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Impact Of Gain-Of-Function Mutations In The Low-Density Lipoprotein Receptor Related Protein 5 (lrp5) On Glucose And Lipid Homeostasis

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Impact of gain-of-function mutations in the low-density lipoprotein receptor related protein 5 (LRP5) on glucose and lipid homeostasis

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IMPACT OF GAIN-OF-FUNCTION MUTATIONS IN THE LOW DENSITY LIPOPROTEIN RECEPTOR RELATED PROTEIN 5 (LRP5) ON GLUCOSE AND LIPID HOMEOSTASIS. Dinah Foer, Meiling Zhu, Christine Simpson, Grace Lee, Rebecca Sullivan, and Karl L. Insogna. Section of Endocrinology, Department of Internal Medicine, Yale University, School of Medicine, New Haven, CT.

Investigations in animal models suggest a physiologic link between LRP5 and glucose and lipid homeostasis. However, the data in human studies is far from consensus. While LRP5 has been shown to be critical to Wnt signaling, the molecular mechanism for the effect on metabolism is unknown. We hypothesize that a high bone mass (HBM) mutation in LRP5 will lead to improved glucose and lipid homeostasis. We aim to measure the impact of these mutated receptors on human glucose and lipid metabolism and to investigate the pathway(s) by which Wnt may affect glucose metabolism *in vitro*. Eleven subjects with HBM-causing LRP5 mutations were matched by gender, age, and body mass index (BMI) to 22 unrelated control subjects. Hemoglobin A1c (HbA1c), estimated average glucose (eAG), HOMA-*B*, HOMA-IR, and lipid panel were analyzed. An oral glucose tolerance test and ^{1}HMRS study of liver and skeletal muscle were performed on a subset of affected patients. Primary hepatocytes and HepG2 cells were treated *in vitro* with Wnt3a, and PEPCK-C mRNA expression was measured by QPCR. INS-1 cells and human pancreatic islet *beta*-cells were assayed for glucose-stimulated insulin secretion using an ELISA technique. Statistical differences were analyzed using the unpaired Student t-test and one-way ANOVA. There were no statistically significant differences between affected and control populations for HbA1c, eAG, insulin, HOMA-*B* and HOMA-IR. Differences in total cholesterol, triglycerides, and HDL, were not significant. However, LDL levels were significantly lower in affected subjects ($p=0.04$). Wnt did not effect PEPCK-C expression. Wnt did not significantly effect glucose-stimulated insulin secretion in INS-1 cells and human islets. In summary, this is the first investigation on the metabolic consequences of LRP5 mutations in human kindreds with HBM-causing mutations. Our data does not support the hypothesis that LRP5 improves glucose metabolism in these individuals. The data does suggest that there may be a specific, beneficial effect of LRP5 on LDL cholesterol, however additional studies need to be done to confirm the effect and elucidate the mechanism.

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This thesis work is dedicated to Meiling Zhu, a truly humble, and gifted teacher.

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INTRODUCTION

The low-density lipoprotein related protein 5 (LRP5) came to clinical attention in 2001 when Gong and coworkers reported that loss of function mutations in this receptor caused profound osteoporosis and fractures (Gong et al., 2001). In 2002, Yale University investigators reported that a mutation in LRP5 that conferred resistance to endogenous inhibitors such as Dkk1, caused another dramatic skeletal phenotype. A kindred was identified with an inherited syndrome of high bone mass that was phenotypically characterized by a reduced angle of the jaw, torus palatinus, and extremely high bone density when measured by dual energy x-ray absorptiometry (Boyden et al., 2002). The syndrome was inherited in an autosomal dominant pattern. Interestingly, members of the kindred reported difficulty staying afloat while swimming. Affected family members were shown to have a single base pair mutation causing a glycine to valine change at position 171. Glycine 171 is evolutionarily conserved in LRP5 and in its homologues, indicating a critical role for this residue (Hey et al., 1998). Subsequently additional high bone mass (HBM)-causing mutations in LRP5 have been identified in other kindreds (Van Wesenbeeck et al., 2003; Whyte, Reinus, & Mumm, 2004). The exact mechanism by which these high bone mass causing mutations lead to the phenotype described is not completely understood. However as noted, the LRP5 G171V mutation is resistant to the actions of the endogenous inhibitor Dickopf 1 (Dkk1). Thus, while the receptor does not signal in the absence of ligand and appears to signal normally in the presence of ligand, when Dkk1 is added ligand-dependent signaling is largely unaffected (Boyden et al., 2002).

The role of LRP5 skeletal homeostasis is now well established (Williams & Insogna, 2009). More recently, animal models with both HBM and low bone mass (LBM)-causing mutations, as well as human studies in populations with LBM-causing mutations, implicate a role for LRP5 in glucose metabolism and lipid homeostasis (Fujino et al., 2003; Saarinen et al., 2010). This introduction will review LRP5's function and its key signaling pathways, providing a background for understanding the potential role of LRP5 in human metabolism.

LRP5. The LRP5 gene is located on chromosome 11q13.4. The encoded protein in vertebrates is a single pass transmembrane molecule that is a receptor for low-density-lipoprotein (LDL) (Galora et al., 2013). The protein also serves as a co-receptor, along with its homologue LRP6 and the family of Frizzled transmembrane receptors, for the wingless-type MMTV integration site family of glycoproteins, commonly denoted as Wnt proteins. The Wnt genes, of which there are currently nineteen, encode secreted glycoproteins, generating signal transduction cascades that are critical to nearly every event during development (Moon, 2005). Wnt signaling persists postnatally to regulate expression of specific target genes in self-renewing adult tissues such as gut, hair follicle, bone, and the hematopoietic system. Mutations in Wnt signaling have subsequently been identified in oncogenesis, notably in colon cancer and liquid cancers (Reya & Clevers, 2005), and is an active area of cancer research.

Wnt signaling pathways. Three pathways are activated following Wnt receptor engagement: the canonical Wnt pathway, the non-canonical planar cell polarity pathway (PCP), and the Wnt/Ca^{2+} pathway. The canonical pathway is the best studied of these (Clevers, 2006). In this pathway, Wnt ligands bind to a Frizzled/LRP complex, interacting with Dash and Axin proteins, and preventing the proteosomal degradation of *beta*-catenin, which plays a major role in the canonical pathway. As just noted, in the absence of Wnt signaling, *beta*-catenin is targeted for phosphorylation-dependent destruction. Stabilized and unphosphorylated, *beta*-catenin accumulates in the cytoplasm and translocates to the nucleus where it binds transcription factors and transcriptionally activates a wide range of target genes (Tamai et al., 2004). Secreted Dkk proteins inhibit Wnt signaling by direct binding to LRP5/6, promoting internalization and inactivation of the receptors (Tamai et al., 2000).

Bone. The role of LRP5 in bone accrual is well-established. In osteoblasts LRP5 transduces Wnt signaling via the canonical pathway. LRP5-deficient mice demonstrate low bone mass as a result of impaired bone formation attributable to defects in osteoblast proliferation and maturation (Kato et al., 2002). Of note, LRP5-deficient mice also demonstrate persistent embryonic eye vascularization as a result of failed macrophage- mediated apoptosis. Conversely, transgenic mice expressing a gain-of-function LRP5 allele in osteoblasts have increased bone density and increased osteoblast activity (Babij et al., 2003).

As noted, humans homozygous for loss-of-function mutations in LRP5 are consistent with observations in animal models. These individuals have an autosomal recessive disorder, Osteoporosis Pseudoglioma (OPPG) syndrome, characterized by severe osteoporosis, childhood fractures and blindness due to abnormal vitreous development (Gong et al., 2001). Common polymorphic variants in LRP5 in a variety of human ethnic populations and age groups have been shown to influence bone density in the general population, indicating that in addition to its role in the rare inherited syndromes just described, it has an important role in regulating normal skeletal metabolism (Jiang et al., 2010; Saarinen et al., 2010; Zenibayashi et al., 2008).

Consistent with a critical role of Wnt signaling in bone homeostasis are the skeletal phenotypes observed with loss of function mutations in the endogenous inhibitors of Wnt signaling Dkk1 (described above) and sclerostin. Heterozygous loss of function mutations in Dkk1 lead to high bone mass in mice (Morvan et al., 2006). In humans, loss-of-function mutations in sclerostin lead to a condition called sclerosteosis, characterized by massive and progressive increases in bone mass resulting ultimately in nerve entrapment (Li et al., 2005; Semenov, Tamai, & He, 2005).

Lipid Homeostasis. Animal models of LRP5 deficiency demonstrate impaired lipid homeostasis under certain experimental conditions. LRP5 deficient mice fed a high fat diet developed high plasma cholesterol levels, marked by impaired hepatic

clearance of chylomicron remnants. When fed a normal diet, these mice had normal plasma lipid levels (Fujino et al., 2003). However, mice deficient in both ApoE and LRP5 fed a normal diet exhibited approximately 60% higher plasma cholesterol levels compared with ApoE knockout mice. Analysis of lipid components showed that plasma clearance of diet-derived triglycerides was severely impaired by LRP5 but not ApoE deficiency. These data suggest that in a murine model, LRP5 mediates catabolism of plasma lipoproteins through ApoE dependent and independent mechanisms (Magoori et al., 2003).

In human studies, an impact of LRP5 on lipid metabolism is less well established. Two single nucleotide polymorphisms in LRP5 have been associated with a higher risk for of abdominal aortic aneurysm. Specifically, these polymorphisms result in reduced expression of LRP5 causing impaired clearance of lipoprotein (a), an atherothrombotic risk factor (Giusti et al., 2009). A study of a Chinese Han population found an association between two single nucleotide polymorphisms in LRP5 and increased circulating levels of total cholesterol (Jiang et al., 2010). In a prepubertal Finnish population, variants in LRP5 were associated with higher total and LDL cholesterol levels (Lappalainen et al., 2009). However, in contrast, a study of 13 Finnish individuals from one family with three different LBM-causing LRP5 mutations did not demonstrate hypercholesterolemia or elevated triglyceride levels (Saarinen et al., 2010).

The findings of abnormal lipid metabolism in murine models of LRP5 deficiency and

in some human studies, in combination with the role of LRP5 in osteoporosis, have prompted investigation of LRP5 as a candidate gene underlying the metabolic syndrome. Findings from one study in a Caucasian population suggested an association of SNPs and haplotypes in the LRP5 gene, with obesity, although the molecular mechanism was not defined (Guo et al., 2006). In contrast, a study in a Japanese population, which included the same SNP, did not find an association between body mass index (BMI) and that same polymorphism or haplotypes comprising it. Furthermore, other studies have found no evidence of an association between LRP5 polymorphisms and BMI, homeostatic model assessment of estimated beta-cell function (HOMA-*B*), estimated insulin resistance (HOMA-IR), or serum lipids in diabetic versus non-diabetic controls (Zenibayashi et al., 2008). Consistent with this, Jing et al (mentioned above) found no association of LRP5 polymorphisms with BMI (Jiang et al., 2010).

Glucose metabolism. A potential role for LRP5 in glucose metabolism was first reported in an animal model. LRP5-deficient mice, fed a normal diet, were found to have impaired glucose tolerance and blunted glucose-induced insulin secretion by isolated pancreatic beta cells. Islets isolated from these mice had reduced intracellular levels ATP and $Ca^{2+,}$ and decreased mRNA levels of insulin signaling targets including glucokinase and HNF4 under stimulatory conditions. Wnt3a failed to induce insulin secretion in LRP5-deficient islets, while in wild type islets both Wnt3a and Wnt 5a stimulated glucose-induced insulin secretion (Fujino et al., 2003).

The suggested link between LRP5 and glucose metabolism prompted a range of human studies. However, findings to date have been less definitive than those in animal models. No statistically significant associations have been found between genetic polymorphisms in LRP5 and glucose metabolism, as assessed by Hemoglobin A1c (HbA1c) (Jiang et al., 2010), HOMA-*B*, or HOMA-IR, was found in individuals with type 2 diabetes or in non-diabetic subjects (Lappalainen et al., 2009; Zenibayashi et al., 2008). However, the single nucleotide polymorphisms that give rise to the HBM phenotypes have never been included in any of these analyses for the obvious reason that their frequency in the general population is exceedingly rare. A study of Finnish individuals who were homozygous, compound heterozygous, or heterozygous for three different deletional LBM-causing LRP5 mutations, found that approximately 50% of these subjects had diabetes, with abnormalities reported in either blood HbA1c levels or in oral glucose tolerance tests (OGTT) (Saarinen et al., 2010). Those with abnormal OGTTs had low first phase insulin release but were not insulin resistant. These interesting observations are limited by the lack of a control population. For example, this population included overweight patients as well as patients with significant comorbidities, that could have confounded the result. The study also lacked statistical power to detect potential differences in metabolic parameters.

LRP6. While this thesis focuses on LRP5 and its role in human metabolism, recent studies have elucidated a role for LRP6 in fuel metabolism. Mani et al. identified a non-conservative loss of function mutation in LRP6 that attenuates transcription

factor 7 like-2 (TCF7L2)-dependent transcription of the insulin receptor gene (Singh et al., 2013). Affected individuals have a syndrome of early-onset type 2 diabetes, coronary artery disease, metabolic syndrome, and osteoporosis inherited in an autosomal dominant pattern. Carriers exhibit hyperinsulinemia and reduced insulin sensitivity in peripheral tissues, particularly skeletal muscle, which can be detected prior to the development of impaired glucose tolerance (Mani et al., 2007).

STATEMENT OF PURPOSE, HYPOTHESIS, SPECIFIC AIMS OF THESIS

As reviewed in the Introduction, studies in animals suggest a physiologic link between LRP5 and metabolism. However, there is no consensus opinion in human studies. In addition there have been no human studies on the metabolic consequences of HBM-causing LRP5 mutations. This thesis explores the role of LRP5 in human glucose and lipid metabolism by studying volunteers with HBM-causing LRP5 mutations. Studies in this population could provide insights into LRP5's role, if any, in common diseases such as diabetes and hypercholesterolemia.

We hypothesize that the LRP5 pathway has a similar role in humans, as in mice, and that HBM-causing LRP5 mutations in humans will lead to improved glucose homeostasis and lipid metabolism compared to matched controls. The specific aims of this thesis are to:

1. Measure the impact of HBM-causing mutations in LRP5 on human glucose and lipid metabolism
2. Investigate the pathway(s) by which Wnt may affect glucose metabolism *in vitro*

METHODS

Study Subjects. Nine subjects with the G171V HBM-causing LRP5 mutation were recruited from two previously reported kindreds (Fig.1A, Fig.1B) (Boyden et al., 2002; Lee et al., 2013). Two subjects with an N198S (asparagine to serine) mutation in LRP5 associated with HBM were also studied (Lee et al., 2013). We also recruited 22 gender, age, race, and body mass index (BMI)-matched individuals unrelated to the kindred members to serve as controls.

HbA1c and insulin were measured in all participants. Two control subjects matched for gender, age, and BMI were recruited for every study subject. Five subjects, in total, from the affected and control groups reported taking lipid lowering medications and were excluded from the lipid analysis portion of the study. Recent data suggest a link between HMG-CoA reductase inhibitor use and type 2 diabetes (Carter et al., 2013). As this association has not established causality, we chose not to exclude these five subjects from the glucose analysis. Three subjects agreed to undergo an OGTT, and two underwent MRS studies. These subjects were matched for age, race, BMI, and activity level to a large control group (N=54).

The study complied with the World Medical Association Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects. The study was approved by the Yale Human Research Protection Program IRB. All subjects gave written consent to participate in the study after the purpose, nature and potential risks of the studies were explained.

I Human Studies

The author of this thesis recruited, enrolled and studied all volunteers whose data are reported here unless otherwise specified.

Hemoglobin A1c. Participants were instructed to fast from midnight onward. All blood sampling took place between 7am-10am on the Yale New Haven Hospital Research Unit. HbA1c was measured in whole blood by the Yale New Haven Clinical Immunology Lab, using Premier Hb9210 boronate affinity high-performance liquid chromatography with eluates measured at 413±2nM absorbance according to manufacturers instructions (Trinity Biotech; Jamestown, NY).

Insulin. Plasma concentrations of insulin were measured with a Human Insulin Specific RIA Kit (Millipore; St. Charles, MO) in the Yale Mineral Metabolism Laboratory. HOMA-*B* and HOMA-IR were mathematically derived from the measured insulin value and the estimated average glucose (eAG) from the same blood sample (Nathan et al., 2008).

OGTT. Whole body insulin sensitivity was assessed in collaboration with Dr. Kitt Falk Petersen (Yale University, Department of Internal Medicine, Section of Endocrinology; New Haven, CT) with a 3-hr OGTT. Subjects were fasted from 8pm the night prior to testing. Twenty minutes after insertion of an antecubital IV line, fasting blood samples were collected for determination of plasma glucose. The 75g dextrose drink (Glucola; Curtin Matheson Scientific, Houston, Tx) was then

administered, and blood samples collected at 10, 20, 30, 60, 90, 120, 150, and 180 min for determination of plasma glucose and insulin concentrations. The Insulin Sensitivity Index (ISI) was used to estimate insulin sensitivity (Petersen et al., 2006). Plasma glucose concentrations during the OGTT were measured using a YSI STAT 2700 Analyzer (Yellow Springs Instrument Co., Yellow Springs, CA). Insulin concentrations were measured using a Linco RIA kit (EMD Millipore; Billerica, Massachusetts).

Lipid panel. Total cholesterol, high density lipoprotein (HDL), and triglycerides (TG) concentrations were measured on an Ace Clinical Chemistry System autoanalyzer (Alfa Wassermann; West Caldwell, NJ). Low density lipoprotein (LDL) concentrations were calculated from TC, HDL, and TG with the Friedewald formula (Friedewald, Levy, & Fredrickson, 1972).

^{1}H MRS Assessment of Intramyocellular, Extramyocellular, and Hepatic Lipid Content. Intramyocellular (IMCL) and extramyocellular (EMCL) and hepatic lipid contents were measured using ^{1}H MRS also in collaboration with Dr. Kitt Falk Petersen as previously described (Petersen et al., 2006). Muscle lipid content was measured in the soleus muscle using an 8.5 cm diameter circular ^{13}C surface coil with twin, orthogonal circular 13-cm ^{1}H quadrature coils. The probe was tuned and matched and scout images of the lower leg were obtained to ensure correct positioning of the subject and to define an adequate volume for localized shimming using the FASTMAP procedure (Gruetter, 1993). The liver TG content was measure by ^{1}H

respiratory-gated STEAM spectroscopy in a 15x15x15 mm^3 voxel. Acquisition was synchronized to the respiratory cycle and triggered at the end of expiration. A water-suppressed lipid spectrum and a lipid-suppressed water spectrum were acquired in three different locations of the liver to account for liver inhomogeneity. A minimum of six spectra was acquired for each subject and the total lipid content was averaged and calculated. In addition, hepatic lipid content was corrected for transverse relaxation, using the transverse relaxation times of 22 ms for water and 44 ms for lipid, as previously described (Rabol, Petersen, Dufour, Flannery, & Shulman, 2011).

II *In vitro* studies

The author of this thesis designed and conducted all the *in vitro* experiments described here, unless otherwise specified.

Materials. Recombinant murine Wnt3a and human Wnt3a were purchased from R&D Systems (Minneapolis, MN). References to "Wnt" in these experiments will only refer to Wnt3a use. Insulin Humalog ND1048 was from Eli Lilly and Company (Indianapolis, IN). Glucagon and dexamethasone were purchased from Sigma-Aldrich (St. Louis, MO). DMEM, heat-inactivated fetal bovine serum (FBS), and Opti-MEM GlutaMAX were all purchased from Gibco-Life Technologies (Carlsbad, CA). All reagents used for INS-1 cell cultures were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Cells. Primary hepatocytes isolated from Sprague Dawley (SD) rats were purchased from Life Technologies (Carlsbad, CA). HepG2 cells (p76) were purchased from American Type Culture Collection (ATCC; Manassas, VA). Expression levels of LRP5 transcripts were measured in tissues isolated from SD rat purchased from Charles River (Wilmington, MA). The INS-1 832/13 rat insulinoma cell line was generously provided by Dr. Richard Kibbey (Yale University; New Haven, CT), and initial stocks were originally received from Christopher Newgard (Duke University; Durham, North Carolina) (Hohmeier et al., 2000).

Primary Rat Islets. Sprague Dawley rat pancreatic islets were prepared by Tamara Dlugos in the Yale Diabetes Endocrinology Research Center.

Primary Hepatocyte Cell Cultures. Pelleted cells were re-suspended twice in media containing high glucose DMEM, 10% FBS, 1nM dexamethasone, 1nM insulin, and P/S (1:100). Cells were plated at an initial density of $5x10^5$ in collagen-coated 6-well plates and incubated for four hours at 37°C. Cells were then washed 1x with phosphate buffered saline (PBS) and incubated at 37°C overnight in OptiMEM-GlutaMAX. The next morning cells were washed 1x with PBS and incubated for two hours in glucose-free DMEM containing sodium bicarbonate. Cells were washed again with PBS, and then received one of the following treatments in glucose-free, bicarbonate-free DMEM:

1. 500 nM dexamethasone
2. 100 nM glucagon with 1mM sodium pyruvate

3. 10 nM insulin, Wnt (100 ng/mL)
4. Wnt and dexamethasone
5. Wnt and glucagon with 1mM sodium pyruvate
6. dexamethasone and insulin
7. glucagon and insulin

Media was sampled at 1, 3, 5, 12, and 24 hrs after treatment was initiated. The 24 hour time point data were used for the analyses reported here. Cells were washed with PBS and RNA extracted using the RNeasy kit (Qiagen, Valencia, CA).

HepG2 Cell culture. Cells were thawed and centrifuged according to the ATCC recommended protocol. They were then treated with the same reagents under identical conditions as described above in the section on Primary Hepatocyte Cell Cultures.

INS-1 Cell Culture. This experiment was performed in collaboration with Dr. Richard Kibbey (Yale University; New Haven, CT) and Rebecca Pongratz from his laboratory. INS-1 cells were maintained as monolayers in RPMI 1640 complete medium with 11.1mM D-glucose supplemented with 10% (v/v) fetal bovine serum, antibiotics (10,000 units/ml penicillin and 10 mg/ml streptomycin), 10mM HEPES, 4mM L-glutamine, 1mM sodium pyruvate, and 50mM beta-mercaptoethanol. For experiments, cells were plated into 6-well plates at a density of 2.0 x10^5 cells per well and cultured at 37°C until 80% confluent. Cells were then randomly assigned to

three conditions: Wnt pretreatment, no Wnt pretreatment, or no Wnt treatment. For pretreatment Wnt (100ng/mL) was added the night prior to glucose stimulation. Equivalent amounts of PBS were added to the non-pretreatment cells to ensure equivalent volumes of media. On the day of treatment cells were washed with PBS and then pre-incubated in basal media for 90 minutes. Basal media was glucose-free DMEM containing 3.7g/L NaHCO3, 4 mM glutamine, 10 mM HEPES, 2.5 mM dextrose and 0.2% 96% fatty acid-free bovine serum albumin. Wnt was added to the media of cells selected for Wnt treatment. Media was then changed to basal media containing either 2.5mM dextrose ±Wnt, or stimulation media containing 9mM dextrose ±Wnt for 45 minutes. Following the incubation, media fractions were collected and placed on ice until analyzed for insulin content. The remaining media was aspirated, cells were washed with PBS x2 and then 1 ml of ice cold 0.1% Triton X100 was added. Cell lysates were collected and assayed for protein.

INS-1 insulin analysis. Insulin concentration in the conditioned media was measured using the High Range Rat Insulin ELISA kit in 96-well plates (ALPCO Diagnostics, Salem, NH). All media extracts were normalized to a cell protein concentration using a Micro-BCA Protein Assay Kit (Thermo Scientific; Waltham, MA) and the manufacturer's recommended protocol.

Human islet perfusion. This experiment was also performed in collaboration with Dr. Kibbey and Ms. Pongratz. Human islets were obtained from the University of Alberta Islet Core, Alberta Diabetes Institute, Edmonton (Pigeau et al., 2009). Islets were

cultured in complete CMRL media at 37°C with 5% CO_2, 95% air until the time of the experiment. Prior to perfusion, approximately 160 islets (40 islets/per replicate) were pretreated with Wnt (75ng/mL) for 19 hours. Another 160 islets were designated as the control (no Wnt pretreatment). Islets were perfused with DMEM basal media containing 2.5mM glucose (D5030, Sigma). Forty islets per chamber were perifused with basal media for 45 minutes to equilibrate the islets to the column and instrument. After the pre-incubation, islets were perifused with the different treatments and 100ul of perifusate collected per minute. The perifusion protocol consisted of a basal period of 2.5mM glucose for 20 min in which Wnt (75ng/mL) was added back to the treatment group islets after 10min and maintained for the duration of the perifusion. The basal period was followed by a 9mM glucose stimulation for 45min. The glucose concentration was then reduced to 2.5 mM glucose for 15 min followed by a second stimulation with 30 mM KCl at 2.5mM glucose (5min.) to evaluate insulin secretion independent of glucose metabolism. The islets were then allowed to recover in basal media for 5 min. Insulin levels in the perfustate were measured with an ELISA for human insulin (80-INSHU-E01, ALPCO Diagnostics, Salem, NH) and normalized to the DNA content of the cell layer (P7589, Molecular Probes).

Quantitative Real Time PCR. Total RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA) per the manufacturers instructions. cDNA synthesis was carried out at 43° C for 45 min followed by denaturation at 95° C for five minutes using a cDNA synthesis kit (Stratagene, La Jolla, CA). Quantitative Real Time PCR (qPCR) was

performed with TaqMan PCR reagent kits (Stratagene, La Jolla, CA) using 40 ng of RNA with a reaction volume of 20uL using the Opticon 2 DNA Engine Continuous Fluorescence Detection System (MJR, Waltham, MA). Specific primer/probe sets for target genes were purchased from Applied Biosystems-Life Technologies (Carlsbad, CA) (see Table 1). All qPCR reactions were performed in duplicate, and cycling conditions were 95° C for 20s and 60° C for 1 min for 39 cycles. The amplification signal from the target gene was normalized to a *beta*-glucuronidase signal in the same reaction. The relative expression levels were determined using the comparative CT method (also known as the 2CT method). Data are presented as the relative mRNA levels.

Statistical Analyses. We estimated we could recruit 11 subjects from the affected kindreds suitable for analysis to be paired with 22 matched controls in a 2:1 ratio. Based on preliminary control data, we estimated that the standard deviation (SD) for HbA1c would be 0.22(%). Since we did not know, *a priori*, what the SD would be for HbA1c in the affected individuals we conservatively estimated it at 0.44(%). Using these estimates for SDs, we would have 99% power to see a difference in HbA1c values of 0.5% or greater, and 80% power to see a difference of 0.3% or greater (GraphPad StatMate 2.0: GraphPad Software Inc., La Jolla, CA).

Statistical differences were analyzed using the unpaired Student t test, and one-way ANOVA where appropriate (GraphPad Prism version 6.00 for Windows, GraphPad Software; La Jolla California USA, www.graphpad.com). Data presented are mean

±standard error of the mean (SEM). Differences were considered statistically significant when $p<0.05$.

RESULTS

I Human Studies

Baseline characteristics of the affected subjects and the age, gender, and BMI-matched control group are shown in Table 2. Mean BMI of these groups were 27.4±1.8 and 27.6±1.1, respectively.

HbA1c is not significantly different in individuals with HBM mutations in LRP5

The parameters of glucose metabolism measured in the study subjects are presented in Table 3. Two affected subjects were known diabetics taking insulin. These subjects and their four matched controls were excluded from analysis. With 9 affected individuals and 18 controls, we had 99% power to see a 1.0% difference and 80% power to see a 0.6% difference in HbA1c values. Changes of this magnitude would have been clinically meaningful. However, we did not observe any difference in this parameter. There were no statistically significant differences between affected and control populations for HbA1c ($p=0.06$), eAG ($p=0.06$), insulin ($p=0.82$), HOMA-*B* ($p=0.34$) and HOMA-IR ($p=0.66$). Mean HbA1c for affected and control groups were 5.8±0.3% and 5.4±0.5% respectively. One affected subject and one control subject were found to have elevated HbA1c levels in the diabetic range (≥6.5) when tested for the purposes of this study. HbA1c values are depicted in Figure 2.

OGTT results were not significantly different in individuals with HBM LRP5 mutations

Three subjects with the HBM LRP5 mutation underwent an OGTT. Blood glucose (Fig. 3A), insulin (Fig. 3B) and c-peptide (Fig. 3C) levels were not consistently different from the control group, suggesting that there is no difference in insulin production as well as in insulin sensitivity in these three individuals when compared to a large control group.

IMCL and liver TG is not significantly different in individuals with HBM mutations in LRP5

As part of the planned investigation into the role of LRP5 in glucose metabolism, we also measured intramyocyte lipid as an index of insulin sensitivity. In particular, there is a substantial literature on assessing intramyocellular lipid (IMCL) content as a biomarker of insulin sensitivity: Magnetic resonance spectroscopy (MRS) studies in diabetic and non-diabetic patients have shown that insulin resistance can be attributed to post-receptor defects in insulin signaling, for example, reduced Glut-4 transporter activity caused by metabolites of intracellular lipids (Morino, Petersen, & Shulman, 2006; Petersen et al., 2007). Studies in a healthy, lean elderly individuals, lean and obese adolescents, and lean, insulin resistant offspring of patients with type 2 diabetes (Petersen et al., 2003; Petersen, Dufour, Befroy, Garcia, & Shulman, 2004; Sinha et al., 2002), all support the conclusion that insulin resistance results from the intracellular accumulation of fatty-acyl CoAs, and diacylglycerol in skeletal myocytes and hepatocytes. These adverse effects can be attenuated with weight loss (Petersen et al., 2005; Petersen et al., 2012; Rabol et al.,

2011).

Two of the LRP5 affected subjects underwent MRS of their skeletal muscle and liver (Table 4). The Insulin Sensitivity Index (ISI) was predictably higher in Subject B who had a lower BMI, lower percent body fat, and higher activity level (not shown) than Subject A. The ISI of both subjects compared to the control groups varies predictably with BMI and body fat percentage, with Subject A having a lower percent body fat and higher ISI compared with controls, and Subject B having a higher BMI and lower ISI compared with controls. The IMCL in both subjects was not significantly different compared to the control group. There was also no consistent trend in liver TG towards higher or lower percentage lipids compared with controls.

LDL cholesterol levels are lower in affected study subjects

Lipid profiles for the affected individuals are presented in Table 5. Three affected and two control subjects were known to have hypercholesterolemia and were taking cholesterol-controlling medications at the time of study. These subjects, and their matched controls, were excluded from the analysis.

There were no significant differences in total cholesterol, TGs, and HDL, between control and affected subjects (Figs. 4A-C). However as shown in Figure 4D, LDL levels were significantly lower in affected subjects ($p=0.04$).

II *In vitro* studies

Tissue survey

As an initial step to explore the role of Wnt signaling in glucose metabolism, we surveyed the expression of LRP5 in several tissues involved in fuel metabolism. LRP5 expression was highest in liver, and lowest in bone (Fig. 5). These data largely agree with previously published tissue expression surveys of LRP5 (Hey et al., 1998).

Testing the hypothesis that Wnt suppresses gluconeogenesis by inhibiting PEPCK-C

HBM mutations in LRP5 are posited to exert their effect on the skeleton by causing a functional increase in Wnt signaling. This is not due to ligand-independent receptor signaling, but rather due to a resistance of these mutant receptors to endogenous inhibitors such as DKK1. Thus, in vivo, these mutant receptors transmit a greater signal in the presence of endogenous inhibitors than the wild type receptor. From a functional standpoint, therefore, these mutations constitute gain-of-function in Wnt signaling. We therefore sought to determine the effect of Wnt signaling on glucose metabolism by testing two hypotheses.

The first was that Wnt signaling would suppress gluconeogenesis by inhibiting one of the key enzymes in the gluconeogenic pathway, the cytosolic form of phosphoenolpyruvate carboxykinase (PEPCK-C). PEPCK-C catalyzes the first committed step of gluconeogenesis, specifically the conversion of oxaloacetate+GTP –> phosphoenolypyruvate +GDP+$C0_2$. It is not regulated post-translationally or

allosterically but entirely transcriptionally such that enzyme activity is directly proportional to the level of transcript expressed in the liver. (Chakravarty, Cassuto, Reshef, & Hanson, 2005). Previous work in primary hepatocytes, hepatoma cell lines, and animal models has explored the effect of canonical Wnt signaling on PEPCK-C and modulation of the gluconeogenic response (Hall, Wang, George, Koch, & Granner, 2007; Liu et al., 2011). While the PEPCK promoter has no known canonical Wnt-binding motifs, PEPCK-C is inhibited by glucagon synthase kinase 3 (GSK3) a downstream target for canonical Wnt signaling. Activation of Wnt signaling suppresses GSK3 activity (Frame & Cohen, 2001). Therefore we hypothesized that Wnt signaling would suppress gluconeogenesis, by inhibiting PEPCK-C expression.

Wnt does not effect PEPCK-C expression in primary hepatocytes

As depicted in Figures 6A-C, and as expected, PEPCK expression in primary hepatocytes was suppressed by insulin, and induced by dexamethasone and glucagon. PEPCK induction by glucagon was blunted by insulin. These are all known effects of insulin, dexamethasone and glucagon and confirm that our *in vitro* assay is sensitive enough to detect changes induced by hormones and cytokines. However, the addition of Wnt did not have a significant suppressive effect on PEPCK-C expression as compared to the control (Fig. 7A) nor did it blunt the effect of glucagon (Fig. 7C). Unexpectedly, Wnt seemed to enhance the effect of dexamethasone though the change was not statistically significant (Fig. 7B). These results were confirmed in experiments under the same conditions using cells from the HepG2 cell line.

Testing the hypothesis that Wnt augments glucose-stimulated insulin secretion in pancreatic islets

The effect of Wnt on insulin release from pancreatic islet *beta*-cells was also explored as an alternative mechanism by which Wnt signaling could affect glucose metabolism. As noted earlier, Wnt fails to stimulate insulin secretion from islets isolated from LPR5 knock out mice; conversely, exposure of wild type islets to Wnt stimulated glucose-induced insulin secretion (Fujino et al., 2003).

We initially undertook studies using the INS-1 832/23 cell line since these cells have important similarities to pancreatic *beta*-cells including those related to insulin secretion (Cline, Lepine, Papas, Kibbey, & Shulman, 2004; Hohmeier et al., 2000; Lu et al., 2002; Pongratz, Kibbey, Shulman, & Cline, 2007). Therefore we expected that the results in these cells would likely mirror those in primary human islets.

Wnt does not augment glucose-stimulated insulin secretion in INS-1 cells

9.0 mM glucose stimulated insulin secretion in INS-1 cell two-fold compared to the secretory rate under basal conditions (2.5mM Glucose). Adding Wnt to either basal or to stimulatory glucose concentrations had no statistically significant effect on insulin secretion (Fig.8).

Given prior reports that Wnt is active in pancreatic *beta*-cells we next sought to confirm LRP5 expression in the INS-1 832/23 cells. For these studies the level of LRP5 transcript expression in the INS-1 832/23 cells was compared to that in

primary rat islets. LRP5 expression is significantly lower in the INS-1 832/23 cells when compared to the primary cells (Fig. 9). This suggests that Wnt does not affect insulin secretion in INS-1 832/23 cells perhaps in part because the level of receptor expression is too low.

Wnt does not augment glucose-stimulated insulin secretion in primary human islets

In view of the finding that LRP5 was only expressed at a low level in INS-1 832/23 cells, we next examined the effect of Wnt on insulin section in primary human islets. Human islets are known to have robust expression of LRP5 (Hey et al., 1998). That notwithstanding, glucose-induced insulin secretion in primary human islets was not affected by Wnt (Fig. 10). While these data are consistent with our finding in the INS-1 cells, they are at variance with prior studies using murine islets in which Wnt treatment led to a doubling of glucose-induced insulin secretion (Fujino et al., 2003).

DISCUSSION

The prevalence and reach of diabetes across generations and populations is well known. Over eight percent of the United States population has diabetes. In particular, there is an increased incidence among adolescent and non-white populations (http://www.cdc.gov/diabetes/pubs/pdf/ndfs_2011.pdf). Hospital and health systems face soaring direct medical costs, which topped $176 billion in 2012. The average annual cost of caring for a diabetic is 2.3 times higher than a non-diabetic, multiplying the cost of the disease across hospitalizations and outpatient visits ("Economic costs of diabetes in the U.S. in 2012," 2013). Notably, these

estimates do not account for the additional, significant cost of reduced productivity and caregiver burden.

Wnt signaling through LRP5 represents a newly appreciated metabolic pathway that might be a target for drug discovery in type 2 diabetes. In preadipocytes Wnt and insulin signaling have been shown to interact with LRP5 exerting a positive effect on insulin signaling (Palsgaard et al., 2012). Studies in rodents support the conclusion that LRP5 mediates glucose and lipid metabolism (Fujino et al., 2003; Magoori et al., 2003). However, data from human studies in LBM-causing mutations populations has not been as consistent, with some studies suggesting that these individuals have impaired glucose metabolism and others failing to find a similar trend (Saarinen et al., 2010; Zenibayashi et al., 2008).

In view of these conflicting data and because a variety of methodologies have been used to measure glucose and lipid metabolism we revisited the relationships in a human population that has not yet been studied: Those with HBM-causing-mutant LRP5 receptors.

Our data show that compared to matched controls, individuals with HBM-causing LRP5 mutations did not demonstrate improved glucose homeostasis. HbA1c, a common clinical test for diagnosis and clinical monitoring of diabetes, was not significantly different between control and affected populations. Similarly, there was no difference in eAG, insulin levels, Homa-*B*, and Homa-IR scores. As noted, our

study included members of multiple kindreds, encompassing two different HBM-causing mutations in LRP5. While the study was sufficiently powered to detect a difference in HbA1c between the two study groups, it was not possible to do a meaningful sub-analysis of the small number of individuals with the N198S mutation to determine if their response differed from that in the individuals with the G171V mutations. However, a sub-analysis of G171V mutation kindred members alone also demonstrated no significant difference from the control group ($p=0.07$) (data not shown). In other studies in these kindreds it has been suggested that the in vivo actions of these two mutation differed (Lee et al., 2013).

A smaller sample of affected LRP5 subjects underwent an OGTT and MRS of liver and skeletal muscle. In agreement with the data in the larger study group, blood glucose, insulin levels, and c-peptide levels were no different in these three individuals compared to controls. The ISI varied appropriately with BMI, percent body fat and activity level, and there was no trend to indicate improved insulin sensitivity in the two affected individuals who participated in these analyses. Previous studies have found a strong relationship between IMCL content and muscle insulin resistance, suggesting that intracellular lipid metabolites might be a causal factor in its pathogenesis. MRS measures of TG in muscle and liver are useful proxies for assessing specific fatty acid content (i.e. diacylglycerols) that could otherwise only be evaluated with invasive techniques such as muscle biopsy. We hypothesized that one mechanism by which LRP5 affected individuals could have improved glucose metabolism was through improved insulin sensitivity resulting

from reduced IMCL content. Given our small sample size, we would have needed to see a very robust difference between the findings in the two study subjects and the entire control group. However, insulin sensitivity in these two study subjects was no different from that of the control subjects. It is worth recalling that two affected subjects were taking insulin and were excluded from these analyses. A third affected subject was found to have a serum HbA1c in the diabetic range. These observations support the findings that HBM-causing mutations do not impact glucose homeostasis. Consistent with that, our data also indicate that HBM-causing LRP5 mutations do not improve insulin sensitivity or insulin production. If LRP5 plays a role in glucose homeostasis, then this study suggests that its impact is minimal in comparison to more dominant factors thus keeping us from observing it in a small population.

In addition to effects on glucose homeostasis, animal studies also point to a role for LRP5 in lipid homeostasis. LRP5$^{-/-}$ mice fed high fat diets had elevated cholesterol, and impaired plasma clearance of diet-derived triglycerides implying a possible ApoE-independent role for LRP5 in triglyceride hydrolysis (Magoori et al., 2003). However, studies in human LBM-causing mutation populations, have not found differences in lipid levels. Of note, these studies were not control-matched, and one study included multiple subjects on lipid lowering drugs. (Saarinen et al., 2010; Zenibayashi et al., 2008).

Our analysis excluded individuals on lipid lowering drugs and controlled for gender, age, and BMI. Total cholesterol, triglycerides and HDL were not different between

affected and controls populations. However, LDL was significantly lower in the affected subjects. This finding is particularly interesting as the LRP5 receptor is known to bind ApoE-containing lipoproteins *in vitro* (Kim et al., 1998).

LRP5 is known to be highly expressed in the liver and in hepatocytes (Hey et al., 1998; Kim et al., 1998). We confirmed this in the current study. We hypothesized that the addition of Wnt would inhibit PEPCK-C, a rate-limiting step in the gluconeogenic pathway. Since accelerated gluconeogenesis plays an important role in type 2 diabetes, this hypothesis had considerable clinical relevance. However our *in vitro* studies consistently showed no effect Wnt on PEPCK-C in primary hepatocytes. Neither the induction of PEPCK by dexamethasone or glucagon or the suppression by insulin was affected by Wnt. It remains possible that Wnt signaling through LRP5 has targets genes other than PEPCK to modulate gluconeogenesis, that were not considered in the current study.

We also examined the effect of Wnt treatment in INS-1 cells and human islets to assess the impact this has on insulin secretion. Rat INS-1 cells, considered a valid system to study insulin secretion dynamics (Hohmeier et al., 2000), responded appropriately to glucose stimulation with a robust increase in insulin secretion. However, they did not further increase insulin production in response to Wnt treatment. Since these cells had low levels of LRP5 compared with primary rat islets, these studies were extended to human islets. Wnt treatment for 16 hours did not change human insulin production under basal or glucose-stimulated culture

conditions. These findings are at odds with the finding that Wnt increased glucose-induced insulin secretion in murine islets (Fujino et al., 2003). The reason for these divergent findings is not clear. One obvious possibility is that murine and human islets respond differently to Wnt. Why then does murine bone apparently faithfully recapitulate the effects of Wnt on the human skeleton? This may be because the macroarchitecture of bone as well as the interactions of bone cells are very similar in mouse and man. In contrast, the architecture of the murine islet as well as its cellular composition is different from the human islet (Steiner et al., 2010), and so may be the biological mechanism involved in the development and maintenance of these tissues.

Conclusion

Studies of LBM-causing mutations in LRP5 in both experimental models and in humans with OPPG suggest that loss of function in this receptor is associated with impaired glucose tolerance. However these studies are limited by the lack of control groups and small numbers of subjects studied. Our study, the first in humans with HBM-causing mutations in LRP5, included a carefully matched control group and a larger number of affected individuals. These findings provide strong evidence for a limited role of LRP5 in glucose metabolism as we could not demonstrate an interaction between the two.

The HBM-causing mutations studied in our kindreds are not tissue-specific in their actions (as demonstrated by our expression survey), and should change Wnt

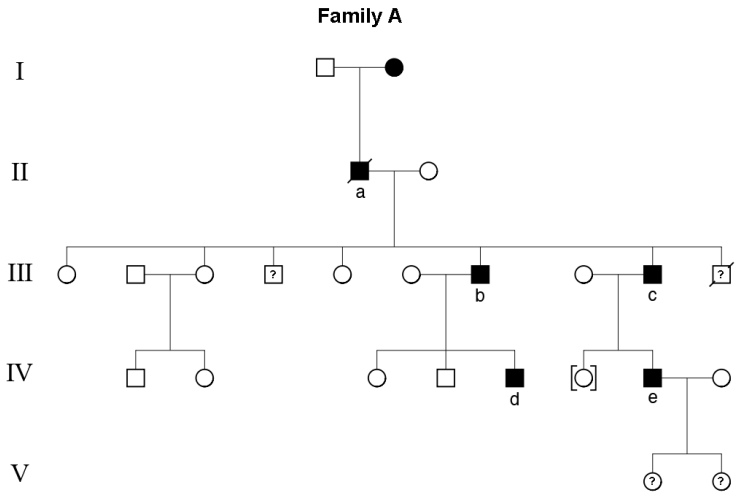
signaling anywhere that Wnt acts. Our study population included two HBM-causing mutations, each with extreme skeletal phenotypes. Given the robust phenotype in bone, we anticipated a considerable phenotype in glucose homeostasis. Although the sample size for the OGTT and MRS sub-analyses were too small to demonstrate statistical significance, we could identify no trend toward increased insulin sensitivity or altered insulin production. In fact, three of the kindred members screened for inclusion in this study had diabetes and/or insulin resistance. These results are in direct contradiction to the findings in previously reported animal models, underscoring the potential limitations in extending some findings in mice to humans.

While our findings do not directly evaluate the role of loss of function mutations in LRP5 on glucose metabolism, they do make LRP5 a less attractive target for drug discovery in diabetes. However, it does appear that there is an effect on LDL metabolism, a major risk factor for coronary artery disease. To detect a statistically significant effect on LDL even with our small sample, certainly suggests that the molecular mechanism underpinning this effect warrants further study.

In summary, our data does not support the hypothesis that LRP5 improves glucose metabolism in individuals with HBM-causing LRP5 mutations. The data suggest that there may be a specific, beneficial effect of LRP5 on LDL cholesterol. Additional studies should focus on clarifying the cellular and molecular mechanisms by which these occur.

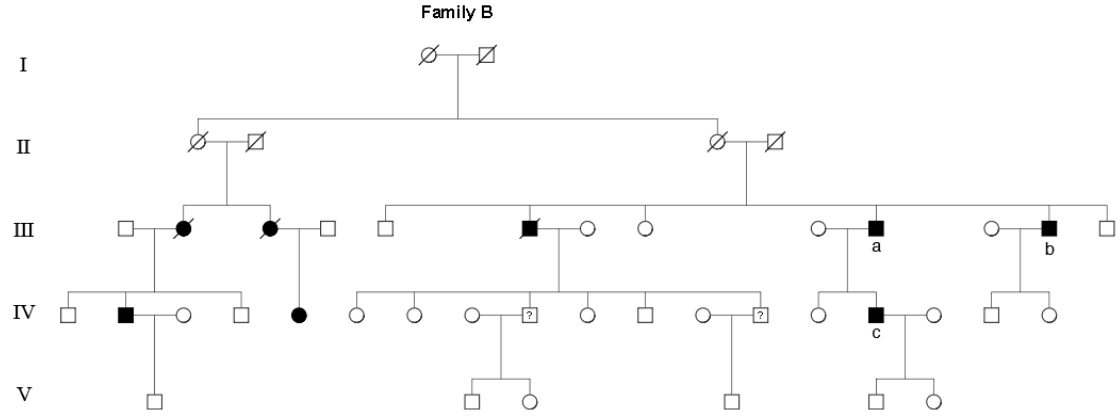
FIGURES & TABLES

Figure 1A. G171V Kindred Pedigree of Family A



Affected kindred members, indicated by dark symbols, have a single base pair mutation causing a glycine to valine change at position 171 in the low density lipoprotein related receptor protein 5 (LRP5). This results in a high bone mass (HBM) phenotype.

Figure 1B. G171V Kindred Pedigree of Family B



Affected kindred members, indicated by dark symbols, have a single base pair mutation causing a glycine to valine change at position 171 in the low density lipoprotein related receptor protein 5 (LRP5). This results in a high bone mass (HBM) phenotype.

Table 1. TaqMan assays used for qPCR reactions.

Gene Symbol	NCBI gene reference	TaqMan assay ID
Pck1	NM_198780.3	Rn01529014
Alp1	NM_007431.2	Rn01516028
LRP5	NM_001106321.2	Rn01451428
Actb	NM_031144.2	Rn00667869

QPCR was used to analyze PEPCK-C mRNA expression in experiments using RNA from primary hepatocytes and HepG2 cells. LRP5 mRNA expression was analyzed in a variety of rat tissues, INS-1 cells, and primary rat islets.

Table 2. Baseline characteristics of all study participants

Variable	Affected (n=11)	Matched Controls (n=22)	p value
Age (years)	63±7	63±4	0.96
Male (n, %)	9 (82%)	18 (82%)	n/a
Female (n, %)	2 (18%)	4 (18%)	n/a
BMI (kg/m^2)	27.4 ±1.8	27.6±1.1	0.92

The term “affected” refers to those study subjects with the HBM-causing LRP5 mutations. There were no statistical differences in age and BMI between the two groups. There were equivalent numbers of males and females enrolled in both groups. This table demonstrates that the control group was appropriately matched for these characteristics to the affected individuals. Data is shown as mean±SEM.

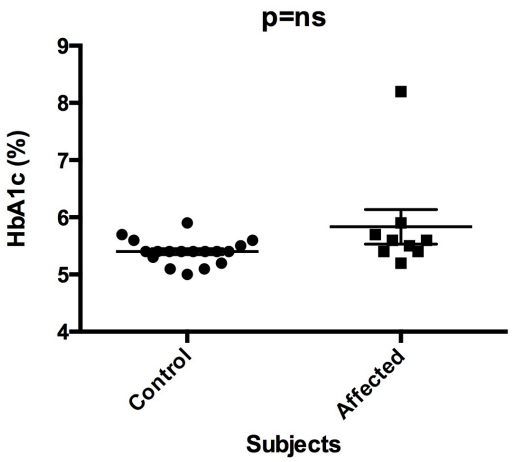
Table 3. Measurements of glucose metabolism in affected study participants

Pt#	LRP5 genotype	BMI (kg/m^2)	HbA1c (%)	eAG[A]	Insulin (uU/mL)	HOMA[B]-IR	HOMA-*B*
1*	g171v	22.3	6.4	137	5.5	1.9	26.8
2	g171v	20.8	5.6	114	8.0	2.3	56.5
3	g171v	35.0	8.2	188.6	14.9	6.9	42.7
4	g171v	34.0	5.2	102.5	17.8	4.5	162.2
5	g171v	29.4	5.6	114	10.6	3.0	74.8
6	g171v	24.8	5.4	108.3	9.9	2.7	78.7
7	g171v	32.7	5.9	122.6	24.4	7.4	147.4
8	g171v	26.7	5.5	111.2	5.8	1.6	43.3
9*	g171v	34.7	6.8	148.5	12.1	4.4	51.0
10	n198s	21.2	5.7	116.9	12.4	3.6	82.8
11	n198s	20.1	5.4	108.3	7.0	1.9	55.6
Mean±SEM		*27.4±1.8*	*6.0±0.3*	*124.7±7.6*	*11.7±1.7*	*3.6±0.6*	*74.7±13.0*

[A] Estimated average glucose= 28.7xHbA1c-46.7 (Nathan et al., 2008)
[B] Homeostatic Model Assessment
* Subject takes insulin

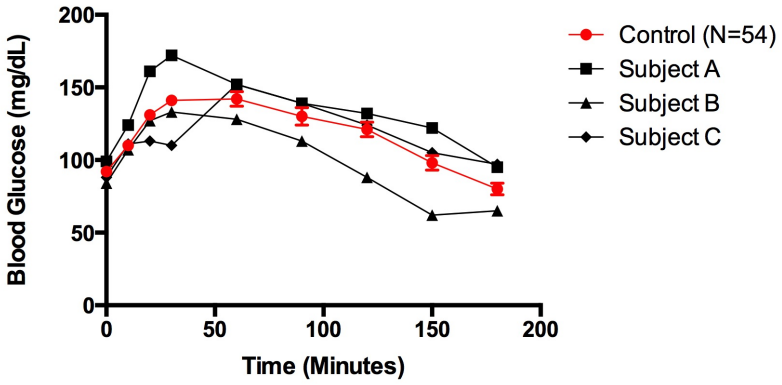
This table summarizes the results of the studies of glucose metabolism in the affected individuals. Two subjects using insulin were excluded from subsequent analysis.

Figure 2. HbA1c results in study participants



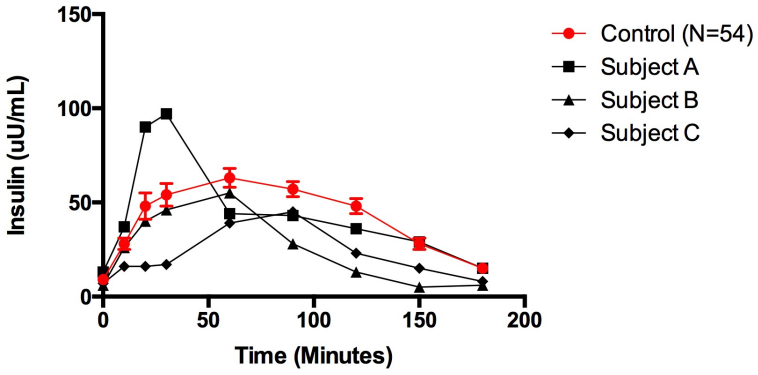
HbA1c values are not significantly different between LRP5 affected individuals and age, gender, and BMI matched controls (p=0.06). Error bars represent the standard error of the mean.

Figure 3A. Changes in blood glucose during the OGTT in three study participants



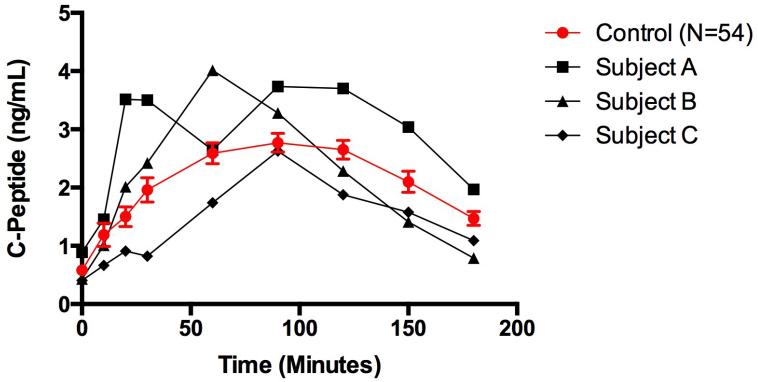
Three affected subjects underwent an OGTT. There was no consistent difference in glucose levels in these three individuals compared to a group of previously studied age, race, BMI, and activity-matched controls (N=54). The testing and control data were generously provided by Dr. Kitt Falk Petersen.

Figure 3B. Changes in insulin levels during the OGTT in three study participants



Three affected subjects underwent an OGTT. There was no consistent difference in insulin levels in these three individuals compared to a group of previously studied age, race, BMI, and activity-matched controls (N=54). The testing and control data were kindly provided by Dr. Kitt Falk Petersen. Subject B has more intramyocyte lipid and is likely somewhat insulin resistant (see Table 4).

Figure 3C. Changes in C-peptide levels during the OGTT in three study participants



Three affected subjects underwent an OGTT. There was no consistent difference in c-peptide levels in these three individuals compared to a group of previously studied age, race, BMI, and activity-matched controls (N=54). The testing and control data was kindly provided by Dr. Kitt Falk Petersen.

Table 4. Intramyocellular lipid and hepatic lipid triglyceride content by ^{1}HMRS

Subject	BMI (kg/m^2)	Body Fat (%)	ISI	IMCL (%)	Liver TG (%)
Subject A	27.8	25.3	3.1	1.4	4.3
Subject B	25.7	19.4	6.6	1.7	0.6
Control (n=54)	23.8±0.4	27.6±1.4	4.7±0.2	1.3±0.1	1.5±0.3

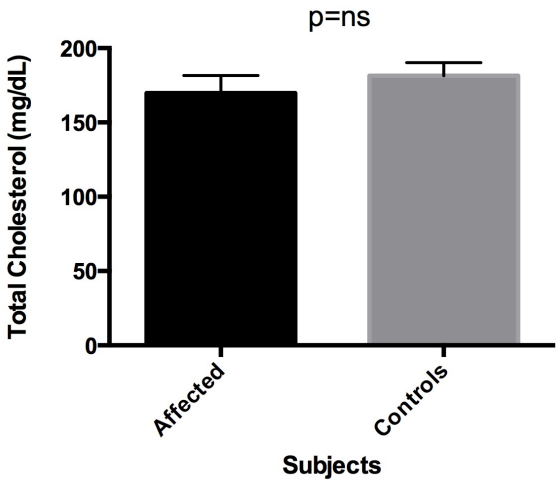
Two affected subjects underwent ^{1}HMRS of skeletal muscle and liver for lipid content. Subjects were compared to a control group (n=54) matched for age, race, BMI, and activity level. While the sample is too small for statistical analysis, there was no trend observed in the data towards higher or lower percent lipid content, which has been shown to be an important marker of insulin resistance (Petersen et al., 2007). ^{1}HMRS testing was generously conducted by Dr. Kitt Falk Petersen. Data is shown as mean±SEM.

Table 5. Lipid levels in affected individuals

Pt#[A,B]	BMI (kg/m^2)	Total Cholesterol (mg/dL)	TG (mg/dL)	HDL (mg/dL)	LDL (mg/dL)
2	20.8	115	49	40	65.2
4	34.0	180	112	58	99.6
6	24.8	185	66	71	100.8
7	32.7	163	104	45	97.2
8	26.7	149	44	68	72.2
9	34.7	143	140	49	66.0
10	21.2	209	132	101	81.6
11	20.1	212	96	80	112.8
Mean±SEM	*26.9±2.2*	*169.5±11.8*	*92.9±12.9*	*64.0±7.2*	*86.9±6.4*

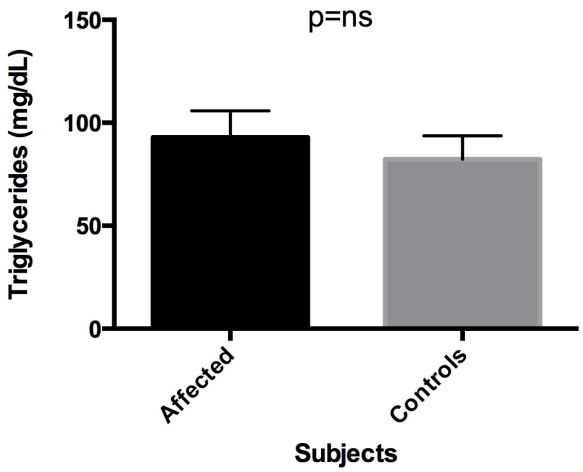
[A] Genotypes as listed in Table 3.
[B] Study subjects #1, #3, #5 were excluded for reasons described in text.
[C] LDL=TC-HDL-(TG/5) (Friedewald et al., 1972).

Figure 4A. Comparison of total blood cholesterol in affected and control individuals



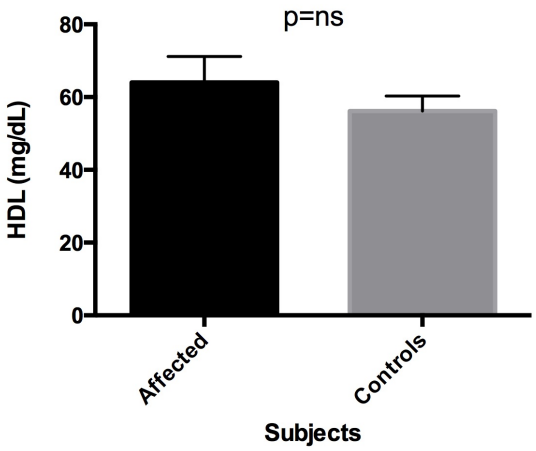
Individuals taking lipid lowering drugs were excluded from the analysis. Total cholesterol levels were not significantly different (p=0.43). Data is shown as mean±SEM.

Figure 4B. Comparison of blood triglyceride levels in affected and control individuals



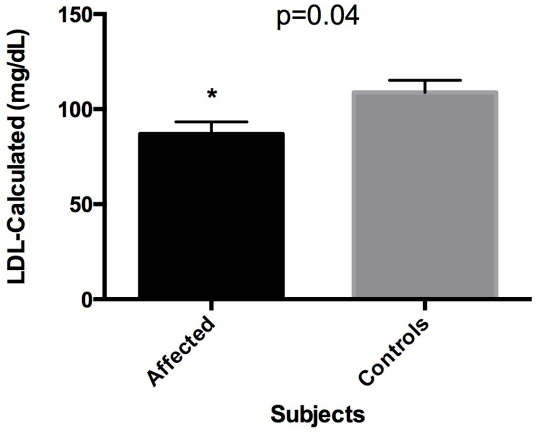
Individuals taking lipid lowering drugs were excluded from the analysis. Triglyceride levels were not significantly different (p=0.56). Data is shown as mean±SEM.

Figure 4C. Comparison of high density lipoprotein (HDL) level in affected and control individuals



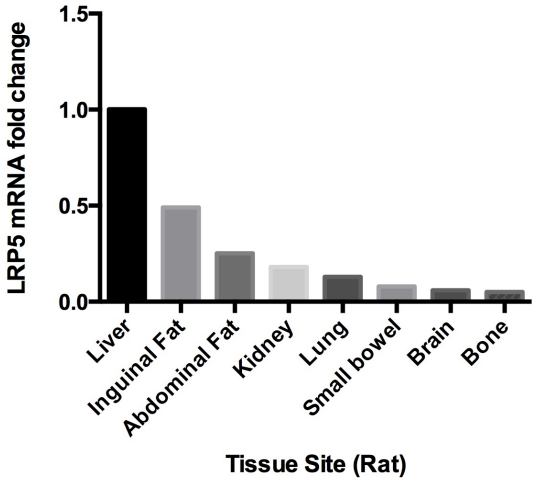
Individuals taking lipid lowering drugs were excluded from the analysis.. HDL levels were not significantly different (p=0.32). Data is shown as mean±SEM.

Figure 4D. Comparison of low density lipoprotein (LDL) levels in affected and control individuals



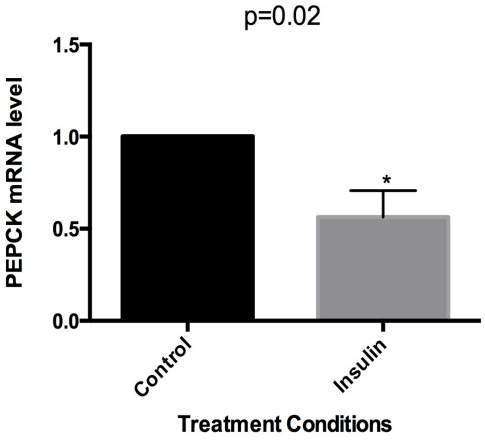
Individuals taking lipid lowering drugs were excluded from the analysis. LDL levels were significantly lower in affected individuals (p=0.04). Data is shown as mean±SEM.

Figure 5. Relative expression of LRP5 in rat tissues



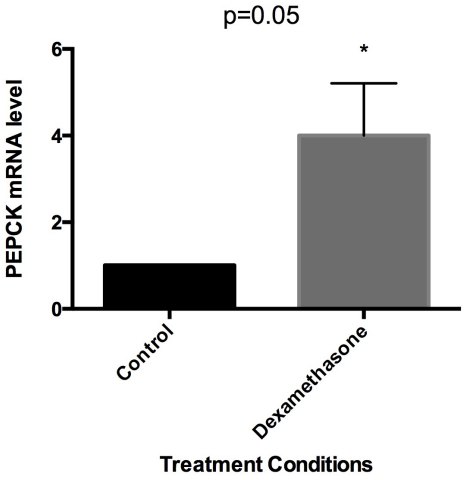
LRP5 expression was highest in the liver, and lowest in bone. Transcript expression is defined as fold change in comparison to liver.

Figure 6A. Regulation of PEPCK-C expression in primary rat hepatocytes by insulin



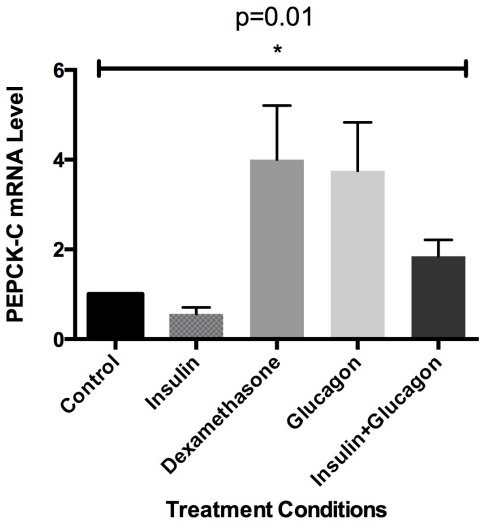
Phosphoenolpyruvate carboxykinase (cytosolic form) (PEPCK-C) expression in primary rat hepatocytes was measured in the presence of known inducers and inhibitors to confirm the sensitivity of our *in vitro* assay. Insulin predictably suppressed PEPCK-C expression compared to the control (p=0.02). Data is shown as mean±SEM.

Figure 6B. Regulation of PEPCK-C expression in primary rat hepatocytes by dexamethasone



PEPCK-C expression in primary rat hepatocytes was measured in the presence of known inducers and inhibitors to confirm the sensitivity of our *in vitro* assay. Dexamethasone predictably induced PEPCK-C expression compared to the control (p=0.05). Data is shown as mean±SEM.

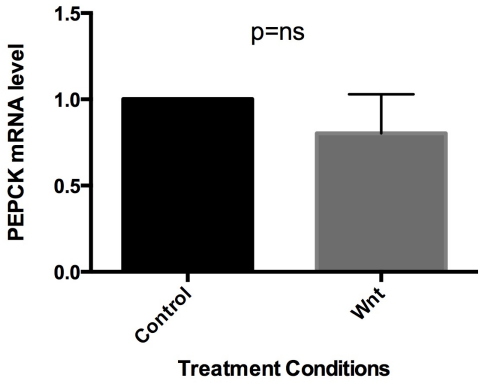
Figure 6C. Regulation of PEPCK-C expression in primary rat hepatocytes by glucagon, insulin and dexamethasone



PEPCK-C expression in primary rat hepatocytes was measured in the presence of known inducers and inhibitors to confirm the sensitivity of our *in vitro* assay. Glucagon predictably induced PEPCK-C expression compared to the control. This experiment confirmed the induction of PEPCK-C by dexamethasone, and its

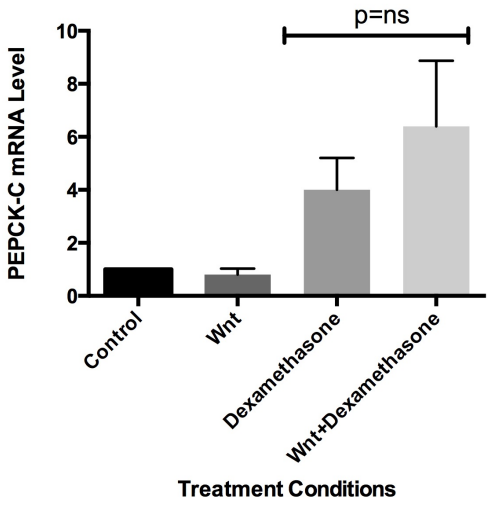
suppression by insulin relative to control. The effect of glucagon was blunted by insulin (p=0.01). Data analyzed using one-way ANOVA. Data is shown as mean±SEM.

Figure 7A. Effect of Wnt on PEPCK-C expression in primary rat hepatocytes



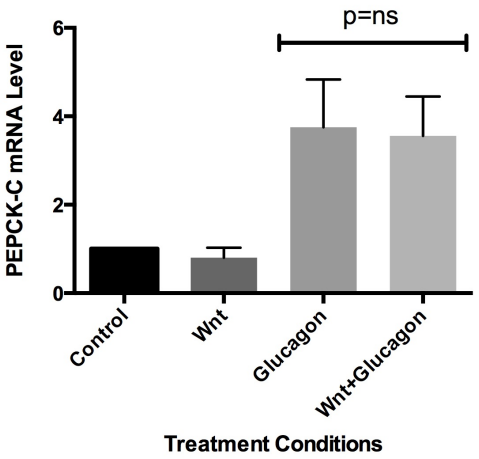
PEPCK-C levels were measured after treatment for 24 hours with 100ng/ml of Wnt. Wnt had no effect on PEPCK-C transcript expression (p=0.42). Data is shown as mean±SEM.

Figure 7B. Effect of Wnt and Dexamethasone-induced expression of PEPCK-C in primary rat hepatocytes



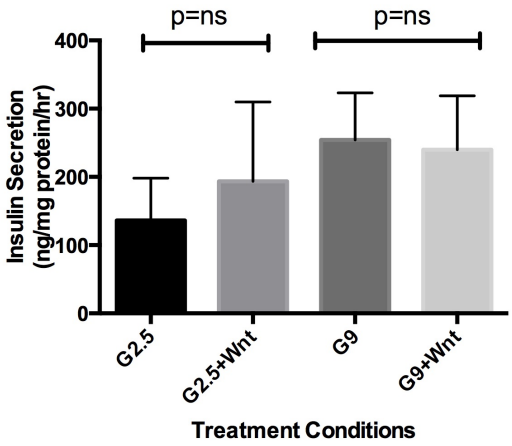
PEPCK-C transcript levels were measured after treatment with Wnt or Wnt in combination Dexamethasone. Dexamethasone alone induced PEPCK-C expression. The addition of Wnt did not significantly change the effect of dexamethasone (p=0.42). Data is shown as mean±SEM.

Figure 7C. Effect of Wnt and glucagon on PEPCK-C expression in primary rat hepatocytes



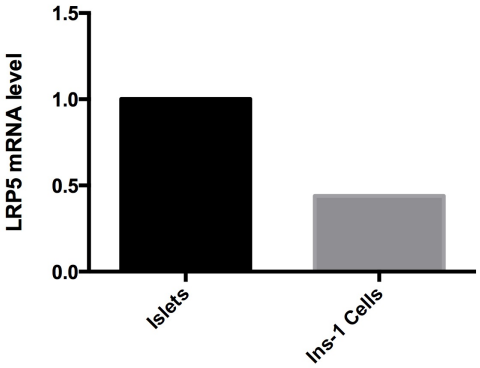
PEPCK-C levels were measured in after treatment with Wnt or in combination with glucagon. Glucagon alone induced PEPCK-C expression. The addition of Wnt did not significantly alter the response to glucagon (p=0.90). Data is shown as mean±SEM.

Figure 8. The effect of Wnt on glucose-induced insulin secretion in INS-1 cells



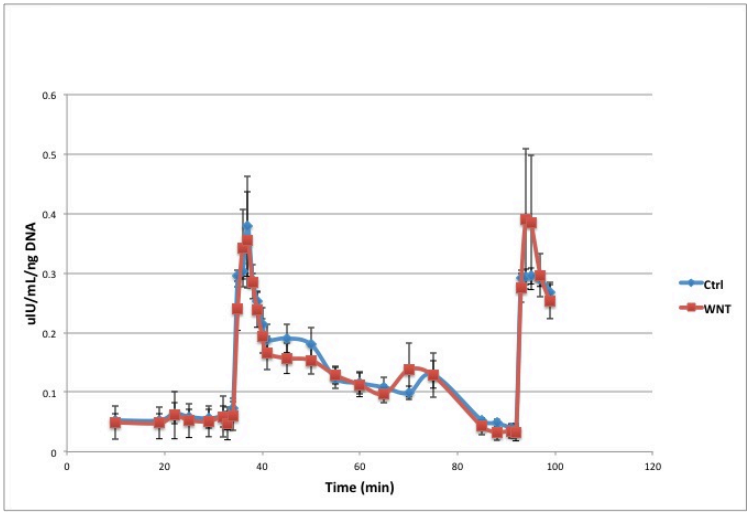
INS-1 cells were treated with Wnt in the presence of basal (2.5mM) and stimulatory (9.0 mM) levels of glucose) High extracellular glucose significantly increased insulin secretion. However, the addition of Wnt did not change basal or glucose-stimulated insulin secretion. Data is shown as mean±SEM.

Figure 9. LRP5 transcript expression in INS-1 cells and primary rat islets.



LRP5 transcript expression in the INS-1 cells was compared to that in primary rat islets. LRP5 expression in INS-1 cells was less than half of that in the primary rat islets.

Figure 10. Effect of Wnt on glucose-induced insulin release from primary human islets



Human islets were perifused with Wnt and exposed to basal (2.5mM) and stimulatory (9.0mM) concentrations of glucose. Glucose-induced insulin secretion was not affected by Wnt treatment.

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