

## Ossabaw Pig – A Model for Studying Alzheimer’s Disease in Type 2 Diabetic Patients

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The rising obesity epidemic has led to an increase in type 2 diabetes, which is characterized by insulin resistance and can cause hyperinsulinemia. Hyperinsulinemia, defined by excess insulin levels, is also regarded as a possible risk factor for Alzheimer’s disease, leading us to suspect a close relationship between type 2 diabetes and Alzheimer’s disease. Furthermore, insulin contributes to A Beta accumulation by competing for insulin degrading enzyme (IDE), which degrades both insulin and A Beta. Thus a high level of insulin in the brain may lead to a build up of A Beta and the onset of the amyloid plaques and neurofibrillary tangles characteristic of Alzheimer’s disease. To date, this hypothesis remains to be tested in valid animal models. Ossabaw pigs have “thrifty” phenotype, which allows them to store large amounts of fat during feasting and then survive periods of famine. Interestingly, obese Ossabaw pigs resemble the human “metabolic syndrome”, also known as “pre-diabetes”. We hope to show that this Alzheimer’s pathology is present in Ossabaw pigs expressing metabolic syndrome, thus linking type 2 diabetes and Alzheimer’s disease. These pigs, which were fed a high fat diet for 58.6 weeks, showed hyperinsulinemia and weighed twice as much as their control fed counterparts. Because of this, these pigs provide an excellent model for studying the molecular mechanisms and potential therapeutic intervention for Alzheimer’s Disease in type 2 diabetic patients.

## Determining How Hypoxia and Specific Substrate Deficiencies Limit Fetal Growth via a Chronically Catherized Fetal Sheep Model for Uterine Blood Flow Reduction

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Intrauterine Growth Restriction (IUGR) is a significant cause of perinatal morbidity and mortality. We are attempting to discover the molecular mechanisms through which inadequate oxygen and nutrient supply to the fetus modifies fetal metabolism and protein translation initiation in ways that ensure fetal survival while sacrificing growth. The hypothesis is that oxygen, glucose, and amino acids are important in regulating the rate of growth and protein accretion in the fetus, but have differing degrees of importance relative to one another upon selective deprivation. Surgery was performed on 12 pregnant ewes in late gestation involving placement of a vascular occluder, a crown-rump measuring device, and catheters in both the ewe and fetus through which blood samples were drawn and infusates were transfused throughout the study. Ewes were divided into four groups: controls, occluded, occluded plus maternal oxygen and glucose, and occluded plus maternal oxygen and amino acids. On study day 7, baseline metabolic studies were done on all animals. Experimental groups underwent uterine blood flow reduction and infusates beginning on study day 8, with the fetuses then remaining hypoxic for ~7 days. Data derived from the studies show that occluded animals had a 35% greater reduction in linear growth rate over control animals, as well as a significant reduction in fetal arterial oxygen content ( $p= 0.003$ ) and skeletal muscle protein fractional synthetic rate ( $p= 0.02$ ). Western blotting revealed that occluded animals' translation initiation factor 4EBP1 was hypophosphorylated, causing it to stay bound to eIF4E and thereby inhibit protein translation. The groups with supplements afforded numbers lower than the controls, but not significantly so. Our preliminary data support the hypothesis that supplementing oxygen, glucose, and amino acids can help avoid IUGR, but more data will be needed to determine which factor is most important and elucidate its pathway of action.

**CARDIOMYOGENIC DIFFERENTIATION OF MURINE SKELETAL MUSCLE-DERIVED COMMON PLURIPOTENT STEM CELLS.** Darryl Finkton, Neelima Sanakkayala, Tamara Horvath, Edward F. Srouf. Department of Pediatrics, Section of Hematology/Oncology, Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, Cancer Research Institute, 1044 W. Walnut, Indianapolis IN 46202

There is evidence that adult stem cells exhibit high levels of plasticity, enabling them to differentiate into multiple cell lineages. Our laboratory has previously demonstrated that a phenotypically identical common pluripotent stem cell (CoPSC) may exist in multiple adult tissues. We also established that CoPSC from readily available skeletal muscle (SM) are capable of differentiating into other cell types. It remains to be determined however if SM-derived CoPSC have the potential to differentiate into cardiomyocytes. Skeletal muscle and cardiac cells from 8-week old C57BL/6 mice expressing the phenotype Sca-1<sup>+</sup>/ CD45<sup>-</sup>/ c-kit<sup>-</sup>/ CD90<sup>+</sup> (CoPSC) were isolated by flow cytometric cell sorting along with Sca-1<sup>-</sup>/ CD45<sup>-</sup>/ c-kit<sup>-</sup>/ CD90<sup>-</sup> control cells. Samples of freshly isolated CoPSC and control cells were used to determine the baseline expression of cardiac-specific genes. Both CoPSC and control cells from SM and cardiac tissues were cultured in multilineage adult progenitor cell (MAPC) media containing 10 ng/mL of EGF, PDGF, and LIF to promote undifferentiated expansion of the cells or in cardiomyogenic differentiation medium consisting of DMEM plus 10% FBS plus 1% Pen Strep, 1µg/mL insulin and 10 ng/mL IGF1. On day 1, SM-derived CoPSC and control cells did not express any of the 4 cardiomyogenic specific markers gata4, nkx2.5, mef2c, and myl2. In contrast, cardiac CoPSC expressed all 4 genes except myl2. After 8 days of culture in MAPC medium, skeletal muscle-derived CoPSC expressed mef2c only whereas under cardiomyogenic conditions, these cells expressed all 4 genes. In addition, SM-derived CoPSC cultured under cardiomyogenic conditions acquired cardiomyogenic morphological characteristics. These results suggest that, under appropriate conditions, SM-derived CoPSC may differentiate into morphologically cardiomyogenic-like cells expressing cardiomyogenic-specific molecular differentiation markers. These results suggest that in response to specific differentiation signals, it may be possible to differentiate skeletal muscle-derived CoPSC into potentially functional cardiomyocytes.

## Role of T-bet in the Generation of Pulmonary Atopic Inflammation

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Interleukin-17 (IL-17) has been implicated in the pathology of asthma, suggesting a possible role for IL-17-secreting T helper cells (Th17) as effectors in allergic airway inflammation. Development of Th17 cells is negatively regulated by the transcription factor T-box expressed in T cells (T-bet), which also promotes the production of interferon-gamma (IFN- $\gamma$ ). Increased incidence of airway hyperresponsiveness has been reported in T-bet-deficient mice on mixed and BALB/c backgrounds, a phenomenon that could result from the lack of suppression of Th17 development. To test this hypothesis, T-bet-deficient and wild type mice on the C57BL/6 background were sensitized to induce atopic pulmonary reactivity and analyzed for production of IL-17, the Th1 cytokine IFN- $\gamma$ , and the Th2 cytokines IL-4, IL-5, and IL-13. T-bet-deficient mice displayed increased IL-17 production in comparison to wild type mice but had depressed severity of several inflammatory parameters. The histopathology of the lung tissue and cellular populations of bronchoalveolar lavage fluid were examined and revealed a decreased incidence of eosinophil infiltration in T-bet-deficient mice compared to wild type mice. A concomitant reduction in serum IgE levels in T-bet-deficient mice as compared to wild type mice also was observed. These results suggest that the effects of T-bet deficiency on the development of pulmonary infiltration are dependent on the genetic background of the mice. Furthermore, these results showed that increased development of IL-17-secreting cells is not sufficient to generate airway infiltration.

## **ELF-3 and ELF-1 uterine expression in normal and abnormal mouse models of parturition.**

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The Ets family of transcription factors regulates genes involved in cellular growth and differentiation. Ets-like factor-3 (ELF-3) is an Ets transcription factor that is involved in the regulation of cyclooxygenase-1 (COX-1) expression in vitro. COX-1 is critical for normally timed parturition in mice (19.5 days). ELF-3 and COX-1 are both regulated near the end of pregnancy in the mouse endometrium. We hypothesized that ELF-3 also up-regulates COX-1 in vivo. COX-1 knockout mice deliver their pups two days late at day 21.5 but ELF-3-knockout mice deliver normally (19.5 days). Based on this evidence, we also hypothesized that the regulation of COX-1 is being mediated by another factor in the absence of ELF-3. Preliminary data suggested that ELF-1, another Ets transcription factor, was up-regulated in the ELF-3 knockout mouse. We hypothesized that in the absence of ELF-3, ELF-1 regulates COX-1. We examined the RNA and protein levels of ELF-3, COX-1, and, ELF-1 in gravid uterus tissue of wild-type, COX-1 knockout, and ELF-3 knockout mice. We found that ELF-3 protein expression is decreased 41% in the COX-1 knockout mice when compared to the wild-type ( $p=0.050$ ). ELF-1 RNA expression in ELF-3 knockout mice showed a 49% increase over WT expression, but this was not statistically significant ( $p=0.075$ ). In addition, ELF-1 RNA levels increased 96% in COX-1 knockout mice, showing statistical significance ( $p=0.046$ ) when compared to the WT. COX-1 levels in ELF-3 knockout mice were not significantly different from WT mice at day 19.0 of pregnancy in both protein and RNA analysis. Our data suggests that ELF-3 plays a role in normal mouse parturition but is not critical. Our findings are also consistent with the possibility that ELF-1 can act as a replacement for ELF-3.

## Purification and Inhibition of Recombinant Vaccinia Virus H1 Protein

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Variola, a member of the poxvirus family is the causative agent of smallpox and has long been recognized has a potential threat in biowarfare. Vaccinia virus is a highly related poxvirus and is used as the vaccine for smallpox. Since smallpox has been largely eradicated worldwide, infants and adults are no longer routinely immunized. Both Variola and Vaccinia employ a number of immune evasion mechanisms including blocking interferon (IFN) signaling that is required for optimal viral clearance. VH1 is a Vaccinia encoded phosphatase that is important for the viral life cycle and disrupts IFN signaling in part by dephosphorylating tyrosine residues of STAT proteins. Previous attempts to purify recombinant VH1 have yielded inactive enzyme. We have employed an altered protocol in the attempt to obtain purified active enzyme that can be used for in vitro assays. We also tested the efficacy of phosphatase inhibitors in an assay for VH1 activity using recombinant protein bound to the purification resin with the rationale that inhibition of the VH1 phosphatase could be a treatment for poxvirus infection. We tested various inhibitors using the *p*-Nitrophenyl Phosphate (pNPP) colorimetric assay. Six inhibitors of known phosphatase targets were tested. Significant inhibition was only observed with a broad spectrum inhibitor. An inhibitor of the related mammalian kinase VHR did not have an effect. Purified VH1 will be tested with additional inhibitors and used for additional in vitro assays.

Phosphoinositide-3-kinase (PI3K) plays a critical role in mesenchymal stem cell differentiation

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Mesenchymal stem cells (MSCs) are pluripotent progenitor cells which present within the bone marrow cavity. MSCs exhibit self-renewal and multilineage differentiation capacity and serve as a reservoir for the continuous renewal of various mesenchymal tissues, including adipose tissue, bone, cartilage, tendon, and muscle. In addition, MSCs provide supportive microenvironment for growth, differentiation, and proper function of the hematopoietic stem cells and their progeny. However, little is known about the molecular basis of MSC differentiation. Recently, utilizing 2T3 cell line, Ghosh-Choudhury et al. indicate that PI3-K is important for MSC differentiation into osteoblasts. In the present study, utilizing genetic approach, we investigate the role of PI3-K in mediating osteoblast differentiation from MSCs *in vivo* and *in vitro* using *p85 $\alpha$*  deficient (-/-) mice, which lack the regulatory subunit of PI3-K. Bone marrow mononuclear cells were harvested from wildtype (WT) and *p85 $\alpha$*  -/- mice. Following 6 weeks of culture in a specialized MSC culture media, the cells were phenotypically analyzed and confirmed as MSCs. The purified MSCs were then stimulated with insulin-like growth factor-1 (IGF-1), a known osteoblastic stimulating factor. Despite the increased [<sup>3</sup>H]thymidine incorporation in *p85 $\alpha$*  -/- MSCs as compared to WT cells, a dramatic reduction of osteoblast differentiation was observed in *p85 $\alpha$*  -/- MSCs as compared to that in WT cells. To confirm the reduced osteoblast differentiation is the consequence of deletion of the regulatory subunit of PI3-K, we added PI3-K inhibitor, Ly294002, and osteoblast differentiation was repeated. Addition of Ly294002, but not PD98059 (a MEK inhibitor) was sufficient to block the osteoblast differentiation from MSCs. Our study provides strong evidence that PI3-K is critical for osteoblast differentiation from MSCs.

267 word count.

## The Effects of Hyperglycemia on Newborn Endothelial Progenitor Cells

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Individuals with diabetes are at higher risk of developing vascular diseases. Endothelial progenitor cells (EPCs) have been shown to have a role in endothelial repair and angiogenesis. We hypothesize that hyperglycemia induced damage to EPCs may reduce the ability of a vascular system to regenerate and repair itself, and therefore lead to vascular disease. The aims of my studies were to test the proliferative potential of EPCs treated under hyperglycemic conditions; to determine whether hyperglycemia induces apoptosis and/or senescence of EPCs; and to examine if hyperglycemia impacts tube forming capabilities of EPCs. I found that the proliferative potential of EPCs decreased with increased dosages of dextrose as determined by a population doubling assay. To further examine the effect of hyperglycemia on the proliferative capacity of EPCs, I had single EPCs sorted into each well of a 96 well tissue culture dish using flow cytometry and quantitated the number of single cells that were high-proliferative potential endothelial colony forming cells (HPP-ECFCs), an EPCs that forms colonies containing >2000 cells. These studies demonstrated that dextrose treatment significantly reduced the number of HPP-ECFCs compared to untreated controls. Given these data, we questioned whether hyperglycemia would also increase the proportion of EPCs undergoing apoptosis and/or senescence. My data showed that EPCs treated with dextrose exhibited increased rates of senescence and increased rates of apoptosis. To determine if the functional capacity of EPCs was altered by hyperglycemia, I measured the tube forming ability of hyperglycemia treated EPCs. My data showed that EPCs exhibited decreased tube forming capabilities in response to dextrose treatment as compared to euglycemic controls. This study provides insight into how diabetes affects the functionality of EPCs and the health of a vascular system.

Joal Beane

Wildtype c-kit activation by its ligand, stem cell factor, results in the recruitment of phosphoinositol 3-kinase (PI3K) via p85 $\alpha$  regulatory subunit. Upon binding to c-kitY719 a conformational change occurs in PI3K whereby it acquires its catalytic activity necessary for normal growth and proliferation. Previous research has demonstrated that mast cells display decreased proliferation in the absence of p85 $\alpha$ . In order to elucidate which domains of p85 $\alpha$  are important for its function in mast cell proliferation, we transduce mast cells taken from p85 $\alpha$  knockout mice with various p85 $\alpha$  chimeras and perform proliferation assays. In this study we support previous findings that loss of p85 $\alpha$  decreases proliferation and demonstrate that the n-terminus of p85 $\alpha$  plays a pivotal role in this observation. This study further implicates p85 $\alpha$  as a possible drug target for the treatment of mastocytosis, acute myeloid leukemia (AML), and other diseases caused by activation mutations of c-kit.

## **The effect of cryo-preservation on the production of endothelial colony forming cells from human umbilical cord blood CD34<sup>+</sup> stem cells.**

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The process of cryo-preserving purified stem cell isolations has been thought to disrupt the ability of endothelial progenitor cells (EPCs) to form colonies when plated in endothelial colony forming cell (ECFC) cultures following the thawing process. CD34 is a known EPC marker and we isolated CD34<sup>+</sup> cells from CB utilizing the magnetic cell sorting (MACS) system. We hypothesized that frozen CD34<sup>+</sup> cells would form only a slightly lower number of ECFCs as compared to freshly isolated CD34<sup>+</sup> cells. By demonstrating colony forming ability of frozen human umbilical cord blood (CB) CD34<sup>+</sup> cells, we could then freeze down CD34<sup>+</sup> cells until there are enough to pool and sort for rarer cell sub populations that express several EPC markers. We compared the frequency of ECFC's in freshly isolated CD34<sup>+</sup> cells to that of frozen and thawed CD34<sup>+</sup> cells. Mononuclear cells (MNCs) from each sample were used as positive controls. The same number of cells were plated both pre and post freeze in order to provide an accurate comparison. After the CB MNCs and CD34<sup>+</sup> cells were plated in complete endothelial growth media (cEGM-2), colony counts and morphology were checked daily. Our data demonstrated that while in most instances the number of endothelial colonies formed from the frozen CD34<sup>+</sup> cells were lower than that observed in the freshly isolated CD34<sup>+</sup> cell cultures, a substantial number of endothelial colonies can be grown from frozen CD34<sup>+</sup> stem cells (SCs). In some instances the morphology of the colonies from the frozen CD34<sup>+</sup> SCs was abnormal, but most colonies retained a normal morphology post freezing. Flow cytometric analysis pre and post freezing showed that the CD34<sup>+</sup> purity was actually enhanced by the freezing process, although this enhanced purity did not lead to increased colony formation. Only 40%-68% of the ECFCs from the frozen CD34<sup>+</sup> cells were able to be recovered compared to that of freshly isolated CD34<sup>+</sup> cells.

## **Isolating Endothelial Colony Forming Cells by MACS Separation of CD105<sup>+</sup> and CD146<sup>+</sup> Cells in Cord Blood and Bone Marrow**

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Currently, endothelial colony forming cells (ECFCs) are identified *in vitro* by culturing mononuclear cells (MNC) isolated from either adult peripheral blood (PB) or human umbilical cord blood (CB). We are attempting to show that ECFCs can be identified by fluorescence activated cell sorting (FACS) utilizing the cell surface antigens CD105 and CD146, two markers commonly expressed by mature endothelial cells. This would provide a simple, one step method of obtaining the frequency of ECFCs within CB or bone marrow (BM), which could serve as a possible biomarker for vascular disorders. Utilizing the Magnetic Cell Sorting (MACS) system, CB MNC were CD45 depleted to prevent hematopoietic cell contamination. The CD45<sup>-</sup> cells were then either CD105 or CD146 enriched with the MACS system. The positive cell fractions were then plated on collagen coated 12 well plates in complete endothelial growth media (cEGM-2). Both the CD105<sup>+</sup> and the CD146<sup>+</sup> cultures yielded ECFC colonies, showing that these markers are in fact present on endothelial progenitor cells. However, the frequency of these colonies was greatly decreased when compared to the frequency of colonies in a control of MNC, showing that the method of culturing cells to obtain the true frequency of ECFCs in CB is inefficient. In addition, BM MNC were also CD45 depleted and subsequently CD105 or CD146 enriched. Neither the BM CD105<sup>+</sup> nor CD146<sup>+</sup> cultures produced ECFC colonies, but instead both produced individual mesenchymal cells as well as mesenchymal colonies. This data implies that ECFCs do not reside in the BM. Further studies may lead to more accurate frequency readings, as well as the true location of ECFCs in the bone.

# **The Relative Roles of the Platelet Activating Factor Receptor and Peroxisome Proliferator Activated Receptor- $\gamma$ in UVB-mediated Prostaglandin E<sub>2</sub> Formation**

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Cyclooxygenase-2 (COX-2) and prostaglandins exert important biological effects on human skin, and have been proposed to be involved in ultraviolet B (UVB) radiation-induced immunosuppression, cytokine production, and photocarcinogenesis. Previous studies demonstrated that activation of the skin platelet activating factor receptor (PAF-R) induces the COX-2 gene expression and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production. Likewise, the activation of epidermal peroxisome proliferator activated receptor  $\gamma$  (PPAR- $\gamma$ ) upregulates expression of COX-2 and PGE<sub>2</sub>. It has been identified that oxidative stressors, including UVB, can generate oxidized glycerophosphocholines that can act as either PAF-R or PPAR $\gamma$  agonists. The present study was designed to determine the relative roles of the PAF-R and PPAR- $\gamma$  in the mechanism for UVB-induced COX-2 expression and PGE<sub>2</sub> production using the immortalized human keratinocyte cell line n-TERT. First, it was shown that the production of PGE<sub>2</sub> in n-TERT cells was significantly increased via the activation of PAF-R or PPAR- $\gamma$  by administration of the specific PAF-R agonist, CPAF, or PPAR $\gamma$  agonist, ciglitazone, respectively. This augmentation of PGE<sub>2</sub> production was inhibited by pre-treatment with specific PAF-R antagonist, WEB2086, or PPAR $\gamma$  antagonist, GW9662. Then, we investigated the relative roles of PAF-R and PPAR- $\gamma$  in UVB irradiation-induced PGE<sub>2</sub> production. It was shown that the PPAR- $\gamma$  antagonist, GW9662, significantly reduced the UVB-induced PGE<sub>2</sub> production in n-TERT cells, while the PAF-R antagonist, WEB2086, revealed lesser inhibitory effects. These studies indicate that the activation of PAF-R or PPAR- $\gamma$  could upregulate PGE<sub>2</sub> production. This study also exhibits that the PPAR- $\gamma$  pathway is the primary mechanism for UVB-mediated PGE<sub>2</sub> formation and COX-2 gene expression.

Effect of human apurinic/aprimidinic endonuclease DNA base excision repair enzyme/redox factor (APE1/Ref-1) on NT2 GCT (germ cell tumor) cells after exposure to cis-Diamminedichloroplatinum.

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APE1 is a base excision DNA repair enzyme that is involved in the repair of abasic sites in DNA, and has two functional domains, a repair domain and a redox domain. Certain high risk patients with germ cell tumors (GCT's) have been shown to have heightened levels of apurinic/aprimidinic (AP) endonuclease (APE1). Previous experiments have shown that GCT cells which over-express APE1 are resistant to bleomycin and radiation cancer therapy. In the present study we examined cis-Diamminedichloroplatinum (cisplatin), which is used widely in the treatment of GCT's. We hypothesized that over-expression of APE1 in GCT cells would render these cells resistant to cisplatin as was observed with bleomycin and radiation. Although over-expression of APE1 was expected to provide a degree of protection, MTS and LDH assays demonstrated that NT2 cells over-expressing APE1 actually became more sensitive to cisplatin exposure, showing enhanced cytotoxicity. To determine whether the redox domain of APE1 was important in the sensitivity of GCT cells to cisplatin, we over-expressed the redox domain-mutated C65 APE1, which rendered the cells' redox function ineffective, in NT2 cells and examined their response to cisplatin. MTS experiments showed these cells experiencing decreased cell killing in response to cisplatin compared to wild type APE1 and empty vector transduced NT2 cells, which would suggest that the redox domain plays a role in cisplatin cytotoxicity. We conclude that 1) APE1 sensitizes GCT cells to cisplatin and 2) that this effect appears to involve a redox based mechanism. Future experiments could include testing with the H309 repair mutant to confirm the role of the redox domain and the use of the redox inhibitor E3330 with APE1 to confirm the protective effect observed in the C65 NT2 cells.

## Delineation of temozolomide-mediated apoptotic mechanisms in hematopoietic progenitor cells

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The aim of the study is to discover ways to protect normal tissue cells during high dose chemotherapy while killing cancer cells. Temozolomide (TMZ) is a therapeutic drug used in chemotherapy of many cancers. It is an alkylating agent that destroys cells by inducing O6-methylguanine lesions in the DNA (O6-MeG). However, O6-MeG DNA methyltransferase (MGMT) is known to protect the cells against alkylating agents by removing the methyl group from the O6 position of guanine. Hematopoietic progenitor cells such as CD34+ cells have very low levels of MGMT compared to cancer cells, which makes them more susceptible to TMZ mediated cytotoxicity. This means TMZ would be more effective in killing progenitor cells than cancer cells. If TMZ were used in high doses, a large proportion of progenitor cells would be destroyed by the drug, and the patient's life would be threatened by possibilities for infections. Our recent work is aimed at observing cell reactions toward TMZ by itself compared to TMZ in conjunction with a MGMT suppressor, BG. Umbilical cord blood progenitor cells and various cancer cell-lines are used in the experiment. Here, we probe the cell cycle arrest behaviors and caspase activities of these cells under TMZ treatments. A basic understanding of apoptotic mechanisms will help in setting the groundwork for future design of novel therapies that specifically kill cancer cells but do not cause toxicity to normal tissues.

## **Analysis of the Effect of Ape1 Knockdown and Temozolomide Treatment on DNA Polymerase Beta and X-Ray Cross Complementing Group 1 Protein Levels**

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Base damage within a DNA strand can be mended by the cell's Base Excision Repair (BER) pathway. This involves the combined efforts of several repair enzymes endogenous to the cell. A DNA glycosylase excises the damaged base creating an abasic site (AP site). AP endonuclease 1 (Ape1) then cleaves the AP site, preparing it for DNA synthesis by DNA Polymerase  $\beta$  (pol  $\beta$ ). X-ray cross complementing group 1 (XRCC1), which is complexed to DNA ligase, interacts with pol  $\beta$ , allowing ligation of DNA strand. We wanted to determine if knocking down the expression of Ape1 using siRNA would affect the expression levels of XRCC1 or pol  $\beta$ . We transfected an ovarian cancer cell line, SKOV-3X, with 50nM Ape1 siRNA and collected cells one to five days after transfection to analyze via Western Analysis. The Ape1 levels were down 4.3-fold three days after transfection. Preliminary results indicate a direct correlation between Ape1 knockdown and decreased pol  $\beta$  expression. Pol  $\beta$  levels were down 2.1-fold three days after transfection. XRCC1 results were inconclusive. We also wanted to generate DNA damage by treating the cells with Temozolomide (TMZ), an alkylating drug, in order to determine if Ape1, XRCC1 or pol  $\beta$  expression would be induced. Eighty percent of the lesions caused by TMZ, N<sup>7</sup>-methylguanine (N7mG), and N<sup>3</sup>-methyladenine (N3mA), are repaired by the BER pathway. We treated SKOV-3X cells with 2mM and 4mM TMZ for 4h and 24h. We then investigated the resulting levels of Ape1, pol  $\beta$ , and XRCC1 via Western analysis. The data on Ape1 expression was inconclusive. However, preliminary results indicated that pol  $\beta$  expression increased up to 3.0-fold and XRCC1 up to 1.8-fold with 4h treatment of 4mM TMZ. In conclusion, these preliminary results demonstrate that pol  $\beta$  protein levels decrease when Ape1 is knocked down in SKOV-3X cells. This could indicate that one of Ape1's functions may be to signal pol  $\beta$  or, conversely, that removing Ape1 causes pol  $\beta$  to destabilized and degrade. Furthermore, preliminary results also demonstrate that pol  $\beta$  and XRCC1 levels increase when DNA damage is induced. This indicates an up-regulation of pol  $\beta$  and XRCC1 in response to DNA damage. These experiments will be repeated to determine the significance of these results. The next step will be to treat the SKOV-3X cells with TMZ while the Ape1 is knocked down. Based on our preliminary results, we would hypothesize that, because Ape1 would not be available to signal or stabilize pol  $\beta$ , the BER pathway would be impaired. Therefore, we would expect to see less up-regulation of pol  $\beta$  expression and greater cytotoxicity after TMZ exposure in the cells with the Ape1 knockdown as compared to the control cells with endogenous Ape1.

## **Isolation and identification of ECFCs from porcine peripheral blood mononuclear cells with a CD45- selection.**

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Porcine endothelial progenitor cells have not been well studied so far, which makes their characterization and identification difficult. However, recently we isolated and identified endothelial colonies forming cells (ECFCs) from porcine peripheral blood. They have a typical cobblestone morphology. Immunostaining analysis showed that ECFCs do not express CD45 and SLA-DR, two common leukocyte markers, when compared with the mononuclear cell (MNCs), from which they are grown. Also, we demonstrated that a hierarchy of circulating ECFCs exists in the swine using a single cell assay.

Based on these observations, we hypothesized that ECFCs may be enriched in CD45- cells. Our strategy was to use a hematopoietic marker: CD45, to separate the MNCs into two populations: the CD45+, containing the hematopoietic cells, and the CD45-, and grow these two cells population to test which one enriches the ECFCs. To isolate the MNCs, we used Histopaque 1119. Then, we stained the MNCs with the primary antibody: mouse anti porcine CD45, and the secondary antibody: rat anti mouse's IgG1 coupled with iron beads. After incubation, we used the Magnetic Activated Cell Sorting (MACS) to separate the MNCs in a CD45 negative and positive population.

The results showed that before MACS, there were 90% of CD45+ cells and 10% of CD45- cells in the MNCs. However, two distinct populations, with 98% of purification for both, were obtained after MACS. Noticeably, the frequency of ECFCs was about 1 colony per  $20 \times 10^3$  CD45- cells, a significant increase in comparison to that from MNCs (1 colony per  $80 \times 10^6$  cells). The ECFCs grown from the CD45- population had typical cobblestone morphology, and FACS analysis showed that they were CD45 and SLA-DR negative. Furthermore, they were able to uptake Low Density Lipoprotein (LDL) and did not phagocytose E.Coli. Thus, the data showed that the ECFCs isolated using a CD45-selection were the same as the ECFCs grown directly from the MNCs.

In conclusion, we have seen that the pig ECFCs are enriched (4000 fold) in CD45-MNCs. Also, we have seen that the ECFCs isolated from the CD45- MNCs display the same phenotypic and functional properties as those derived from the entire population of MNCs. In future work, we will use CD45 and CD31, an endothelial cell marker, to obtain a better enrichment.

Impaired homing causes engraftment defect in transduced lineage-depleted marrow in submyeloablated murine hosts.

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Our laboratory previously demonstrated that engraftment of murine whole bone marrow (WBM) transduced with an oncoretroviral vector using an optimized 5-fluorouracil (5-FU)-based transduction protocol is reduced ~3-fold, compared to fresh unmanipulated WBM, upon transplantation into sublethally-irradiated hosts. We hypothesized that  $lin^{-}$  marrow cells transduced in the absence of 5-FU would lead to improved engraftment in submyeloablated hosts. Lineage-depleted cells were transduced as described by Li et al. (Exp. Hematol. 31:1206, 2003). Cell recovery from the MACS column,  $lin^{-}$  cell purity, and bulk transduction efficiency was similar to that previously described. Transplantation of  $10^6$   $lin^{-}$  transduced cells into 300 cGy-conditioned congenic hosts, however, produced only  $3\pm 0.5\%$  donor chimerism 4-6 months post-transplant (N=13 hosts), significantly lower than that observed using fresh  $lin^{-}$  cells ( $29\pm 18.8\%$ ). The percentage of vector-marked cells ( $43\pm 6.8\%$ ) in the donor population indicates that the stem cells were efficiently marked but engrafted poorly. We hypothesized that increased conditioning may improve donor engraftment. Transplantation of  $10^6$   $lin^{-}$  transduced cells into 550 cGy-conditioned congenic hosts produced  $40.7\pm 20\%$  donor chimerism 4-6 months post-transplant, compared to  $90.2\pm 0.6\%$  chimerism using fresh  $lin^{-}$  cells (N=5-7 hosts;  $p < 0.0001$ ). These data suggest that the marrow cells acquired an engraftment defect during the transduction process. Studies to determine potential mechanisms responsible for the engraftment defect induced by transduction included investigation of homing efficiency. 20 hour homing assays showed that freshly isolated  $lin^{-}$  cells home to the marrow more efficiently than transduced  $lin^{-}$  cells ( $1.5\pm 0.5\%$  vs.  $0.2\pm 0.1\%$ ; N=8;  $p < 0.0001$ ). Analyses of the host marrow  $lin^{-}$  population show that fresh  $lin^{-}$  donor cells comprise  $6.5\pm 2.3\%$  of the marrow 20 hours post-transplant, while transduced donor cells account for only  $1.8\pm 0.7\%$  ( $p = 0.0005$ ). Thus, we have identified impaired homing as one mechanism by which retrovirus-transduced marrow engrafts less successfully than freshly-isolated marrow in submyeloablated hosts.

Inhibition of PDGF- $\beta$  and TGF- $\beta$  mediated proliferation, migration, and collagen synthesis in *Nf1*<sup>+/-</sup> fibroblasts using Sunitinib Malate (SU11248)

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*NF1* encodes neurofibromin, a GTPase activating protein (GAP) responsible for p21<sup>ras</sup> (Ras). Mutations in the *NF1* gene cause Neurofibromatosis Type 1 (NF1). NF1 is characterized by the genesis of neurofibromas, which are composed of Schwann cells, endothelial cells, mast cells, and fibroblasts. Genetic studies demonstrated that tumorigenesis requires nullizygous loss of *NF1* in Schwann cells and haploinsufficient non-neuronal cells. Fibroblasts account for up to 60% of all cells in neurofibromas, and produce excessive amounts of collagen. Previous research by Yang, *et al.* using a murine *Nf1* model demonstrated that at concentrations secreted by mast cells the receptor tyrosine kinase (RTK), TGF- $\beta$ , mediates increased proliferation, migration, and collagen synthesis of *Nf1*<sup>+/-</sup> fibroblasts as compared to WT syngeneic fibroblasts. This same research also demonstrated that TGF- $\beta$  mediates hyperactivation of Ras via neurofibromin's function as a RasGAP in both murine and human fibroblasts. Sunitinib (sunitinib malate; SU11248; SUTENT, Pfizer) is an FDA approved small molecule class III/V receptor tyrosine kinase inhibitor of platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), KIT, and FLK3. The effects of PDGF- $\beta$  on *Nf1*<sup>+/-</sup> fibroblasts are unknown. Furthermore, while Sunitinib has been shown to have biological effects on many RTKs, its effect on TGF- $\beta$  has not been examined in vitro. This study treated *Nf1*<sup>+/-</sup> fibroblasts and WT syngeneic fibroblasts with either TGF- $\beta$  or PDGF- $\beta$  alone or in combination with varying concentrations of Sunitinib to assess the ability of Sunitinib to inhibit the tumorigenic activity of *Nf1*<sup>+/-</sup> fibroblasts. To test migration fibroblasts were first plated to confluence and treated with mitomycin c to inactivate the mitotic cell machinery. An established 'wound healing' assay was used to administer the treatments and measure cell migration. Proliferation and collagen synthesis were assessed using previously established protocols that measured tritiated thymidine and proline, respectively. Results were not conclusive overall, but some preliminary data suggest that Sunitinib may inhibit both PDGF- $\beta$  and TGF- $\beta$  mediated proliferation, migration, and collagen synthesis in both *Nf1*<sup>+/-</sup> and WT fibroblasts.

## Rac2<sup>-/-</sup> *PTPN11* Mutant Bone Marrow Cells Exhibit Modest Reduction in GM-CSF Stimulated Hyperproliferation

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Juvenile Myelomonocytic Leukemia (JMML) is a rare and lethal leukemia of early childhood with increased incidence among patients with Noonan Syndrome. Approximately 35% of children with JMML bear mutations of *PTPN11*, which encodes for the protein tyrosine phosphatase Shp-2. We have previously demonstrated that *PTPN11* mutants induce hyperproliferation of hematopoietic progenitors and increased phospho-extracellular signal-related kinase (p-Erk) levels upon stimulation with the cytokine GM-CSF. Erk is a downstream effector of the Shp-2-mediated Ras signaling axis recently suggested to crosstalk with the PI3K pathway and its effectors, Rac2 and Akt. Here we hypothesize that knocking out Rac2 in murine *PTPN11* mutant-bearing bone marrow cells reduces hyperactivation of the Shp-2-mediated Ras and PI3K pathways. To test this hypothesis, murine WT and Rac2<sup>-/-</sup> low density bone marrow cells were transduced with MSCV-based bicistronic retroviral vector PMIEG3 containing either WT Shp-2 or two Shp-2 mutants, D61Y and E76K, in tandem with the enhanced green fluorescent protein (EGFP). Transduced cells were collected using fluorescence activated cell sorting (FACS), starved for 24 hours, and stimulated with 0-10 ng/ml GM-CSF in a progenitor assay. Rac2<sup>-/-</sup> cells transduced with Shp2 mutants E76K and D61Y demonstrated decreased colony counts compared to WT cells, supporting the hypothesis that blocking Rac2 aids in the relief of GM-CSF stimulated hyperproliferation of hematopoietic progenitors. In addition, transduced cells were cultured in 50 ng/ml M-CSF to generate macrophage progenitors, starved for 24 hours, and stimulated with 50 ng/ml GM-CSF for 0-60 minutes. Protein extracts were collected and analyzed for p-Erk and p-Akt using Western Blot analysis. Rac2<sup>-/-</sup> mutants consistently illustrated a modest decrease in p-Erk and p-Akt levels at 60 minutes as compared to WT cells. These data support Rac2 as an effector of activating Shp-2 mutant-induced hypersensitivity to GM-CSF. Further experimentation is ongoing by utilizing a double knockout of Rac2 and p85 $\alpha$ , the regulatory subunit of class 1A PI3K, to further elucidate rational molecular targets for novel therapies in JMML.

Linda Yu  
Dr. Wei Lei  
Abstract

### The role of ROCK-1 in cardiomyocyte apoptosis and heart failure

Cleavage of Rho-associated kinase (ROCK-1) by activated caspase-3 is crucial to the execution of apoptosis, and caspase-3 activation is known to be associated with cardiomyocyte loss through the apoptotic cascade in heart failure. While cleavage of ROCK-1 during apoptosis has been demonstrated in Jurkat and NIH3T3 cells, this phenomenon remains to be tested in cardiomyocytes. We examined ROCK-1 cleavage in apoptotic neonatal rat cardiomyocyte induced by doxorubicin. Doxorubicin-treated cardiomyocytes resulted in the 130 kDa ROCK-1 cleavage fragment whereas the non-treated cardiomyocyte lacked the cleavage fragment. However, when treated with doxorubicin and caspase-3 inhibitor, Z-VAD-fmk, the ROCK-1 130 kDa fragment was absent, reinforcing the fact that ROCK-1 cleavage is caspase-3-dependent. A constitutively active ROCK-1 mutant was transfected into cardiomyocytes, resulting in a three-fold increase in caspase-3 activity. This suggests the presence of a positive feedback loop between cleaved ROCK-1 and caspase-3 activation. Deleting ROCK-1 in mice using siRNA application showed a reduction in apoptosis associated with pressure overload. Similarly, ROCK-1 <sup>-/-</sup> mice under pressure overload showed a decrease in the number of TUNEL-positive cardiomyocytes compared to wild-type mice. Therefore, our data demonstrate that ROCK-1 plays an important role in cardiomyocyte apoptosis, where therapeutic inhibition of ROCK-1 may be a possible method to treat heart failure. Future experiments include examining the ability of transgenic wild-type ROCK-1 and a mutant ROCK-1-D1113A in ROCK-1 <sup>-/-</sup> knock-out mice to maintain cardiac-protection from pressure overload.

## The binding of fbx29 to p193 and its role in cardiomyocyte cell cycle reentry through targeted protein degradation

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The ability to induce cardiomyocyte cell cycle reentry can be of therapeutic benefit in diseased hearts. p193 (also known as CUL7) is a member of an E3 ubiquitin ligase complex. p193 forms an SCF-like, E3 ubiquitin ligase complex consisting of skp1, Fbx 29, and ROC1. Although the targets of this ligase complex remain unknown, previous studies showed that expression of dominant interfering p193 mutants induced cardiomyocyte cell cycle reentry following myocardial injury. To determine if modulating the activities of other proteins in the p193 E3 ligase complex could also induce cardiomyocyte cell cycle activity, transgenic mice expressing a dominant interfering, myc-tagged Fbx29 protein were generated. Preliminary studies established that these "Fbx" mice express Fbx29 in the myocardium. To monitor cell cycle activity, the Fbx mice were crossed with "nLAC" mice, which express a nuclear localized beta-galactosidase reporter exclusively in cardiomyocytes. Cardiomyocyte cell cycle activity in the resulting Fbx/nLAC double transgenic mice and the nLAC single transgenic mice were compared following two forms of cardiac injury (namely, isoproterenol-induced cardiac hypertrophy or myocardial infarction). To monitor cardiomyocyte cell cycle activity after injury, the mice received an injection of tritiated thymidine and were sacrificed. The hearts were frozen and sectioned at 10 microns. The sections were then incubated with X-GAL (which gives rise to a blue signal in the presence of beta-galactosidase) and then subjected to autoradiography. Cardiomyocyte DNA synthesis was then scored by the presence of silver grains over blue nuclei. Although the Fbx/nLAC mice showed higher levels of atrial cardiomyocyte cell cycle activity following injury as compared to the nLAC mice, no difference in ventricular cardiomyocyte cell cycle activity was seen. This data suggests that Fbx29 may be an important regulator of atrial cardiomyocyte cell cycle activity.

Improvement of the physiological quality of vasculature grown *in vivo* from endothelial colony forming cells (ECFC's) through co-culturing with pericytes and mesenchymal cells

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Previous work has shown that endothelial colony forming cells (ECFC's) will grow into a network of tube-like structures in a collagen-based gel, developing into a primitive vasculature *in vivo*. While these vessels carry blood, the vessels are primitive – lacking many features of mature vasculature. The purpose of this project was to improve the physiological characteristics of this vascular model. We hypothesized that co-culturing the ECFC's with either pericytes or mesenchymal cells within the collagen matrix may promote vascular development and maturation. The pericytes used in co-culture were derived from adipose stromal cells and are hypothesized to be a vessel-supporting cell. Mesenchymal cells were derived from primary cultures and were phenotyped by flow cytometry.

Co-cultures of ECFC's with pericytes and mesenchymal cells, along with control gels seeded with individual cell lines, were implanted into mice and allowed to develop over a two-week period. The gels were then excised and, after a visual inspection (Phase 1 Analysis), the gels were analyzed via immunohistochemistry for anti-human CD31 (PECAM) and scored for vessel density (Phase 2 Analysis). Thus far, Phase 1 Analysis has shown that the co-culture of pericytes with ECFC's *in vivo* greatly supports vasculogenesis, while co-culturing ECFC's with mesenchymal cells had no effect on vasculogenesis, contrary to prior reports.

#### Phase 1 Analysis

Lines	5.15-6.1	5.30-6.15	6.13-6.29	7.11-7.25	7.18-8.1	7.21-8.4
CB ECFC	3.5	2.25	2.5	2.1	1.5	3.0
ASC					1.0	
CB ECFC & ASC					4.0	
MSC						1.0
CB ECFC & MC						2.1

## Characterization of 5.0kb *CRP1*-Cre mice using the ROSA26R reporter line

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*Cysteine-Rich Protein 1 (CRP1)* is expressed in all muscle cell types during embryogenesis but is found mostly in vascular and visceral smooth muscle cells in the adult. *CRP1* plays a role in actin filament bundling by directly cross-linking actin filaments and stabilizing the interaction of  $\alpha$ -actinin and actin filaments. Previously, it has been shown that a 5.0kb enhancer within the *CRP1* gene is sufficient to drive expression in arterial but not venous or visceral smooth muscle cells in transgenic mice. Using this enhancer, we generated 5.0kb *CRP1*-Cre mice that will be used in future *Cre/loxP* conditional mutagenesis and lineage mapping studies. By crossing the 5.0kb *CRP1*-Cre mice to *ROSA26R* indicator mice that permanently mark Cre recombinase-expressing cells, we are able to lineage trace the arterial smooth muscle cells in transgenic mice.

Analysis of a developmental series revealed that lacZ reporter expression can initially be detected within the E10 outflow tract of the heart and around the dorsal aorta. As development progresses, lacZ expression becomes evident in the face, somites/limb muscles, hindbrain and within isolated ventricular cells of the heart. Using marker immunohistochemistry, we determined that *CRP1*-Cre x *ROSA26R* cells co-localize with  $\alpha$ -smooth muscle actin expression, demonstrating that the expanded lacZ staining around the vessels is smooth muscle and not endothelial in origin.

## Xigris Stimulates the Proliferation of Endothelial Progenitor Cells

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Our lab has identified a hierarchy of endothelial colony forming cells (ECFCs) based on their proliferative potential: high proliferative potential-endothelial colony-forming cells (HPP-ECFCs) form the largest colonies and can be re-plated to form secondary and tertiary colonies, low proliferative potential-endothelial colony-forming cells (LPP-ECFCs) form smaller colonies but fail to be re-plated, endothelial cell clusters contain fewer than fifty cells that are typically larger than HPP cells, and mature cells do not divide.

Xigris is a recombinant version of human Activated Protein C (APC) used to treat adult patients with severe sepsis. Activated Protein C is the thrombin-activated form of protein C that acts as a feedback inhibitor of coagulation. Once protein C binds to the endothelial cell protein C receptor (EPCR) on the endothelial cell surface, it is converted to APC. The APC-EPCR complex activates the protease activated receptor (PAR-1) on the endothelial cells thereby activating mitogen-activated protein kinase (MAPK) pathway. It has been postulated that through this pathway APC effects HUVEC proliferation. Based on these findings, we hypothesize that since xigris is a form of recombinant APC, it will stimulate circulating ECFCs to proliferate, leading to the production of more HPP-ECFCs.

Human endothelial cells were derived from cord blood and cultured to early passages. First, FACS analysis using the EPCR antibody showed that the cord-blood-derived ECFCs expressed EPCR. After the cells proved to be EPCR- positive, we performed a proliferation assay to determine the optimal concentration and time point for xigris to stimulate the ECFCs' proliferation. Five to ten thousand cells were plated on a 24-well plate with different concentrations for 24, 48, 72, and 96 hours. After comparing the results, we found 100ng/mL of xigris to be the most effect treatment for ECFC proliferation. Finally, we plated the ECFCs with the treatment on a single cell level to show how xigris would affect the ECFC hierarchy. We anticipate that the drug will have an effect to increase the number of progenitors and may alter the distribution of ECFC (data pending). Our preliminary results suggest that the human ECFC express the EPCR and respond to xigris with increased proliferation. Additional experiments will determine which stage of ECFC is most affected.

## **Prolonged Continuous Positive Airway Pressure (CPAP) is still effective after 24h of reducing respiratory system responsiveness in rabbits.**

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In a former study rabbits that used **High CPAP (6 cmH<sub>2</sub>O)** had lower responsiveness to acetylcholine (Ach) when compared to **Low CPAP (0 cmH<sub>2</sub>O)** ( $p < 0.05$ ). This present study used CPAP to investigate the potential of the intermittent CPAP (use of high CPAP at night) and also to establish if the effects were still active after 24h without CPAP in rabbits. Active, nonanesthetized, tracheostomized rabbits used CPAP for 5 days divided in 3 groups **L-CPAP** (low CPAP), **CPAP-H/L** (high at night, low at day), and High CPAP for 4 days and without CPAP for 24h (**CPAP-H4+L1**). After 5 days respiratory resistance was measured by forced oscillation. The Rabbits were challenged with increasing concentrations of Ach (1.0, 3.3, 10, 20, 33, and 50 mg/ml) and measured during mechanical ventilation by Flexi Vent, SCIREQ. Respiratory system resistance was calculated, flow and volume signals recorded during a volume 1-Hz oscillation signal. **Results:** CPAP-H/L had a significantly smaller increase in resistance than L-CPAP and this effect was consistent even after 24h without CPAP (CPAP-H4+L1 group). Intermittent CPAP was proved to be efficient with possible clinical correlations. The curve response of Ach of the CPAP-H/L and CPAP-H4+L1 shows an increased resistance with the last 3 higher doses, but at a smaller scale compared to the L-CPAP. Both CPAP-H/L and CPAP-H4+L1 were effective and have similar results suggesting that intermittent CPAP might be a better clinical option in some cases. Further studies are in development in order to establish an optimum usage of CPAP.

## The Role of FKBP4 in Prostate Growth and Development

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Growth and development of the prostate begin at 17 days of gestation for mice and are complete at sexual maturation. The initial event of prostate development starts with outgrowth of solid epithelial buds from the urogenital sinus into the surrounding mesenchyme. From this earliest stage of development through embryonic and neonatal growth and the maturation of secretory function at puberty, the prostate requires androgens and androgen mediated pathways. After puberty, androgens are still needed for cell proliferation when cell proliferation is in equilibrium with cell death. FKBP4 is the best known of several tetratricopeptide repeat proteins that co-chaperone steroid receptors, including androgen receptors. Male FKBP4 mutants have several defects in reproductive tissues consistent with androgen insensitivity such as hypospadias and underdeveloped prostate. Transcription assay analysis using FKBP4-deficient MEF cells further indicate that FKBP4 is critical to maintain androgen receptor transcriptional activity. To determine whether the underdeveloped prostate in FKBP4 deficient male is due to the defect in prostate morphogenesis during embryonic development or due to the defect in its postnatal growth and maturation, in this study, we analyzed prostatic histology of FKBP4 mutants at E17, newborn, and postnatal age of 3 weeks. We found that FKBP4 deficient mice were able to initiate the prostatic development in the embryonic stage; however, branching morphogenesis at three weeks were dramatically reduced when compared to littermate controls. Our data lead to the conclusion that FKBP4 is not required during embryonic morphogenesis, but plays an important role in the postnatal development, possibly via androgen receptor transcriptional activity.

Use of caged fluorescein and two-photon microscopy to study cell to cell communication within atrial heart muscle of an intact mouse heart

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Photoactivatable compounds have been used to study cell to cell communication within several types of tissue. We wanted to develop a method to utilize CMNB caged fluorescein (Molecular Probes) to study cell to cell communication within an intact mouse heart. Caged fluorescein is a non-membrane permeable compound that is non-fluorescent until it is exposed to UV light. When high energy light strikes the compound, it is un-caged and becomes fluorescent. We used microinjection coupled with microelectrode recording to load the caged fluorescein into myocytes in intact mouse atria. The samples were placed on an epifluorescent microscope and exposed to UV light. We observed the appearance of fluorescent spots in the tissue. The un-caged fluorescent compound also diffused rapidly through the tissue. These results proved that microinjection was a feasible and usable delivery method for caged fluorescein and that the un-caged compound moved from cell to cell through gap junctions. In the future this delivery method will be used in combination with two-photon microscopy. With two-photon microscopy, we will be able to un-cage the compound within a single cell. This ability will allow us to observe the diffusion of the fluorescent compound on a cellular level. We hope to use this technique to shed light on the characteristics of gap junction permeability in cellular communication of the heart. We also hope to study hetero-cell coupling and what light it may shed on cardiac processes.