, via a mechanism that may involve direct physical interaction between E1A and the bHLH region of myogenic determination proteins.

P 330 GENETIC HETEROGENEITY IN LONG QT SYNDROME, Jeffrey A. Towbin, Benjamin Siu, Jennifer Robinson, Arthur Moss, J. Fielding Hejtmancik, Departments of Pediatrics and Institute for Molecular Genetics, Baylor College of Medicine, Houston, TX 77030, Department of Medicine, University of Rochester, Rochester, NY, and National Eye Institute/NIH.

Long QT Syndrome is an autosomal dominant inherited disorder characterized by QT interval prolongation and T wave abnormalities on ECG, syncope triggered by stress, and fatal ventricular arrhythmias including torsade de pointes. Keating and coworkers localized a gene responsible for long QT syndrome in 7 families to the short arm of chromosome 11 (11p15.5) at the Harvey-ras-1 (H-ras-1) locus. They proposed that this disorder was likely to be a single gene disorder based on these studies. We have evaluated 20 families with this disorder by the methods of Southern blotting and polymerase chain reaction. In 10/20 families, linkage to the H-ras-1 locus was found with combined LOD score = +8.95. The remaining 10/20 families were not linked, with LOD=-12.40; genetic heterogeneity was confirmed with the HOMOG computer package. We conclude that Long QT Syndrome is linked to the H-ras-1 locus on chromosome 11p in approximately half of the families and that genetic heterogeneity exists for the remaining families. Localization of other genetic loci responsible for this disorder are ongoing.

P 331 ATRIAL DEVELOPMENT IN THE MAMMALIAN HEART

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Using immunohistochemical methods, scanning electron microscopy (SEM) and computer aided reconstruction techniques we have studied the development of the atrial segment in serial sections of human, mouse and rabbit embryonic hearts. In the human heart we were, based on the staining patterns obtained with monoclonal antibodies directed against the M and B isoforms of creatine kinase (CK) and the α and β isoforms of myosin heavy chain (MHC), able to distinguish 4 different populations of atrial cardiocytes. The first group consisted of cells expressing little CK-B and CK-M (e.g. sinus venosus), the second group consisted of cells with a low CK-B expression and a high CK-M expression (e.g. the lateral wall of the right atrium), the third group consisted of cells with a high CK-B and a low CK-M expression (e.g. primary atrial septum) and the fourth group consisted of cells with a high CK-B and a high CK-M expression (e.g. lateral wall of the left atrium). Using the computer aided reconstruction techniques, the spatial organization of these "compartments" was mapped and, to obtain more insight in the relationship between these compartments and the complex morphology of the developing atria, carefully oriented mouse hearts of corresponding developmental stages were studied by SEM. This latter method enabled comparison of immunohistochemically stained sections with SEM preparations. We conclude from our data that the 4 different groups of cells in the atrial myocardium have a distinct developmental history and/or fate. The mechanisms underlying these differential patterns of gene expression in this part of the developing mammalian myocardium remain however to be elucidated.

P 332 DEVELOPMENTAL EXPRESSION OF SMOOTH MUSCLE MYOSIN HEAVY CHAIN ISOFORMS.

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Smooth muscle myosin heavy chain (SMHC) isoforms, referred to as SM1 and SM2, have previously been shown to be produced by alternative splicing of a 39 nt exon, resulting in different carboxyl termini. We have recently demonstrated the presence of additional SMHC isoforms, which differ in the S1-head region of the molecule. Two different SMHC cDNAs have been isolated from rat cDNA libraries which contain a 21 nt divergence located at the 25/50 KD junction, adjacent to the ATP binding site. The SMB isoform contains an additional 21 nt, encoding seven extra amino acids, not present in the SMA isoform. RNase protection analysis demonstrated that these two isoforms are differentially expressed in adult rat smooth muscles. SMB is the predominant mRNA present in bladder and intestine, while the SMA isoform mRNA predominates in aorta, stomach, vein and uterus smooth muscles. RNase protection analysis of rat stomach, bladder, aorta and intestine during development reveals that the isoforms are differentially expressed during development in a tissue-specific manner. The SMB isoform mRNA predominates in all fetal tissues, while SMA mRNA levels increase during neonatal stages. An antibody has been made to the extra seven amino acid region of the SMB isoform and preliminary results confirm that the SMB isoform is present in all developing smooth muscles.

P 333 EFFECT OF RETINOIC ACID ON MYOGENESIS DURING MOUSE EMBRYONIC DEVELOPMENT,

Yonghong Xiao, Uta Grieshammer and Nadia Rosenthal, Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118

Retinoic acid has been shown to be very important in both embryonic development and cell differentiation. During vertebrate development, retinoic acid influences axis specification and limb formation, apparently by shifting Hox gene expression. Retinoic acid also promotes differentiation in many malignant cell lines, including rhabdomyosarcoma derived lines. In order to study the effect of retinoic acid on myogenesis during development, we used transgenic mice that express a muscle-specific reporter gene, the chloromphenicol acetyl transferase (CAT) gene driven by gene regulatory elements from the myosin light chain 1/3 locus. We cultured cells from dissociated somites, limb buds from various developmental stages and leg muscles of new born mice with and without retinoic acid. In all cultures retinoic acid treatment reduced myotube formation as well as MLC-CAT transgene activity. We did not see a similar inhibitory effect on muscle gene expression in the C2 myoblast cell line stably transfected with the same MLC-CAT vector. We conclude that retinoic acid has an inhibitory effect on myogenesis during mouse embryonic development, either by blocking myoblast cell differentiation (in contrast to what has been seen in most tumor cell lines) or by selecting against myogenically committed cells.

Receptors/Signal Transduction

P 400 **CONTROL OF TRANSCRIPTION DURING CARDIAC GROWTH AND DIFFERENTIATION**

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We have used the atrial natriuretic factor (ANF) gene to study the mechanism of transcription during normal and hypertrophic heart development. ANF is the major secretory product of the heart and the ANF promoter directs high level expression in cardiac cells in primary cultures and in transgenic mice. The ANF gene is expressed at a high level in both atria and ventricles at a very early stage of heart development. In the ventricles, expression of the ANF gene is positively correlated with growth stimulation of the myocytes. Primary cardiocytes derived from fetal or neonate rats of various ages were used to study transcription of ANF during normal cardiac development. Using a combination of deletion analysis and *in vitro* DNA binding assays we identified several elements within the proximal promoter region, which are differentially recognized in atrial and ventricular myocytes. At least one element of this region is bound by a cardiac-specific transcription factor and can confer tissue-specific expression to a heterologous promoter. We have also identified an element that plays a dual positive and negative role in ANF transcription dependently on the differentiation stage of the myocytes. Thus the expression of the ANF gene appears to be under control of positive and negative elements which are active in a stage and tissue-specific fashion. Finally during adrenergic mediated hypertrophy, ANF gene transcription is up regulated in the ventricles. Deletion analysis and *in vitro* DNA binding assays have unvailed a novel element, distinct from the cardiac-specific element, that binds an $\alpha 1-$adrenergic induced nuclear protein. Methylation interference and in vitro competition assays indicate that the induced nuclear protein is a member of the zinc finger family of transcription factors.

P 401 DIFFERENTIAL REGULATION OF SLOW-SKELETAL AND CARDIAC TROPONIN I mRNA DURING DEVELOPMENT AND BY THYROID HORMONE IN RAT HEART,

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Thin filament contractile proteins are members of complex muscle gene families, many of which exist as isoform populations under different physiological conditions. During normal rat heart development, troponin I isoform switching from the embryonic to the adult form occurs between 14 to 21 days postnatal. Our studies investigate the correlation of the troponin I (TNI) isoform types during myocardial development and in response to thyroid hormones. Using SDS gel analysis, our laboratory has shown that whereas both euthyroid and hypothyroid rats display a developmental shift toward the adult form of TNI (cTNI) protein, hypothyroid rats displayed a lower percentage of adult TNI at each age studied (1). We have extended these studies to the mRNA levels for cTNI and embryonic or slow-skeletal TNI (ssTNI). Northern and slot blot analysis with cDNA probes for cTNI and ssTNI have been used to examine the expression of these isoforms. Northern blots show that the hypothyroid state delays the expected isoform switching and that the reduced levels of cTNI mRNA in the hypothyroid animal can be reversed by treating with triiodothyronine (T3) on alternate days for 7 days. RNA slot blots show a 6-fold increase in cTNI from 3 day to 21 day postnatal in the euthyroid rat compared to a 3-fold increase in cTNI for the same period in the hypothyroid animal. When 21 day hypothyroid animals were injected with 15ug/100g body weight T3 every 48 hours for 7 days, the cTNI mRNA levels at 28 day postnatal were significantly higher than both hypothyroid and euthyroid animals. On the other hand, ssTNI mRNA levels are consistently higher after 3 days in the hypothyroid state and fall to undetectable levels after 21 days in the hypothyroid animals and after 14 days in the euthyroid animal. ssTNI is absent in the adult and is not re-expressed once the isoform switching occurs. In the 82-120 day adult rat, the cTNI mRNA levels were significantly lower in the hypothyroid state, when compared to both euthyroid and hyperthyroid animals. The thyroid status of postnatal rats was confirmed by thyroxine (T4) RIA and that of the adults by T3 ligand immunoassay. These RNA results support our previous protein data.

1. Dieckman LJ and Solaro RJ: Circ Res 67:344-351, 1990.

P 402 TISSUE-SPECIFIC AND DEVELOPMENTALLY REGULATED ALTERNATIVE SPLICING OF A VISCERAL ISOFORM OF SMOOTH MUSCLE MYOSIN HEAVY CHAIN (MHC). Philip Babij, Department of Physiology, University College London, Gower Street, London WC1E 6BT, England.

Previous work demonstrated that the rabbit smooth muscle myosin heavy chain gene showed sequence divergence at the 25kDa/50kDa junction of the S1 subfragment when compared to chicken gizzard and chicken epithelial nonmuscle myosin. RNase protection analysis with a probe spanning this region detected two partially protected fragments which were not present in RNA from vascular tissue and only found in RNA from visceral tissue. The polymerase chain reaction was used to amplify a 162bp product from rabbit stomach RNA using primers spanning the putative region of divergence. DNA sequence analysis revealed a 21bp insertion encoding seven amino acids which were not previously detected in other characterised cDNA clones. RNase protection analysis using the PCR product as probe showed that the inserted sequence was expressed exclusively in RNA from visceral tissue. Similar RNA analysis showed that the visceral isoform was not expressed in 20 day fetal rabbit smooth muscle tissues. These results indicated that the new visceral isoform was expressed in a tissue-specific and developmentally regulated manner. Genomic DNA sequencing and mapping of the exon-intron boundaries showed that the visceral isoform was the product of cassette-type alternative splicing. cDNA library screening and analysis of several clones >6.0kb in size indicated that the visceral-specific exon was not apparently associated with SM1 type MHC. The location of the visceral isoform near the Mg-ATPase domain and at the 25kDa/50kDa junction suggests that the visceral isoform may be important for myosin function in smooth muscle cells.

Supported by the British Heart Foundation.

P 403 TROPONIN GENE EXPRESSION IN THE DEVELOPING HUMAN HEART. Paul J.R. Barton, Pankaj Bhavsar, Nigel J.Brand, Sabine Sasse, Philip Townsend and Magdi H. Yacoub. National Heart and Lung Institute, Dovehouse Street, London U.K.

The troponin complex lies on the thin filament of striated muscle and is involved in regulating muscle contraction in response to changes in intracellular Ca^{++} concentration. We have examined the expression of both troponin I (TnI) and troponin T (TnT) during human cardiac development at the level of both protein and mRNA from 8 weeks gestation to 9 months postnatal life. In addition we have isolated the human TnIc gene in order to investigate the molecular regulation of its expression.

During fetal cardiac development cardiac TnI (TnIc) is detectable at all stages but the predominant isoform of troponin I expressed is that of the adult skeletal muscle isoform (TnIs). Soon after birth a transition occurs resulting in expression of exclusively TnIc. This occurs by a rapid increase in TnIc mRNA and protein accumulation and a concomitant loss of TnIs mRNA and protein and is highly suggestive of transcriptional regulation for both genes. Interestingly, TnIs mRNA was not detectable in any of 34 end-stage heart failure samples indicating that, unlike many other genes expressed during fetal cardiac growth, TnIs is not re-expressed in adult pathology. The cardiac troponin I gene is composed of 8 exons with no evidence of alternative splicing. The sequence of the promoter region reveals several elements of potential importance including a putative MEF-2 binding site, which are currently being investigated.

In the case of troponin T, a single adult isoform is detectable in normal adult heart. We have isolated a corresponding full length cDNA which provides the first amino acid sequence data for this isoform. PCR-based analysis of cardiac development indicates the adult mRNA is expressed throughout fetal life, but that two additional alternatively spliced mRNAs are present up to 24 weeks gestation. cDNAs encoding the alternatively spliced mRNAs are currently being analysed.

P 404 EXPRESSIONAL ANALYSIS OF AN ANTISENSE mRNA TO β-MHC IN RAT VENTRICLES: IMPLICATIONS FOR GENE REGULATION?, Kenneth R. Boheler, Xavier J. Martin, Anita Buffing and Ketty Schwartz, INSERM U127, Hopital Lariboisiere, 75010 Paris FRANCE

Both α- and β-myosin heavy chain (MHC) gene expressions have been implicated in phenotypic changes important to cardiac function and whose expressions are regulated by a number of *cis* elements and *trans* activating factors. Recently we have described the expression of an antisense mRNA to β-MHC (Boheler et al., J Biol Chem 1992; 267: 12979). This antisense mRNA is transcribed from the same locus as the cardiac MHCs (Southern blot). Its accumulation is not seen during fetal development (in situ hybridizations) and is first detected about 20-25 days after birth (RNase Protection assays). Direct analysis of the transcription of this antisense mRNA by nuclear run on assays indicates that it is first transcribed between 15 and 20 days after birth. Its accumulation is relatively important for a period of several months, but it remains detectable at least until the age of 8 months, the period of time when β-MHC begins to be reexpressed at significant levels. Its transcription can be inhibited by 2.5 μg/ml α-amanitin indicating that it is transcribed by RNA Polymerase II, the enzyme responsible for mRNA transcription. Further experiments indicate that the transcription and accumulation (PCR amplification) of the antisense mRNA are detectable only if the transcription for the β-MHC is at levels too low to measure (run on assays). The effects of thyroid hormone injections on β-MHC and the antisense mRNA expression have also been analyzed in hypothyroid rats. β-MHC is expressed at very high levels in hypothyroid rats but within 18 hours after an injection of thyroxine (1μg/10g body weight), the antisense mRNA accumulates to measureable quantities. These studies show that 1) this antisense mRNA is transcriptionally activated when transcription of the β-MHC has been inhibited during postnatal development and 2) that it is sensitive to thyroid hormone in the adult rat heart, suggesting a possible role in the regulation of the gene for β-MHC.

P 405 ADRENERGIC RECEPTOR STIMULATION OF PREPROENKEPHALIN GENE EXPRESSION IN CARDIAC MYOCYTES AND NON-MYOCYTES. Marvin O. Boluyt, Lydia O'Neill, Jing-Sheng Zheng, Andrea Meredith, Edward G. Lakatta, and Michael T. Crow. Laboratory of Cardiovascular Science, GRC, NIA, NIH, 4940 Eastern Avenue, Baltimore, MD 21224.

Since the PKA and PKC signal transduction pathways act synergistically to induce preproenkephalin (PNK) gene expression in cardiac cells, we tested the hypothesis that adrenergic receptor stimulation would increase PNK mRNA levels in cardiac myocytes and fibroblasts. Neonatal cardiac myocytes were enzymatically dissociated and separated from non-myocytes by differential centrifugation on a Percoll gradient. Cardiac fibroblasts were passaged 3 times to remove residual myocytes. Cells were cultured in 100 mm dishes containing media with 5% fetal calf serum. 24 hours after plating, media was replaced with serum free media and adrenergic agents were added 24 hours later. PNK mRNA levels were assessed qualitatively by in situ hybridization and quantitatively by Northern blot analysis. In cardiac myocytes, norepinephrine (NE) induced a 3-fold increase in PNK mRNA levels by 4 hours, and this effect was eliminated by either α-(Prazosin) or ß-adrenergic (Propranolol) blockade. PNK mRNA was also evident in cardiac fibroblasts in the basal state, and the level was increased 2-fold by NE administration. The increase in cardiac myocyte PNK gene expression induced by NE was potentiated by increasing cell density and by the presence of cardiac fibroblasts, but not by media from NE-stimulated cardiac fibroblasts. These results demonstrate that adrenergic receptor stimulation is sufficient to increase PNK mRNA levels in cardiac myocytes and fibroblasts, and that simultaneous stimulation of both α- and ß-adrenergic receptors is required to increase PNK mRNA levels in cardiac myocytes.

P 406 MEF2 FACTORS CLONED FROM ZEBRAFISH EMBRYOS AND THEIR ROLE IN EARLY CARDIAC DEVELOPMENT,

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Several new transcription factors belonging to the muscle-specific enhancer factor 2 (MEF2) family have been isolated recently from human heart. These highly conserved MADS box proteins trans-activate via DNA binding to the MEF2 site on which multiple cardiac gene promoters and enhancers depend. They are present early in muscle differentiation in culture and almost certainly play a central role in embryonic cardiac development. Detailed investigation of vertebrate cardiogenesis, however, requires a model in which the earliest events can be examined and manipulated in vivo, and which is amenable to genetic analysis. Recently, the zebrafish has been increasingly recognized as a particularly promising system for this purpose. Externally fertilized, rapidly developing, transparent fish embryos can be observed in real-time, with the cardiovascular system defined as early as 8 hours. For genetic study, they are fecund, have a rapid generation time, and can be made to develop as haploid or homozygous diploid embryos. We are using the MEF2 factors as a window into early cardiac development in the zebrafish. We have isolated multiple MEF2-related cDNAs from a 33-36 hour zebrafish embryo library. One of these has been completely sequenced and encodes a protein that is highly homologous to human MEF2, particularly in the amino-terminal MADS/MEF2 domain; other clones are being characterized. The zebrafish protein, translated in vitro, binds the MEF2 DNA site with the same sequence specificity as its human counterpart, and it is expected to activate transcription of appropriate reporter genes via this site as well. We are beginning an analysis of the embryonic expression patterns of zebrafish MEF2 by whole-mount in situ hybridization. We are now also in a position to evaluate the role of MEF2 in cardiovascular development using experimental approaches that prevent its normal expression in zebrafish embryos.

P 407 **THE REGULATION OF HUMAN MYOCARDIAL GLUTATHIONE PEROXIDASE GENE EXPRESSION BY OXYGEN TENSION.**

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Cultured ventricular cardiomyocytes derived from tetralogy of Fallot patients are being used to investigate oxidant injury and contractile failure associated with corrective surgery of congenital cyanotic heart disease. We have previously shown that the antioxidant enzyme glutathione peroxidase (GSH-Px) is regulated by oxygen tension at the level of transcription. Regulation of GSH-Px gene expression by O_2 is reflected in enzyme activities, mRNA levels and nascent transcript synthesis rates. Results were standardized against specific housekeeping and overall gene expression levels. Hypoxia depletes GSH-Px enzyme concentrations and as a consequence cardiomyocytes cultured at a low pO_2 (40 mm Hg) are more susceptible to oxygen mediated free-radical injury than cells cultured at a normal pO_2 (150 mm Hg). We hypothesize that within the vicinity of the GSH-Px gene there lies a *cis*-acting element which binds an oxygen-responsive *trans*-acting factor modulating GSH-Px transcription. The form of GSH-Px expressed in the myocardium has been identified and both cDNA and genomic clones have been purified and sequenced. S1 nuclease analysis determined the transcriptional start site to extend 47 nucleotides upstream of the beginning of translation. In order to locate oxygen responsive *cis*-acting elements (ORE's) an assortment of GSH-Px 5' flanking region deletion constructs were fused to the reporter gene chloramphenicol acetyltransferase (CAT) and transfected into cardiomyocyte cultures equilibrated at a pO_2 of either 40 or 150 mm Hg. Putative ORE's were assessed for the ability to bind regulatory factors *in vitro* utilizing gel mobility shift and footprinting assays. Further experiments will focus on isolating oxygen responsive regulatory factors (ORRF's).

P 408 *Abstract Withdrawn*

P 409 **cAMP REGULATORY ELEMENT BINDING PROTEIN (*creb*) EXPRESSION IS REGULATED BY cAMP BUT NOT BY TENSION IN CARDIAC MYOCYTES.**

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Cardiac cells grow in response to increased work but the mechanism for this mechano-chemical transduction is not known. Our aim is to find mechanisms by which genes respond in a very short time to mechanical changes. Intermediate early genes such as *creb* are likely candidates. Ventricular cells from 10 day embryonic chick hearts are cultured in serum free media for 3 days. cAMP is raised by forskolin (5 or 10 μM) which also increases the force of beating. Forskolin is used with or without tension blockage by 2,3 butanedione monoxime, BDM (5 mM). Responses for time periods from 0, 0.5, 1 and 2 h are assayed with filter hybridization for *creb* or by anti-bodies to myosin and CRE-BINDING PROTEIN (CRE-BP) (M. Greenberg, Harvard). Although cells beat more rapidly with cAMP and are blocked with BDM, we saw no change in cell size, morphology or myosin content in two hours. In control cultures, low CRE-BP staining was found in the nuclei of myosin-positive cells. The intensity increased markedly in myocytes by 1 h after cAMP activation. Quantitative Northern and filter hybridizations (standardized against 18s or actin) show increased mRNA levels in cAMP stimulated myocytes. The maximum *creb* level was at 1 h at 3.28 ± 0.59 fold above control (SE, n = 22). By 2h of cAMP stimulation this declined to 1.67 ± 0.46 (n = 16). BDM alone caused no change in *creb* expression. At 1 h with BDM and forskolin, expression of *creb* remained at 3.34 ± 0.85 (n = 10) above control and so BDM did not affect up-regulation by cAMP activation. Therefore, we conclude that cAMP regulates *creb* expression but that the status of tension plays no role. **Supported by AHA and HL 40880.**

P 410 POSITIVE COOPERATIVITY BETWEEN CIS-ACTING ELEMENTS OF THE MYOGLOBIN PROMOTER RECONSTITUTES MUSCLE SPECIFIC EXPRESSION, Jason Grayson, Rhonda S. Bassel-Duby, R. Sanders Williams, Department of Internal Medicine, University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75235
Previous work in the laboratory identified two cis-acting elements in the 5' flanking region (-373 to +7) of the human myoglobin gene: nucleotide substitutions within either a CCAC or a Mef-2-like motif suppress transcription drastically below that of the wild-type promoter. The CCAC motif binds specifically to a novel 40 kD protein that appears to be expressed ubiquitously. The Mef-2-like site binds specifically to a factor that is abundant in nuclear extracts from differentiated myotubes and can be distinguished from the Mef-2 site of the muscle creatine kinase by competitive binding studies. Concatemerization of the CCAC motif linked upstream of a TATA element leads to at most 30% of wild-type promoter activity in sol 8 myotubes. In addition, such construct, unlike the myoglobin promoter, are expressed at low levels in NIH 3T3 fibroblasts. A single Mef-2-like site linked to a TATA element has only minimal promoter activity in muscle or non-muscle cells, though concatemerization of the Mef-2-like site could generate up to 50% of wild-type promoter activity selectively in sol8 myotubes. Linkage of multimeric CCAC regions with a single Mef-2-like site recapitulates activity of the wild-type promoter and is muscle specific. We conclude that cooperative interactions between proteins binding these two elements are necessary and sufficient to constitute muscle-specific enhancer activity. We speculate that the CCAC binding factor amplifies transcriptional activation, the specificity of which is determined by the factor that recognizes the Mef-2-like motif.

P 411 FUNCTIONAL SIGNIFICANCE OF THE NH2-TERMINAL DOMAIN IN CARDIAC TROPONIN I, Xiaodu Guo, Jonggonnee Wattanapermpool and R. John Solaro, College of Medicine, University of Illinois at Chicago, Chicago IL 60680
Expression of TnI isoforms in heart is developmentally regulated. The slow skeletal TnI (ssTnI) mRNA is present in the immature heart and decreases with increasing age. Cardiac TnI (cTnI) differs from ssTnI in having an additional stretch of 32 amino acids at the NH2-terminus. In this region there are several serines that have been demonstrated to be phosphorylated in vivo. The insensitivity of the neonatal heart to acidic pH compared to adult heart may also be related to the NH2-terminal domain of cTnI. To understand the functional significance of this domain serines 23 and 24 have been converted to alanines by site-directed mutagenesis. Using the PCR reaction, we have also removed the cardiac specific NH2-terminus. Mutant cTnI(s) were cloned into pET expression vector and expressed in E. Coli. To produce large amounts of wild-type recombinant cTnI, mouse cTnI cDNA was obtained by the RNA-PCR method and expressed in the same system. In the presence of Ca^{2+}, purified cTnI(s) formed a complex with cardiac TnC. Preliminary results have shown that without serines 23 and 24 or the entire NH2-terminus, cTnI can not be phosphorylated by protein kinase A in vitro. To test the functions of mutant cTnI(s), we have established a procedure to exchange native cTnI with recombinant cTnI by adding an excess amount of cTnT to heart myofibrils. Experiments are under way to determine the pCa-ATPase relationship in these preparations.

P 412 **IDENTIFICATION OF TWO CIS REGULATORY ELEMENTS (AF-1 AND AF-2) WHICH MEDIATE INDUCIBILITY OF THE ATRIAL NATRIURETIC FACTOR GENE BY ACTIVATED RAS AND HYPERTROPHIC STIMULI,** Adrienne N. Harris, Yiu-Fai Chen*, and Kenneth R. Chien, AHA-Bugher Foundation Center for Molecular Biology, and *Hypertension Research Program, UA Birmingham, Birmingham, AL.

Reactivation of ANF gene expression in the adult ventricular cell serves as a marker for induction of an embryonic gene program in the hypertrophied ventricular myocardium. Using a combination of transfection and microinjection approaches, we have documented that the $alpha_1$-adrenergic agonist phenylephrine (PE) can induce ANF gene expression through a Ras-dependent pathway. To identify the regulatory elements which mediate inducibility, a combination of deletional and site-directed mutations were generated in -638bp of the ANF promoter that confers inducibility of a luciferase reporter gene in transient assays in primary cultures of neonatal rat ventricular myocytes. ANF sequences between -638 and -548 (AF2 element) and between -135 and -70 (AF1 element) can confer a 5-7- and 2-fold induction respectively on a tk promoter luciferase vector following stimulation by PE, Ras, or serum. Gel shift analyses demonstrated that the binding activities to both AF-1 and AF-2 are induced by PE and serum. Point mutations in AF2a and AF2b reduced Ras activation of the full length -638bp ANF promoter. Methylation interference studies of the AF-1 and AF-2 sites are in progress, which should utimately lead to the isolation of the most downstream nuclear factors that link Ras-dependent pathways with external hypertrophic stimuli.

P 413 A NOVEL PROTEIN CONTAINING A *FORK HEAD* DOMAIN BINDS SPECIFICALLY TO A SEQUENCE ELEMENT REQUIRED FOR MUSCLE-SPECIFIC TRANSCRIPTION, Maria D. Hernandez, R. Sanders Williams and Rhonda S. Bassel-Duby, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75235
A 373 nucleotide base pair upstream region of the myoglobin gene is responsible for regulation of muscle-specific expression in both cardiac and skeletal cells. Transient expression assays indicate that a CCCCACCCCC (CCAC box) sequence (-223 to -204) is necessary for muscle-specific transcription. Gel shift assays and Southwestern blot studies demonstrate that a 40 kD protein binds specifically to the CCAC motif. Oligonucleotides corresponding to the CCAC motif were used to select cDNA clones from a λgt11 expression library. One clone encodes a protein (CBF) with a region of homology to a domain, *fork head*, first identified within a Drosophila homeotic gene that promotes terminal development. A similar domain was subsequently found to be the DNA-binding region of the hepatocyte transcription factor, HNF-3. Gel shift studies using purified recombinant CBF verify the binding specificity of CBF to the CCAC motif. By Northern analysis, CBF is expressed in cardiac and skeletal muscle, but its expression is not limited to muscle tissues. The function of CBF as a trans-activator is currently under investigation.

P 414 MYOSIN LIGHT CHAIN KINASE GENE EXPRESSION DURING SKELETAL MUSCLE DEVELOPMENT, B. Paul Herring, Krannert Institute of Cardiology, Indiana University Medical School, 1111 West 10th St. Indianapolis, IN 46202-4800

Myosin light chain kinases are important enzymes involved in regulation of muscle contraction, cell movement, secretion, and cell division. There are two major forms of myosin light chain kinase which can be distinguished biochemically and structurally, a skeletal muscle form and a smooth/non-muscle form. The gene encoding the skeletal muscle myosin light chain kinase is expressed in adult skeletal muscle and perhaps heart. The smooth/non-muscle enzyme is encoded by a distinct gene which is expressed in all smooth and non-muscle tissues. Myosin light chain kinase has been proposed to be involved in cell division and would thus be expressed in all dividing cells. In cultured C_2C_{12} skeletal muscle cells I have shown that the skeletal muscle myosin light chain kinase is only expressed following terminal differentiation and cell fusion and is not detectable in proliferating myoblasts. Preliminary evidence will be presented that suggests that the smooth muscle myosin light chain kinase is expressed in proliferating skeletal muscle myoblast cells. Following cell fusion and myotube formation expression of the smooth muscle kinase is repressed and expression of the skeletal muscle enzyme is activated. The cis-acting regulatory elements within the myosin light chain kinase genes which mediate this expression pattern are being defined.

P 415 ENDOTHELIN STIMULATES MAP KINASE AND S6 KINASE ACTIVITIES IN ADULT FELINE CARDIAC MYOCYTES. Linda G. Jones and Kathryn E. Meier, Departments of Medicine and Pharmacology, Medical University of South Carolina, Charleston, SC 29425

Endothelin (ET) elicits multiple responses in adult cardiac ventricular myocytes including stimulation of phosphoinositide (PI) hydrolysis, increased expression of c-*fos* and c-*zif*, and positive inotropism (Jones et al., Am J Physiol 1992, *in press*). In addition, ET inhibits cAMP accumulation in a dose-dependent manner (manuscript in preparation). Of these responses, the positive inotropic effect and the inhibition of cAMP accumulation are pertussis toxin-sensitive, suggesting the involvement of G_i in these responses. Whether these two events are causally linked is unclear at this time. However, since a role for G_i in the control of cell proliferation and/or differentiation has been suggested, and since PI hydrolysis and protooncogene expression are also correlated with growth events, it is important to know whether ET stimulates intermediate signals, such as activation of protein kinases that are associated with cell growth. We have shown that 100 nM ET stimulates a 2 to 4-fold increase in the activities of both Mitogen-Activated Protein (MAP) kinase (*erk* gene product) and S6 kinase (*rsk* gene product). An increase in MAP kinase activity is observed by 2 minutes of exposure to 100 nM ET and is maximal by 10 minutes. In keeping with its downstream position relative to MAP kinase, S6 kinase activity is not significantly increased in response to 100 nM ET until after five minutes. Maximal activity for S6 kinase is also observed at 10 minutes, after which a sharp decline occurs in activities of both MAP and S6 kinases. Overnight pretreatment with 100 ng/ml pertussis toxin does not block kinase activation in response to ET, suggesting that this effect may be downstream of phospholipase activation. Both *erk* and *rsk* gene products have been shown to be activated in the intracellular transmission of growth factor signals. Activation of these kinases by ET in isolated adult cardiac myocytes further supports the hypothesis that ET has a role in cardiac growth regulation.

P 416 **MITOCHONDRIAL DNA AND COIII mRNA LEVELS FOLLOWING EMBRYONIC EXPOSURE TO HYPOTHERMIA OR ETHANOL.** John M. Kennedy, Susan W. Kelley, Julia M. Meehan, and Steven R. Lobacz. Department of Physiology, University of Illinois, Chicago, IL 60640.

Embryonic chicks were exposed to either hypothermic conditions (32°C) or ethanol from day 11 to day 18 in ovo. Although both models produced cardiac enlargement, the increase in cardiac mass was larger following hypothermic exposure. Cytochrome oxidase (CO) activity was depressed by 63% in hypothermic ventricles and 36% in ethanol-exposed ventricles. However, while citrate synthase (CS) activity was depressed by 56% in hypothermic ventricles, no difference was seen in CS activity following ethanol exposure. The involvement of mitochondrial gene replication and transcription was evaluated using a cDNA clone for the mitochondrially encoded subunit III of cytochrome c oxidase (COIII). Quantitative slot blot analysis revealed that the relative COIII mRNA level was 46% less in hypothermic ventricles. However, the relative mitochondrial DNA level was 176% greater in hypothermic ventricles. These results indicate that the deficit in CO activity in hypothermic ventricles was transcriptionally regulated by a mechanism which was not coupled to a coordinated decrease in mitochondrial DNA copy number. This dissociation of mitochondrial gene replication and transcription may provide a useful model for examining the regulation of these two processes during mitochondrial biogenesis. No differences were seen in either the relative COIII mRNA or mitochodrial DNA levels in ethanol exposed ventricles. However, incorporation of ^{35}S-methionine into mitochondria from ethanol-exposed ventricles was depressed. These results suggest that the ethanol-induced deficit in CO activity was controlled by a post-transcriptional mechanism. This work was supported by grants from the NIAAA (1R29AA08716) and NIH (T32HL07692).

P 417 THE FORMATION OF THE TRICUSPID VALVE IN THE HUMAN HEART. Wouter Lamers[1], Szabolcs Viragh[2], and Antoon Moorman[1], [1]Dept of Anatomy & Embryology, Amsterdam, The Netherlands and [2]Dept of Pathology, Budapest, Hungary.

The formation of the tricuspid valve (TPV) was studied, using antibodies that specifically recognize the myocardium, the endocardial cushions (ECs) and the neural cell marker that identifies the myocardium of the embryonic interventricular junction (IVJ), and scanning EM. In the 5th week the atrium has only access to the embryonic LV. The right AV connection is formed in the 6th week as a result of the tissue remodelling of the IVJ that also characterizes cardiac septation. Both the ECs and the myocardium contribute to the formation of the leaflets of the TPV, the EC material forming the atrial aspect of the leaflet and the myocardium the ventricular aspect. The septal leaflet originates from the inferior EC. The superior part of the anterior leaflet develops from the endocardial ridges of the outflow tract. The remaining part of the anterior leaflet and the posterior leaflet derive from a flap of ventricular muscle that develops in the 5th week at the boundary between the AVC and the RV. This flap becomes prominent in the 6th week to form a myocardial gully that directs atrial blood towards the middle of the developing RV. The anterior papillary muscle develops as a condensation of trabeculae, first seen at 7 weeks. At the same time fenestrations in the gully create a posterior opening in the valve, thereby transforming the anterior boundary of the gully, that contains the RBB of the developing ventricular conduction system, into the septomarginal trabeculation. The remodelling of the embryonic components into the formed valve is a rather slow process, involving a delamination process within the myocardium to produce freely movable leaflets and, subsequently, a fibrous transformation of the delaminated leaflets. Delamination is first seen in the 10-11 week-old embryo. Fibrous transformation is complete at 16 weeks. The new insights can explain the development of congenital malformations, such as tricuspid atresia and Ebstein's anomaly, as developmental arrests. The muscular origin of the TPV, in particular of its lateral part, demonstrates its homology with the permanently muscular valve in birds and reveals that the formation of the tricuspid valve follows an evolutionarily conserved morphogenetic program.

P 418 VENTRICLE-SPECIFIC EXPRESSION OF A STIMULATORY ADENOSINE RECEPTOR SUBTYPE, Bruce T. Liang, Hao Xu, and Brett Haltiwanger, University of Pennsylvania School of Medicine, Philadelphia, PA 19104.

Atrial and ventricular myocytes cultured from chick embryos 14 days in ovo and adult rat ventricular myocytes were used as model systems to study cardiac adenosine receptor subtypes. Modulation of adenylyl cyclase activity and myocyte contractility (as determined by changes in the contractile amplitude with an optico-video motion detection system) were used to gauge the functional responses mediated by the adenosine receptor subtypes. *In both fetal chick and adult rat ventricular* myocytes, adenosine or its agonists caused significant stimulation of both adenylyl cyclase activity (the % increase ranged from 52 to 63 %, n=6) and myocyte contractility (from 55 to 116%, n=4) after blocking the inhibitory adenosine A_1 receptor (A_1AR) pathway with an A_1receptor-selective antagonist (8-cyclopentyl-1,3-dipropylxanthine)(DPCPX) or with pertussis toxin treatment of the myocyte. However, in cultured fetal chick atrial myocytes, the various adenosine agonists had no effect on either the adenylyl cyclase activity or the contractile amplitude after similar blockade of the A_1 receptor. The adenosine A_2 receptor-selective radioligand ([^{3}H] CGS21680; [^{3}H] 2-[p-(2-carboxyethyl)-phenethylamino]5'-N-ehtylcarboxamido adenosine) and a rat A_2 receptor cDNA directly confirmed the expression of A_2 adenosine receptors at both the protein and the mRNA levels in ventricular but not atrial myocytes. The calcium phosphate precipitation method was modified and adapted to transfect the cultured fetal chick myocyte. Up to 45% of cultured myocytes can be transfected with the Lac Z-containing plasmid, pSV-β-gal, as determined by hemocytometer counting of β-gal-positive cells after X-gal staining. Transfection of atrial myocytes with the A_2 receptor cDNA using this modified calcium phosphate method resulted in the expression of an A_2 receptor that can bind to [^{3}H]CGS21680 and that can couple to stimulation of adenylyl cyclase.

Conclusion: The A_2 subtype of adenosine receptor is expressed and mediates stimulatory functions in both fetal and adult ventricular myocytes. Ventricular myocytes containing the native endogenous A_2 receptor as well as the atrial myocyte that is transfected with A_2 receptor cDNA should be useful models to study the cardiac function and regulation of adenosine A_2 receptors.

P 419 TRANSCRIPTIONAL REGULATION OF GENES FOR CYTOCHROME c OXIDASE SUBUNITS VIA-HEART AND VIII-HEART. Margaret I. Lomax, Department of Anatomy and Cell Biology, University of Michigan Medical School, Ann Arbor, MI 48109.

Cytochrome *c* oxidase (COX), the terminal complex of mitochondrial electron transport, is critically important for oxidative metabolism in highly aerobic tissues such as heart and slow-twitch skeletal muscle. Mammalian COX is composed of 13 polypeptide subunits. Subunits I-III are encoded in mitochondrial DNA and carry out the catalytic functions; the 10 smaller subunits are encoded in nuclear DNA and may modulate cytochrome oxidase activity in response to different physiological signals or metabolic environments, although the exact function of these nucleus-encoded subunits is unknown. Bovine COX has tissue-specific isozymes: a heart (H) or contractile muscle isozyme, and a liver (L) or non-muscle isozyme. These isozymes differ because three of the nucleus-encoded COX subunits (VIa, VIIa, and VIII) have isoforms found only in contractile muscle, i.e., heart/muscle isoforms. To understand the transcriptional regulation of genes for these heart/muscle-specific subunits, we have isolated and sequenced the bovine genes for the heart/muscle isoform of COX subunits VIa-heart (*bCOX6AH*) and VIII-heart (*bCOX8H*). Genomic Southern blots indicated that both *bCOX6AH* and *bCOX8H* are single-copy genes. The *bCOX6AH* gene is located within a 6.2-kb HindIII fragment and is composed of three exons contained within a 2.0-kb EcoRI fragment. Exons two and three are separated by a small 96-bp intron. The proximal promoter region contains conserved TATA, CCAAT, and MyoD1 elements, while the distal promoter contains enhancers for respiratory genes. The *bCOX8H* gene is located within a single 4.8-kb *Eco*RI fragment and contains a single 1.2-kb intron. The promoter of the *bCOX8H* gene contains a single TATA element, tandemly duplicated E-boxes, a CarG box and an MEF-2 site. Transient transfection experiments with CAT reporter genes are currently in progress to determine whether these previously described muscle-specific transcription factors also regulate expression of this important class of heart/muscle-specific genes.

P 420 REGULATION OF THE PROXIMAL α-SMOOTH MUSCLE ACTIN PROMOTER IN EMBRYONIC VENTRICULAR MYOCYTES, Tim C. McQuinn, Robert J. Schwartz, Department of Pediatrics and Cell Biology, Baylor College of Medicine, Houston, TX 77030

In the embryonic heart, the α-smooth muscle actin gene is up-regulated prior to looping and turned off during looping. This gene is re-expressed in cardiac myocytes in culture. We have examined the transcriptional regulation provided by the proximal promoter portion of this gene in cardiac ventricular myocytes in culture. A linker scan extending from the TATA box to -212 was constructed and the mutated promoters assayed by transient transfection into ventricular myocytes from d11.5 chick embryos.

In a similar fashion to other α-actin genes in cardiac myocytes, we have found that (two) consensus serum response elements (SRE's) are present in the promoter, that each is independently required for promoter function, and that each binds to SRF (serum response factor) in vitro, although with different affinities. The upstream, high affinity SRE is flanked by a consensus NF-IL6 response element and mutations of this element result in 4 to 5 fold up-regulation of the promoter. Through band-shift assays we have identified a DNA-binding activity that appears to be specific for this SRE-flanking element and we are currently investigating whether SRF and this protein interact with each other while bound to the promoter, or whether there is mutually exclusive binding to their respective elements.

P 421 FUNCTIONALLY DISTINCT ELEMENTS OF AMPD1 REQUIRED FOR MYOCYTE SPECIFIC EXPRESSION, Takayuki Morisaki and Edward W. Holmes, Department of Medicine and Human Genetics, University of Pennsylvania, Philadelphia, PA 19104.

AMP deaminase (AMPD) is an enzyme found in all eukaryotic cells, and plays a central role in energy metabolism especially in skeletal muscle of vertebrates where the AMPD1 gene is expressed at high levels. Tissue-specific and stage-specific isoforms of this enzyme are found in vertebrates, regulated by multiple genes as well as alternative splicing. The AMPD1 gene is expressed predominantly in skeletal muscle where transcript abundance is controlled by stage-specific and fiber-type specific signals. This activity is important in skeletal muscle because the metabolic myopathy develops in individuals with inherited deficiency of AMPD1. Cis- and trans-acting factors that control expression of AMPD1 were studied on rat and human genes. There are at least two *cis-acting elements required for muscle-specific* expression of the AMPD1 gene, located within 100 nucleotides of 5' flanking region from transcriptional start site. One element (-100 to -79) appears to behave like an enhancer element that interacts with protein(s) found in myocyte nuclei and it has A/T rich core sequences similar to MEF2 binding motif but might have another binding character. The other element (-60 to -40) has properties of a muscle specific promoter in that it interacts with protein(s) restricted to differentiated myocyte nuclei and it has rigid spatial constrains with respect to more proximal region of the promoter. *We conclude that those functionally* distinct elements are required for expression of AMPD1 in myocytes.

P 422 THE RETINOID X RECEPTOR MODULATES THE BINDING OF THE THYROID HORMONE RECEPTOR TO THE SKELETAL ACTIN GENE

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Thyroid hormone(T3) has been shown to cause rapid increases in α-actin mRNA in cardiocyte cultures and in the hearts of normal, hypophysectomised and hypothyroid rats. The *cis*-acting sequences between nucleotide positions -432 and -153 are required for T3/thyroid hormone receptor(TR) mediated *trans*-activation of the human skeletal α-actin gene (CGD 3: 31, 1992). We have used transfection experiments and electrophoretic mobility shift assays (EMSA) to characterize the T3 response element (TRE) in the α-actin gene. This TRE is located between nucleotide positions -273 and -249 (5' GGGCAACTGGGTCGGGTCAGGAGGG 3'). This sequence conferred T3 regulation in a TR-dependent manner to an enhancerless SV40 promoter. EMSA experiments showed that *Escherichia coli* expressed/affinity purified TRα and retinoid X receptors (RXRα) bound to the skeletal α-actin TRE as monomers, homodimers and heterodimers. However, increased levels of RXRα decreased the binding of TRα to the α-actin TRE in contrast to promoting TRα binding to the α-myosin heavy chain (MHC). Site specific mutagenesis of each core receptor binding motif inhibited TRα binding. The α-actin TRE, the palindromic TRE (PAL-0), the synthetic direct repeat TRE (DR-4) and the MHC and growth hormone(GH) TREs interacted with an identical nuclear factor *in vitro* in muscle cells.

P 423 CLONING, SEQUENCING AND CHARATERISATION OF A PUTATIVE SMOOTH MUSCLE-SPECIFIC PROMOTER.

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The inappropriate growth of vascular smooth muscle (VSM) cells within vessel lumen is an important clinical problem, resulting in vessel occlusion and myocardial infarction. One potential way of dealing with this problem is the specific expression of growth inhibitory genes within the VSM cells. To this end we have been trying to elucidate the mechanism of VSM-specific gene expression, with a view to using VSM-specific promoter elements in constructs for gene therapy.

A rat SM cell-specific gene, SM22α, has recently been cloned and sequenced by our laboratory (Shanahan *et al.*, Circulation Research, submitted). SM22α is a single copy gene of unknown function, which is easily extracted from the contractile apparatus. The gene is very highly expressed at the mRNA level in rat aortic cells and other smooth muscle tissues, but shows little expression in none smooth muscle tissues such as liver, kidney and brain. Developmental expression has also been examined, and SM22α mRNA was found to be present as early as 2 days post birth and highly expressed by 2 weeks.

A rat genomic library has been screened and a number of overlapping clones containing the SM22α coding region have been isolated. Segments of these clones, including the 5' region of the gene, have been subcloned and sequenced. A number of regions homologous to the E-box consensus sequence first observed in skeletal muscle specific genes have been identified upstream of the gene, and an intron within the 5' untranslated sequence has been found to possess homology to the rat identifier sequence. The SM22α identifier sequence is 98% homologous to previously reported identifier sequences and contains RNA polymerase III recognition boxes. The relative importance of these potential regulatory elements is now being examined using reporter gene constructs.

P 424 **ANGIOTENSIN II CAUSES ACTIVATION OF TYROSINE KINASES, MAP KINASES AND S6 KINASE CASCADE VIA Ca^{2+} DEPENDENT MECHANISM IN CARDIAC MYOCYTES.**

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It is known that angiotensin II (Ang II) activates multiple second messenger systems through G protein coupled receptors, but little is known about protein tyrosine kinase cascades in Ang II-induced signaling in cardiac myocytes. Mitogen activated protein kinases (MAPK) and 90 kDa S6 kinases (RSK) are serine/threonine kinases which are activated by many growth factor stimuli and are important downstream pathways of tyrosine kinases. Therefore, we examined whether Ang II activates this protein kinase cascade in neonatal rat cardiac myocyte. Immunoblotting with phosphotyrosine antibody showed that Ang II rapidly induced tyrosine phosphorylation of multiple proteins including 42 and 44 kDa polypeptides. These 42 and 44 kDa proteins were immunologically related to ERK1 (extracellular signal-regulated kinase-1), a member of MAPK. Immune complex kinase assay showed that Ang II activated ERK-1 related MAPK activity of 42 and 44 kDa proteins within 1 min and their activity reached a peak around 5-10 min. Immune complex kinase assay of RSK, using S6 peptide as a substrate, indicated that Ang II activated RSK immediately after MAPK activation. Phorbol 12 myristate 13 acetate (PMA) and Ca^{2+} ionophore also activated both MAPK and RSK. Interestingly, downregulation of protein kinase C (PKC) by prolonged treatment of PMA caused little or no suppression of Ang II-induced activation of MAPK and RSK. In contrast, chelating intracellular Ca^{2+} by membrane permeable BAPTA significantly suppressed Ang II-induced MAPK and RSK activation. Our results suggest that; (1)Ang II rapidly activates tyrosine kinases, MAPK and RSK in neonatal rat cardiac myocytes. (2) Intracellular Ca^{2+}, rather than PKC, seems to be critical for Ang II-induced activation of MAPK and RSK. (3) Ang II may activate upstream tyrosine kinases via Ca^{2+} dependent mechanisms.

P 425 LOCALIZATION OF FAST SKELETAL MUSCLE REGULATORY ELEMENTS IN HUMAN ALDOLASE A GENE.

Salminen, M., Concordet, J-P., Moch, C., Maire P., Demignon J., Kahn, A. and Daegelen, D.

The human aldolase A gene is transcribed from three alternative promoters clustered within a 1.6 kb DNA domain. A fast muscle-specific promoter pM lies in between two ubiquitous promoters pN and pH, which are preferentially active in heart and skeletal muscle. The optional first exon is noncoding and spliced to a common coding exon.

With a deletion analysis of the promoter region we have shown that there is a strong ubiquitous enhancer lying in a 400 bp fragment upstream of pH. This enhancer is necessary for both pN and pH ubiquitous activity. When this enhancer and pH are deleted, pM and interestingly also pN remain active in transgenic mice, but only in fast skeletal muscle. Thus, pN can be activated by two different regulatory domains in aldolase gene, either by the ubiquitous enhancer or by sequences which confer the expression in fast skeletal muscle.

The elements involved in the activation of the two promoters in fast, glycolytic muscle were further characterized by creating several lines of transgenic mice with different constructs harboring various deletions or mutations in the aldolase A gene. We have delimited two separate regions which could behave like a fast muscle-specific enhancer. Results of additional analyses of these regions with the help of myogenic cells and transgenic mice will be presented.

Interestingly, all constructs tested so far are active in mouse genome in an integration-site independent fashion. Thus, the transgenic mice carrying different aldolase A constructs provide a good model to study both muscle-specific and ubiquitous gene-expression during development as well as to examine the elements involved in the locus control region effect, first described in the beta-globin locus.

P 426 MOLECULAR ANALYSIS OF THE SERCA2 PROMOTER. Junaid Shabbeer, Drew Sukovich, Hirosuke Matsui and Muthu Periasamy. Division of Cardiology, University of Cincinnati College of Medicine, Cincinnati, OH 45267.
The rabbit SERCA2 gene encodes the cardiac/slow twitch muscle calcium ATPase, an important sarcoplasmic reticulum Ca^{2+} transport pump. A sequence including 1.1 kb of upstream SERCA2 DNA has been shown to confer expression upon the CAT reporter gene in transfected C2C12 muscle cells and NIH3T3 fibroblasts. If a 17 bp element, centered around position -275, is deleted from the promoter, then expression is greatly reduced. Gel shift assays demonstrate that this 17 bp element can form a complex with proteins. The binding proteins involved are present in both myoblasts and differentiated myotubes. Southwestern assays indicate that a single protein of approximately 43 kDa, present in myoblasts, myotubes, and to a lesser extent in fibroblasts, interacts with the 17 bp element. When gel shift competition assays are performed with an array of known promoter elements, DNA binding is not abolished. However, the thyroid response element from the alpha myosin heavy chain gene promoter is able to strongly compete for binding. Antibodies against the thyroid receptor, though, do not alter or interfere with the pattern of band shift formation in binding assays.

P 427 A MURINE TRANSCRIPTION FACTOR RELATED TO HUMAN TEF-1 ENCODES A FACTOR THAT BINDS TO THE M-CAT MOTIF OF THE MYOSIN HEAVY CHAIN β GENE. Noriko Shimizu*, Gillian Smith*, and Seigo Izumo*+, *Molecular Medicine Unit and +Cardiovascular Division, Beth Israel Hospital, *Department of Medicine, Harvard Medical School, Boston, MA 02215
The A element, a fourteen base pair sequence in the rabbit myosin heavy chain (HC) β promoter (-276/-263), contains the M-CAT motif, a cis-acting element found in several muscle-specific genes. The A element is essential for muscle-specific transcription of the myosin HCβ gene. Recently, we have identified both muscle-specific and ubiquitous factors (A1 and A2 factors, respectively) that bind to the A element. Since the sequence of the A element is very similar to the GTIIC motif in the SV40 enhancer, we examined the relationship between A-element-binding factors and a GTIIC binding factor TEF-1, recently isolated from HeLa cells. The GTIIC motif was bound by the A1 and A2 factors in muscle nuclear extracts and competed with the A element for DNA-protein complex formation. Antibody against human TEF-1 "supershifted" the ubiquitous A2 factor-DNA complex, but did not alter the mobility of the muscle-specific A1 factor-DNA complex. This suggests that the A2 factor may be related to TEF-1. We isolated a murine cDNA clone (mTEF-1) from a cardiac cDNA library. The clone is highly homologous to Hela cell TEF-1. The *in vitro* transcription/translation product of mTEF-1 cDNA bound to the A element, and the DNA binding property of mTEF-1 was identical to that of the A2 factor. Transfection of mTEF-1 cDNA into muscle and non-muscle cells confirmed that mTEF-1 corresponds to A2, but not to A1 factors. The mTEF-1 mRNA is expressed abundantly in skeletal and cardiac muscles, kidney and lung. It is detectable at lower levels in other tissues as well. Overexpression of mTEF-1 was not sufficient to transactivate the myosin HCβ promoter in muscle and non-muscle cells. These results suggest that mTEF-1 encodes one of the M-CAT binding factors, A2 factor, but the muscle-specific A1 factor is distinct from mTEF-1.

P 428 *CIS*-ACTING ELEMENTS INVOLVED IN THE TRANSCRIPTIONAL REGULATION OF THE RAT SMOOTH MUSCLE α-ACTIN GENE
Richard T. Shimizu[1], Randal S. Blank[1], Coleen A. McNamara[2] and Gary K. Owens[1], Departments of Physiology[1] and Medicine[2], University of Virginia Health Sciences Center, Charlottesville, VA 22908.

There is considerable interest in identifying genes that regulate differentiation of vascular smooth muscle cells (SMC). The goal of the present study was to identify SM-specific transcription factors, which may serve as master regulatory proteins, through studies of the SM α-actin promoter, a gene that is specifically expressed in vascular SMC in the adult animal. A series of rat SM α-actin promoter fragments coupled to the chloramphenicol acetyltransferase (CAT) reporter gene (p125CAT [-125 to +20], 155CAT, p208CAT, p271CAT, p371CAT, p547CAT, p699CAT, & pPromCAT [containing the ~2.8 kb of cloned 5'-flanking region]) were transiently transfected into rat aortic SMC, rat & bovine aortic endothelial cells (EC), and rat L6 skeletal myoblasts. In SMC & both EC, the p125CAT construct exhibited the highest transcriptional activity (approaching the activity of the viral promoter control construct), while the activity of p125CAT in L6 myoblasts was not substantial higher than that of the promoterless control construct. Addition of further upstream promoter sequences resulted in a net decrease in activity in SMC & EC such that pPromCAT exhibited no significant activity in EC above the promoterless construct but remained elevated in SMC. Additional studies tested the regulatory role of two CArG boxes (designated CArG-A and CArG-B), which have the general sequence motif of $CC(A/T)_6GG$ and are 100% conserved between species. Site-directed mutation of either CArG-A or CArG-B to $AA(A/T)_6AA$ within the p271CAT construct resulted in a decrease in transcriptional activity to near baseline levels, and mutation of both CArG elements within the same construct had no additive effect. These data indicate that: (1) both CArG boxes are required for high transcriptional activity, and (2) tissue-specificity may be conferred by one or more negatively acting *cis*-elements upstream of -125 which restrict expression of the SM α-actin gene to SMC.

P 429 FUNCTIONAL ANALYSIS OF MULTIPLE CLONED MUSCLE ENHANCER FACTOR 2 (MEF2)-RELATED TRANSCRIPTION FACTORS. Leslie B. Smoot, Roger E. Breitbart, Yie-Teh Yu, John C. McDermott, Vijak Mahdavi, Bernardo Nadal-Ginard, Children's Hospital, Howard Hughes Medical Institute, Harvard Medical School, Boston, MA 02215
Muscle enhancer factor 2 (MEF2) comprises a group of tissue specific DNA binding and trans-activating factors important for cardiac and skeletal muscle gene expression. We have cloned cDNAs from heart and skeletal muscle corresponding to four related genes encoding transcription factors responsible for MEF2 activity. Transcripts of at least three of these genes are subject to alternative splicing, generating two-four isoforms for each. All contain the highly conserved MADS box DNA binding domain and a contiguous region termed the MEF2 domain. To investigate the potential functional implications for these multiple MEF2-related isoforms, we compared their activities in assays of specific DNA binding and transactivation. The MEF2 site sequence specificities of these factors appear similar, although certain isoforms show distinctly different DNA binding affinities determined by gel shift analysis using invitro translated proteins.
Multiple MEF2 isoforms both with and without specific alternate exons were tested for their ability to transactivate in cell culture. These cDNA isoforms were sub-cloned into eukaryotic expression vectors and cotransfected with a variety of reporter constructs containing native, mutated, or duplicate intact MEF2 binding sites. All MEF2 isoforms tested have produced comparable levels of reporter gene transcription, including those which exhibit tissue specific expression and/or alternative splicing. We are currently testing truncated factors and GAL4 fusion constructs to delineate the functional significance of various domains within these proteins.

P 430 **Structure, Expression, and Genomic Mapping of the Mouse Natriuretic Peptide Type-B Gene,** Mark E. Steinhelper, Vascular Biology and Hypertension Program, Departments of Medicine and Cell Biology, University of Alabama at Birmingham, Birmingham, AL 35294.
The structure of the mouse natriuretic peptide type-B (BNP) gene was determined by isolating and sequencing genomic clones. The mouse BNP gene was structurally similar to other NP genes, and comprised three exons (203 bp, 223 bp, 245 bp) and two introns (194 bp, 434 bp). Initiation of BNP transcription was 31 bp downstream of a consensus TATA box as determined by primer extension analysis of cardiac RNA. Comparative DNA sequence analysis identified several DNA elements with potential transcriptional regulatory function. Ribonuclease protection and northern blot analysis showed that BNP was expressed only in cardiac tissue. Isogenic cDNA clones from a BALB/c heart library were consistant with the genomic sequence. The mouse BNP cDNA encodes a polypeptide of 121 amino acids (Mr 13,746), and includes a 77 bp of 5'-untranslated region, a 363 bp open reading frame, and a 231 bp 3'-untranslated region. A consensus polyadenylation signal was present 34 bp upstream of the poly-A tail. A 69 bp AT rich sequence containing ten ATTTA repeats was found 87 bp downstream of the termination codon. The mouse BNP preprohormone predicted from the cDNA was 79% identical to the rat sequence. However, sequence identity was more conserved in the N-terminal portion of the BNP prohormone (78%) than in the biologically active C-terminal 45 amino acids (64%). The amino acid sequence surrounding the proteolytic processing site (RXXR-S) that generate bioactive BNP's (both BNP-45 and BNP-32) were conserved among all BNP precursors (mouse, rat, pig, dog, human). This site corresponds to the consensus site of furin, a calcium-dependent serine endoprotease. Finally, the BNP gene (*Bnp*) was mapped using recombinant inbred DNA and a polymerase chain reaction-restriction fragment length polymorphism assay to mouse chromosome 4 near the *Anf* locus. No recombination event between *Bnp* and *Anf* was evident in the 39 recombinant inbred and inbred strains examined. This physical linkage between the two NP genes expressed in cardiac tissue may be important for their transcriptional regulation.

P 431 FUNCTIONAL ANALYSIS OF A PUTATIVE NEGATIVE ELEMENT IN THE MOUSE GLYCEROPHOSPHATE DEHYDROGENASE LOCUS,
Claire M. Steppan, Helena M. Madden and Deborah E. Dobson, Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118
The cytoplasmic enzyme glycerophosphate dehydrogenase (GPD) is involved in lipid synthesis and fuel metabolism in many tissues. In elucidating the regulation of GPD, we have identified a putative negative element. Functional analysis of the GPD negative element indicate that it is a strong transcriptional silencer. Transient transfections involving constructs in which the silencer is inserted proximal to two different viral promoters in a constitutively expressed CAT gene show significant downregulation in C2C12 myoblasts, C2C12 myotubes,3T3F442A preadipocytes and 3T3F442A adipocytes. Electrophoretic mobility shift assays indicate nuclear proteins bind to sites within this negative element. Further analysis of our putative silencer is ongoing.

P 432 TRANSGENIC ANALYSIS OF THE THYROID RESPONSIVE ELEMENTS IN THE α-MYOSIN HEAVY CHAIN GENE PROMOTER, Arun Subramaniam, James Gulick, Jon Neuman, Stephanie Knotts and Jeffrey Robbins, Department of Pharmacology and Cell Biophysics and Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati, Cincinnati, Ohio 45267-0575.

The role of two putative, *cis*-acting thyroid hormone responsive elements, TRE_1 and TRE_2, located at -129 to -149 and -102 to -120 respectively on the murine α-myosin heavy chain (MHC) gene, has been investigated in transgenic mice. These motifs are present in a 5.5 kb fragment lying upstream of the mouse α-MHC gene's transcriptional start site: this fragment directs appropriate expression of a reporter gene in transgenic mice. We have, within the context of this active promoter, independently mutated the TRE_1 and TRE_2 elements by base substitution and analyzed transgene expression in the different tissues. Normal levels of transgene expression were observed in euthyroid mice carrying a mutation in TRE_1. Both cardiac compartment- and developmental stage-specific expression of the transgene was maintained at levels approaching or equal to those observed in the wild type lines in euthyroid animals. In contrast to these results, mice in which TRE_2 was mutated showed reduced levels of CAT activity in both the atria and ventricles, suggesting a previously undefined role for this element in the constitutive up regulation of the α-MHC gene. When hypothyroidism was induced in mice carrying either of these mutations, the complete cessation of ventricular expression of the chloramphenicol acetyl transferase transcripts that takes place in the α-5.5 (wild type) animals did not occur. Further analyses identify a previously undefined upstream region on the α-MHC promoter that is involved in mediating thyroid hormone action. Sequence analysis of this region reveals the presence of several consensus TRE half sites.

P 433 PROTEIN INTERACTIONS AND REGULATORY PHENOMENA ASSOCIATED WITH THE CREATINE KINASE GENE TA-RICH REGULATORY REGIONS. Drew Sukovich, Ranjan Mukherjee, Pat Harlow, Linda Santomenna, Anagha Sant and Pamela Benfield.
DuPont Merck Pharmaceuticals, Experimental Station, Wilmington DE 19880-0328.

We have previously described a TA-rich binding factor TARP that appears to play a role in regulation of both the brain and muscle creatine kinase genes. More recently related TA-rich elements have been implicated in the regulation of genes in cardiac tissue Navankasattusas et al. Mol. Cell. Biol. 12: 1469-1479 (1992). We have purified the TARP activity from HeLa cells and show that it represents a multi-protein complex at least one member of which is related to the MADS domain containing proteins (RSRFC4/C9 and MEF -2). Although this TA-rich element appears important for muscle-specific gene expression this element alone appears unable to direct expression to cardiac or skeletal muscle tissue. Transgenic mice that contain the BCK gene driven by 200 base pairs of upstream sequence are still correctly expressed in brain, kidney etc but lose the ability shown by the parent gene to be expressed in adult heart and stomach. Our data further indicate that the element may confer transcriptional responses to serum components. We have demonstrated that estrogen responsiveness of the brain creatine kinase promoter maps to this TA-rich element. Thus it is possible that cross talk may exist between the steroid hormone receptor family and factors that bind to this non-ERE TA-rich segment. MADS domain proteins represent one possible target for steroid hormone receptor interactions important for creatine kinase gene regulation.

P 434 SIGNALTRANSDUCTION AND TRANSCRIPTIONAL REGULATION IN ENDOTHELIAL CELLS: A ROLE FOR c-Ets-1 PROTO-ONCOGENE

Dietmar von der Ahe Claudia Nischan, Jürgen Otte, Bernd Pötzsch, and Bohdan Wasylyk[1]
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The vascular endothelium participates actively in the regulation of the hemostatic processes by its ability to synthesize and express proteins involved in fibrinolysis, coagulation and cell adhesion. Thrombomodulin (TM) is expressed on vascular endothelial cells and plays an important role in protein C anticoagulant pathway by maintaining the thrombo-resistence of the blood wessel wall. We show that in primary human endothelial cells TM gene transcription is repressed by TNFα through a Protein Kinase C independent pathway. TNFα specifically represses high constitutive expression of transfected TM promoter constructs. The -76/-56 5'-flanking region of human TM mediates both high basal expression and TNFα repression. Protein-DNA binding and mutation analysis indicate that TNFα repression is mediated by an endothelial-specific member of the ETS domain protein family. Although c-Ets-1 and c-Ets-2 stimulate the TM promoter through the -76/-56 element, their activity is not suppressed by TNFα. c-Ets-1 can compete and override TNFα repression in a concentration dependent manner. We propose that either a different cell-specific member of the ETS domain protein family , or an Ets-associated co-factor, is the target of the TNFα signaling cascade in endothelial cells. The identification of an endothelial Ets-target gene provides the opportunity to study the regulatory role of Ets proteins in more detail in vascular cells and blood vessel function. This is of fundamental importance because endothelial cells play a major role in normal vascular function and pathology, associated with angiogenesis, developmental processes, wound healing, thrombosis and vascularization of tumors.

P 435 STRUCTURAL ORGANIZATION OF THE RABBIT CARDIAC SR CALSEQUESTRIN GENE. Kenichi Yano and Angel Zarain-Herzberg, Division of Cardiovascular Sciences, St. Boniface Research Centre, University of Manitoba, Winnipeg, Manitoba, R2H 2A6, Canada.

Calsequestrin is the principal Ca^{2+} binding protein localized intraluminally in the terminal cisternae of the sarcoplasmic reticulum (SR) of cardiac muscle and skeletal muscle. The cardiac and fast-twitch skeletal muscle isoforms of calsequestrin are products of two different genes. The fast-twitch SR calsequestrin gene is expressed exclusively in fast-twitch skeletal muscle, whereas the cardiac SR calsequestrin gene is expressed predominantly in the heart and in relatively in less abundance in slow-twitch skeletal muscle but not in smooth musle and non-muscle tissues. The complete amino acid sequence of SR calsequestrin from rabbit skeletal muscle has been deduced from cDNA sequencing, and the complete genomic organization has been elucidated. The cDNA cloning for canine and rabbit cardiac SR calsequestrin has been reported. We have screened a rabbit genomic library constructed in lambda phage using ^{32}P-labeled cDNA restriction fragments containing the coding and non-coding regions of the rabbit cardiac SR calsequestrin cDNA by plaque hybridization cloning techniques. Screening of $1x10^5$ independent recombinant phages yielded two positive clones. The two clones containing genomic DNA inserts $\approx$19 kb in length were subjected to further analysis by restriction enzyme mapping and Southern blotting. By comparing the DNA sequence of the subcloned genomic fragments to the sequence of the rabbit cardiac SR calsequestrin cDNA we have been able to corroborate that both of the genomic clones isolated correspond to the cardiac SR calsequestrin gene. The locations of exons within the rabbit cardiac calsequestrin gene appears to have similar exon/intron organization compared to the rabbit fast-twitch skeletal muscle calsequestrin gene. To date we have located the exon/intron boundaries for exon 1,2,3 and 4 within the cardiac SR calsequestrin gene. Sequences for exons 2 and 3 were found to be 87 and 98 bp long, respectively, similar as the corresponding exons in the fast-twitch skeletal muscle SR calsequestrin gene. Present studies are directed to locate the transcription initiation site of gene, and to analyze by functional assays the putative promoter and 5'-regulatory regions of the gene.

P 436 SARCOPLASMIC RETICULUM mRNA EXPRESSION IN POST ISCHEMIC CONGESTIVE HEART FAILURE. Angel Zarain-Herzberg, Nasir Afzal and Naranjan S. Dhalla. Division of Cardiovascular Sciences, St. Boniface General Hospital Research Centre, University of Manitoba, Winnipeg, Manitoba, R2H 2A6, Canada.

The recent cloning of several genes encoding sarcoplasmic reticulum (SR) proteins, allows a detailed analysis of the molecular mechanisms responsible for hypertrophic and pathologic stimuli. Although alterations in both SR Ca^{2+} uptake and Ca^{2+} release have been identified in different models of heart failure, the molecular basis for altered cardiac SR function remains to be investigated in post-ischemic congestive heart failure (CHF). For this purpose, CHF was induced in rats by occluding the left coronary artery for 4, 8 and 16 weeks. Sham operated animals were used as control. Total RNA was isolated by the guanidine thiocyanate method from the non-ischemic viable left ventricle and the right ventricle at mild, moderate and severe stages of CHF. The relative mRNA levels of the cardiac SR Ca^{2+}-ATPase, phospholamban, ryanodine receptor, and GAPDH were quantitated by using Northern and slot blot techniques. The results demonstrate decreased mRNA levels for the cardiac SR Ca^{2+} -ATPase, phospholamban, ryanodine receptor with respect of GAPDH and rRNA at different stages of CHF. The decreased in mRNA levels for the above proteins was more pronounced in the remaining viable left ventricle as comared with right ventricle of the experimental animals. Our results suggest that the decreased mRNA levels for cardiac SR Ca^{2+}-ATPase, Ca^{2+}-release channel (ryanodine receptor) and phospholamban in the failing myocardial cell may partly explain the defective SR Ca^{2+} transport and subsequent cardiac dysfunction in CHF due to myocardial infarction. (Supported in part by the Heart and Stroke Foundation of Manitoba and Manitoba Healt Research Council).

P 437 STIMULATION OF P_2-PURINERGIC RECEPTORS INCREASES *c-fos*, *c-jun* AND *jun-B* GENE EXPRESSION IN CULTURED NEONATAL CARDIAC VENTRICULAR MYOCYTES. Jing-Sheng Zheng, Marvin O. Boluyt, Lydia O'Neill, Andrea Meredith, Michael T. Crow, and Edward G. Lakatta. Laboratory of Cardiovascular Science, GRC, NIA, NIH, 4940 Eastern Avenue, Baltimore, MD 21224.

In the neonatal model of cardiac hypertrophy, the effects of norepinephrine (NE) on the expression of immediate early genes (e.g. *c-fos*, *c-jun* and *jun-B*) and target genes (e.g. myosin light chain-2 and atrial natriuretic factor) have been well characterized. Since NE and ATP are co-released from sympathetic nerve endings upon nerve stimulation and cardiac myocytes contain ATP receptors, we wished to determine whether ATP also plays a role in cardiac hypertrophy. In this study, we used immunofluorescence, immunohistochemistry and northern blot analysis to study the effects of extracellular ATP on the expression of immediate early genes in neonatal ventricular myocytes. We found that micromolar ATP increased mRNA levels of *c-fos*, *c-jun* and *jun-B* several fold in myocytes that had been cultured overnight in serum-free medium. Under the same conditions, ATP also increased the level of FOS protein. ATP analogues, such as ATPrS, ADPBS and ADP, but not adenosine, also increased FOS protein. Pretreatment of myocytes with the protein kinase C inhibitor, staurosporine, prevented the ATP-induced FOS protein production, while protein kinase C activator, phorbol 12-myristate 13-actate (TPA) (100 nM) mimicked the effect of ATP and increased FOS protein level. Addition of Bay-K 8644 (a Ca^{2+} channel agonist) also increased FOS protein, whereas pretreatment of myocytes with Ca^{2+} chelator, BAPTA-AM inhibited the ATP-induced FOS protein. These results clearly indicate that ATP is sufficient to induce immediate early genes in neonatal cardiac myocytes and suggest that activation of P_2-purinergic receptors increases FOS protein via both protein kinase C- and Ca^{2+}-dependent pathways.

Late Abstracts

COMPARTMENTALIZED CALCIUM AND CALCIUM SENSITIZERS REGULATE MYOCARDIAL GENE INDUCTION DURING ß-ADRENOCEPTOR-MEDIATED HYPERTROPHY. Nanette H. Bishopric, Barbara Sato and Keith Webster, Cardiovascular Research Institute and VAMC, U.C. San Francisco, and SRI International, Menlo Park, CA 94025

Skeletal α-actin (sACT) and atrial natriuretic peptide (ANP) are examples of genes expressed during fetal cardiac development that are reinduced in cardiac myocyte hypertrophy in adult rats. Numerous second messenger pathways influence the expression of these genes in cardiac myocytes *in vitro*, but it is not known which of these is most important during the onset of hypertrophy *in vivo*. Interestingly, regulation of ANP and sACT mRNA *steady state levels is dissociable in vitro*. Whereas α-adrenergic stimulaton upregulates both ANP and sACT mRNAs, only sACT mRNA was increased by ß-adrenergic stimulation (EC_{50} for isoproterenol ~80 nM). The induction of sACT was mimicked by cholera toxin activation of Gs and by ryanodine. ß-adrenergic activation of sACT required calcium entry via the L-type calcium entry channel. However, the calcium ionophore A23187 caused a large reduction in basal levels of sACT and reduced or eliminated its upregulation by agents such as isoproterenol and protein kinase C activating phorbol esters. In contrast, calcium ionophore and phorbol ester had additive effects on ANP mRNA levels. This data is consistent with a model in which sACT levels are regulated selectively by compartmentalized calcium that is coupled to the ß-adrenoceptor via Gs. The induction appears to be largely transcriptional, since isoproterenol induced expression from the transfected sACT promoter, while mRNA half-life studies did not demonstrate any prolongation of sACT mRNA stability from a mean $t_{1/2}$ of 12 hours. The calmodulin inhibitors W7 (10 µM) and chlorpromazine (1 µM) and the protein kinase C inhibitor staurosporine (100 nM) failed to inhibit ß-adrenergic induction of sACT. The protein kinase A selective inhibitor KT5720, while strongly inhibiting Bt_2cAMP stimulation of proenkephalin mRNA, potentiated the ß-adrenergic induction of sACT in a dose-dependent manner. These data suggest that the calcium-dependent induction of sACT does not require activation of these protein kinases, but in fact PK-A may participate in suppression of this pathway. Finally, the calcium sensitizer EMD53998 (20 µM) induced expression of sACT mRNA to the same degree as isoproterenol, while pure phosphodiesterase inhibitors milrinone (100 µM) and theophylline (1 mM) had no effect. Differential response of ANP and sACT mRNAs to these stimuli suggests that the apparently unitary pattern of fetal/embryonic gene re-induction during myocardial hypertrophy may involve multiple signal transduction pathways.

A NEW SERUM RESPONSIVE CARDIAC TISSUE SPECIFIC FACTOR THAT RECOGNIZES THE MEF-2 SITE IN MYOSIN LIGHT CHAIN-2 PROMOTER, Ming-Dong Zhou, Shyamal K. Goswami, Mary Ellen Martin, and M.A.Q. Siddiqui, Department of Anatomy and Cell Biology, SUNY Health Science Center at Brooklyn, Brooklyn, NY 11203

A new serum responsive, cardiac-specific transcription factor, that recognizes an A/T rich sequence (element B), identical to the myocyte enhancer factor (MEF-2) target site, in cardiac myosin light chain-2 (MLC-2) promoter was identified. Deletion of element B (MEF-2-site) sequence alone from the cardiac MLC-2 promoter causes a marked reduction of its transcription. BBF-1 is distinguishable from cardiac MEF-2 by its inability to cross react with an antibody which recognizes MEF-2. Unlike MEF-2, BBF-1 is present exclusively in cardiac tissue and cardiac muscle cells cultured in medium containing high concentration of fetal calf serum. Deprivation of serum in culture medium abolishes BBF-1 activity selectively with a concomitant loss of the positive regulatory effect of element B on MLC-2 gene transcription. The loss of element B-mediated activation of MLC-2 promoter can be reversed by re-feeding cells with serum containing medium. These data, therefore, demonstrate that cardiac muscle cells contain two distinct protein factors, MEF-2 and BBF-1, which bind to the same target site. However, BBF-1 is serum-inducible and cardiac tissue specific, and thus, appears to be a crucial member of the MEF-2 family proteins which can serve as an important tool in understanding the regulatory mechanism(s) underlying cardiogenic differentiation.

ASSEMBLY OF TROPOMYOSIN INTO MICROFILAMENTS IN CULTURED CELLS, T.J. L'Ecuyer, J. Noller, and A.B. Fulton, Departments of Pediatrics and Biochemistry, University of Iowa School of Medicine, Iowa City Iowa, 52242.

Tropomyosin (TM) is a component of microfilaments of most eukaryotic cells; its function is best understood in striated muscle, where, in conjunction with the troponin complex, it regulates the actin-myosin interaction in a calcium-specific manner. Its role in non-muscle and smooth muscle cells is not known, but likely is a structural one. Cultured primary chick cells were used to study assembly of TM into microfilaments of embryonic cardiac and skeletal muscle. Pulse-chase experiments with 35-S methionine were performed, with puromycin included in the chase medium to cause release of nascent polypeptides from ribosomes. Labeled cells were fractionated into soluble and skeletal fractions with Triton, and TM was immunoprecipitated from each fraction with monoclonal antibodies. After electrophoresis of the immunoprecipitates, autoradiography was used to identify the TM region of the gels, which was excised and radioactivity counted. Kinetics of assembly of full-length TM is most consistent with high levels of co-translational assembly in both striated muscle types. Excision of regions of gels beneath full-length TM at pulse and chase times localizes nascent TM chains to the cytoskeleton, confirming extensive co-translational assembly for striated muscle TM isoforms. Pulse labeling with 3-H puromycin, followed by cell fractionation and immunoprecipitation of TM from soluble and skeletal fractions, confirms nascent TM chain association with the cytoskeleton, independent of ribosomes. Preliminary evidence suggests that the degree of co-translational assembly of non-muscle TM isoforms in cultured fibroblasts is significantly less than that of striated muscle TM isoforms, suggesting that the mechanism of assembly of TM is isoform or cell-type specific.

COOPERATION BETWEEN THE PROXIMAL E BOX WITH THE DISTAL E BOX AND MEF_2 ENHANCER ELEMENTS IS REQUIRED FOR HIGH LEVEL OF DESMIN GENE EXPRESSION, Hui Li and Yassemi Capetanaki, Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030, USA.

Desmin, the muscle specific intermediate filament protein, encoded by a single gene, is one of the earliest myogenic markers expressed in all types of muscle including cardiac, skeletal and smooth. It is expressed at low level in myoblasts and increases several fold upon differentiation. Transient transfections of C_2C_{12} muslce cells with various 5' flank deletion mutants fused to the bacterial chloramphenicol acetyltransferase (CAT) gene have revealed both positive and negative elements that seem to be involved in the regulation of desmin expression. The first 81 bp upstream of the transcription initiation site which includes an E box (E1), a myoD binding site, were sufficient to confer muscle specific expression of the desmin gene. The maximal level of expression, however, is due to a 180 bp long enhancer located between nucleotides -798 and -976. This enhancer region contains another E-box (E2) and one potential muscle-specific enhancer factor MEF-2 site at -832 and -864 respectively. Gel mobility shift assays with nuclear extracts from both myoblasts and myotubes showed that both myoD and myogenin can bind to the proximal E1 and distal E2 boxes, and other proteins, most possibly MEF2, bind to the MEF2 site. To understand the in vivo functional significance of these binding sites in desmin gene expression, site-directed mutagenesis was utilized to mutate E1, E2 and MEF-2 respectively and in combination. Mutations at E2 and MEF-2 sites showed that the activity was reduced only 20% in both myoblast and myotube stage. Double mutation of E2 and MEF-2, however, caused the CAT activity to drop 8-fold. This suggested that the desmin enhancer can equally well function with either the E2 box or MEF2 box and mutation at both of them elimilates transcriptional enhancement. On the other hand, mutagenesis of the proximal E1 box showed that this element is essential for desmin gene expression both in myoblast and myotubes. Furthermore, double mutation of E1 with E2 or MEF2 sites suggested that for high expression of the desmin gene, E1 seems to serve as a bridge for E2 or MEF-2 enhancer element to function.

TISSUE SPECIFIC EXPRESSION OF CARDIAC MYOSIN LIGHT CHAIN 2: MOLECULAR PARADIGM OF TRANSCRIPTIONAL REPRESSION, Manya Dhar, Eduardo Mascareno, Shyamal Goswami, and M.A.Q. Siddiqui, Department of Anatomy and Cell Biology, SUNY Health Science Center at Brooklyn, Brooklyn, NY 11203

We have recently reported that the tissue specific expression of chicken cardiac myosin light chain-2 (MLC2) gene is mediated by a negative cis-acting sequence element (CSS) in the proximal promoter and its binding proteins (CSFs). The removal of CSS sequence (-349 to -282) from MLC2 promoter relieves the repression of cardiac MLC2 transcription in skeletal muscle cells. The effect of CSS on heterologous promoters was examined by introducing the CSS element in muscle creatine kinase (MCK) and, non-muscle angiotensinogen (ANG) gene promoters. CSS when placed at -246 bp (MCK246CSSCAT) in the MCK promoter caused a 90% inhibition of the promoter activity but was ineffective when introduced at -2.5 kb, suggesting that CSS-mediated repression of transcription is position-dependent. Likewise, the CSS-ANG promoter recombinants repressed transcription in a position-dependent manner. The CSS-binding nuclear proteins from cardiac and skeletal muscle tissues showed qualitative differences when compared by gel mobility shift assay. A DNA/protein complex (CSF-1) was formed with adult skeletal muscle nuclear extract, but not with cardiac extracts. In stretch-induced hypertrophied skeletal muscle (ALD), which promotes the cardiac MLC2 gene expression, there was a progressive change in the CSF binding pattern similar to that of cardiac muscle pattern. There was also an increase in an activator nuclear protein (IRE) in the hypertrophied ALD muscle compared with the control ALD. Thus, the modulation in CSS and IRE specific transcription factors in cardiac and skeletal muscles are consistent with the expression pattern of cardiac MLC2 in different pathophysiological conditions.

INDEPENDENT ROLES OF PAIRED E BOXES.
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Despite the shared expression of numerous contractile protein genes in cardiac and skeletal muscle, there is little information indicating whether cis-acting elements are similarly utilized by trans-acting factors in these two types of striated muscle. Although skeletal muscle cells differentiate in the presence of a family of cell restricted transcription factors that share a basic-helix-loop-helix (bHLH) protein motif, extensive *in situ* analysis indicates that these factors are not detected in cardiac anlage at any stage. During development, cardiac actin is initially expressed in both cardiac and skeletal muscle precursors and is later down-regulated in skeletal tissue while remaining at relatively high levels in the heart. We therefore anticipated differences in the levels of expression of the cardiac actin promoter in heart and skeletal primary tissue culture systems. A mutational analysis of the chick cardiac actin promoter element that contains paired bHLH binding sites (E boxes) in these two cell types indicates that these E boxes are utilized differently in skeletal v. heart cell cultures. Although paired E boxes appear to cooperate in the expression of the muscle creatine kinase enhancer, we found that the cardiac actin E boxes acquired independent roles in skeletal v. cardiac primary cultures. Only the proximal site was capable of binding *in vitro* expressed bHLH proteins. In addition, gel shifts using nuclear extracts from these two sources reveal the binding of distinct complexes to the cardiac actin E box element as determined by competition as well as antibody supershift analysis.

ISOLATION AND DEVELOPMENTAL EXPRESSION OF GENES HIGHLY EXPRESSED IN VASCULAR SMOOTH MUSCLE CELLS, Catherine M. Shanahan, Peter L. Weissberg* and James C. Metcalfe, Department of Biochemistry and *Clinical School, University of Cambridge, Cambridge, UK.

Smooth muscle is a major cellular component of a variety of tissues including bladder, vas deferens, uterus, the alimentary tract and the vasculature. Smooth muscle cells from different developmental lineages and cellular environments exhibit differential gene expression and proliferative capacity. Additionally, within vascular smooth muscle tissue there are large changes in protein content during foetal/neonatal development while in the adult, the protein content of smooth muscle cells from different regions of the vessel (media versus intima) are not equivalent. It has been postulated that cells that are contractile in the vessel media are differentiated while those undergoing proliferation in the intima are de-differentiated as they lose protein markers indicative of the contractile phenotype. The de-differentiated state is similar to that of foetal/neonatal vascular smooth muscle cells (VSMCs) which has led to speculation that the cells that proliferate either undergo phenotypic modulation and take on neonatal-like characteristics or indeed are remnant cells retaining neonatal properties that move into the adult intima following stimulation to proliferate.
We have utilized a rat tissue culture model of VSMC de-differentiation and differential screening of a rat cDNA library to isolate genes highly expressed in differentiated and proliferating VSMCs. Three classes of genes showing differential regulation in tissue culture were isolated: 1) genes highly expressed in aortic cells that had not undergone proliferation in culture, 2) genes highly expressed in cells that had not been passaged in culture and 3) genes highly expressed in VSMCs that had undergone proliferation. The expression of these genes during neonatal aortic development was studied with the aim of identifying specific differences in the expression patterns between adult and neonatal VSMC phenotypes and to test the hypothesis that intimal and neonatal cells have similar properties. Additionally the tissue specific expression patterns of these genes revealed substantial heterogeneity in gene expression between smooth muscle cells derived from different tissues both in the adult and during development.

INDUCTION OF IMMEDIATE-EARLY GENES AND HYPERTROPHIC MARKER TRANSCRIPTS BY HYPOXIA-REOXYGENATION CYCLES IN RODENT CARDIAC MYOCYTES, Keith A Webster, Daryl J. Discher, Barbara Sato, Ilona Bodi and Nanette H. Bishopric, Department of Cell and Molecular Biology, SRI International, Menlo Park, CA 94025.
Neonatal rat cardiac myocytes that were contracting spontaneously at an initial rate of > 250 beats per min were subjected to repeated cycles of 4h hypoxia and 2h re-oxygenation under conditions of limiting glucose and mild acidosis to simulate an ischemia-reperfusion-like state. Loss of contractility during hypoxia was gradual and progressive, becoming complete within 4h during the first cycle and more rapidly during subsequent cycles, but was fully reversible on re-oxygenation. Release of pre-loaded [^{3}H]-arachidonic acid confirmed that the cells were not irreversibly damaged by hypoxia. During each of the hypoxic periods the transcript levels of the immediate early genes *c-fos*, *c-jun*, *jun-B* and *jun-D* increased by 5 to 10 fold and declined during re-oxygenation. Immunostaining using anti-Fos and anti-Jun antibodies demonstrated accumulations of these proteins in the nuclei of hypoxic myocytes at levels equal to those induced by the protein kinase-C activating phorbol ester, PMA. Gel mobility shift assays using nuclear extract from aerobic and hypoxic cultures demonstrated several fold enhanced levels of protein binding to an oligonucleotide containing the AP-1 consensus sequence with the hypoxic cell extract. These studies suggest that AP-1 proteins are induced in response to the hypoxia-reoxygenation cycles. To determine whether the elevated AP-1 proteins were functional cardiocytes were transfected with a chloramphenicol acetyltransferase expression plasmid linked to -73 to +63 of the collagenase promoter, which contains an intact AP1 binding site. The level of CAT expression in cells subjected to one cycle of hypoxia was elevated by 2 to 3 fold compared with aerobic cultures. A construct truncated 5' to -60 of the collagenase promoter, lacking the AP-1 site, was not induced. Finally the transcript levels of the skeletal α-actin and atrial natiuretic peptide genes, both of which are positively regulated by AP-1 and are induced early during myocardial hypertrophy *in vivo* and *in vitro* in the rat were measured by Northern analyses in RNA from aerobic and hypoxia-cycled cells. Elevated expression of both transcripts followed hypoxia-reoxygenation cycles which supports our proposal that AP-1 can be activated by hypoxic or ischemic stress. To conclude we provide evidence that hypoxic stress may augment or even pre-empt previously described factors such as work overload, stretch, and neurohormonal stimuli in providing the early signals that trigger compensatory myocardial cell hypertrophy.

CELLULAR BASIS FOR THE NEGATIVE INOTROPIC EFFECTS OF TUMOR NECROSIS FACTOR-ALPHA IN THE ADULT HEART.

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Recent studies have suggested that certain pro-inflammatory cytokines may play a pathogenetic role in cardiac disease states. Tumor necrosis factor-alpha (TNFα) is a pro-inflammatory cytokine which has been implicated in a variety of cardiac pathophysiological conditions including, septic shock, acute viral myocarditis, cardiac allograft rejection, subacute myocardial infarction, and congestive heart failure. However, the exact mechanism(s) for any potential TNFα-induced effects in the intact heart is now known. Moreover, given the complex admixture of cell types in the heart, each of which may respond differently to the pleiotropic effects of TNFα, it has not been possible to determine whether TNFα has a direct effect on the cardiac myocyte itself, or whether instead this cytokine exerts its effect(s) indirectly, by releasing one or more soluble factors from non-myocyte cell types residing within the myocardium. Accordingly, the purpose of this study was to systematically examine the functional effects of TNFα *in vitro* at both the tissue and cellular levels. Studies in the intact ventricle showed that TNFα exerted a concentration and time-dependent negative inotropic effect that was fully reversible upon removal of this cytokine, thus obviating a decline in contractile performance of the experimental model or a cytokine-induced effect on cardiac myocyte viability. Studies in isolated myocytes also demonstrated the TNFα exerted a concentration and time-dependent negative inotropic effect which was reversible upon removal of the cytokine from the superfusate, thus providing straightforward evidence that TNFα exerts its effects directly at the level of the adult cardiac myocyte, and not necessarily indirectly by releasing soluble factor(s) from non-myocyte cell types residing within the myocardium. A mechanistic basis for the negative inotropic effects of TNFα was provided by the studies which showed that treatment with TNFα altered the levels of peak intracellular calcium curing cardiocyte contraction. Moreover, this TNFα-induced defect in cell shortening was not apparent during tetanization of isolated cardiac myocytes. Further studies showed that increased levels of nitric oxide, *de novo* protein synthesis, and metabolites of the arachidonic acid pathway were unlikely to be responsible for the TNFα-induced abnormalities in contractile function. Thus, these studies constitute the initial demonstration that the negative inotropic effects of TNFα are the direct result of alterations in intracellular calcium homeostasis in the adult cardiac myocyte.