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Using Neonatal Skin to Study the Developmental Programming of Aging

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Abstract

Numerous studies have examined how both negative and positive maternal exposures (environmental contaminants, nutrition, exercise, etc.) impact offspring risk for age-associated diseases such as obesity, type 2 diabetes, hypertension, and others. The purpose of this study was to introduce the foreskin as a novel model to examine developmental programming in human neonates, particularly in regards to adipogenesis and insulin receptor signaling, major contributors to age-associated diseases such as obesity and diabetes. Neonatal foreskin was collected following circumcision and primary dermal fibroblasts were isolated to perform adipocyte differentiation and insulin stimulation experiments. Human neonatal foreskin primary fibroblasts take up lipid when stimulated with a differentiation cocktail and demonstrate insulin signaling when stimulated with insulin. Thus, we propose that foreskin tissue can be used to study developmental exposures and programming that occur in the neonate as it relates to age associated diseases such as obesity and diabetes.

Keywords

epigenetics; insulin sensitivity; obesity; pregnancy; adipogenesis; DOHaD

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CONFLICTS OF INTEREST:

The authors have no conflicts of interest to report.

1.0 INTRODUCTION

The Barker hypothesis supports that maternal under-nutrition during pregnancy reprograms offspring to be at greater risk for developing cardiovascular and metabolic disease later in life (Barker et al., 1993). Since this early work, the Developmental Origins of Health and Disease (DOHaD) hypothesis has been expanded to include a large number of intrauterine exposures during fetal and early postnatal development (Wadhwa et al., 2009). Numerous studies have examined how negative and even positive maternal exposures (environmental contaminants, nutrition, exercise, etc.) impact fetal development and long-term offspring health. Studies report altered risk of obesity, type 2 diabetes, hypertension, and others in offspring from mothers who are exposed to a number of environmental influences (Alfaradhi et al., 2014; Carter et al., 2012; Carter et al., 2013; Ino, 2010; Rashid et al., 2013; Swanson et al., 2009; Wang et al., 2002; Zhang et al., 2014). Further, obesity and type 2 diabetes are associated with early death (Kitahara et al., 2014; Leal et al., 2009). Thus, understanding how to prevent and treat obesity and type 2 diabetes is crucial to healthy aging.

The Dutch Famine of 1944 presented a unique time to study developmental programming and specifically the impact of undernutrition during pregnancy on offspring late life disease risk. Roseboom *et al.* found that adult children born to mothers undernourished during pregnancy were smaller at birth and had increased prevalence of cardiovascular disease morbidity and mortality compared to adult children who were not exposed to undernutrition *in utero* (Roseboom et al., 2001). Heijmans *et al.*, (Heijmans et al., 2008) followed up on these studies and demonstrated that whole blood samples from adults who were exposed to the famine 6 decades earlier had reduced insulin-like growth factor-2 (IGF2) differentially methylated region (DMR) compared to their same sex siblings not exposed to famine. This study was one of the first to provide empirical evidence that undernutrition during gestation epigenetically alters individuals. Since these landmark studies, over 126 articles have been published using the key phrases “developmental programing aging” on PubMed, expanding upon the idea that maternal environmental exposures can impact long-term healthy aging.

Gavrilov and Gavrilova put forth the High Initial Damage Load Hypothesis which suggests that high amounts of damage early in development contribute to the accumulated damage associated with aging later in life, giving these events a large impact on lifespan (Gavrilov and Gavrilova, 2004). The basis of their hypothesis stems from studies suggesting that human paternal age can influence aging rates in female offspring (Gavrilov et al., 1997), and the season of birth can affect human longevity (Gavrilov and Gavrilova, 1999). Using animal models, Ozanne and Hales found that offspring born to female mice fed a protein restricted diet had decreased longevity when fed a normal or high fat diet after birth (Ozanne and Hales, 2004; Ozanne and Hales, 2005). These mice were born lighter than offspring from standard diet fed females, but they achieved rapid “catch-up” growth early in life and aged more rapidly. Miller *et al.* have been studying the importance of growth trajectories and found that smaller mice, at young and old age, generally live longer in the absence of the catch-up growth seen in the studies by Ozanne (Miller et al., 2000; Miller et al., 2002). In addition, Sun *et al.* found that increasing litter size from 8 to 12 pups during nursing increased lifespan of the offspring due to decreased growth (Sun et al., 2009). Together these data suggest that age-related mechanisms and lifespan can be influenced by the prenatal or

early postnatal environment. Given that pregnancy in humans is only ~40 weeks and the entire lifespan of a human living in the United States of America is on average 78.8 years (CDC, 2015), the relative investment in time during pregnancy is low to make positive modifiable behavior changes which can impact offspring health for years to follow. However, examining the mechanisms of how maternal behaviors/exposures during pregnancy directly impact offspring health in humans is a major challenge.

Globally, many laboratories are investigating molecular and physiologic features in accessible newborn tissues, such as human placenta and cord blood to explore developmental changes induced by maternal parameters, and these samples have provided valuable information about *in utero* exposures. However, these provide a cross-sectional snapshot of potential variation accumulated over the course of pregnancy, and generally, do not allow for experimental manipulation to determine the functional relevance of these characteristics. In order to overcome these challenges, we propose to use the foreskin as a model to study developmental programming in humans. The skin is readily available via circumcision at birth and through skin punches later allowing for lifelong aging comparisons. This reason sets it apart from the placenta and cord blood, which represent unique samples available at birth. The foreskin is generally considered a waste tissue and thus is readily available where circumcisions are performed. Further, there is ample tissue (roughly 400 mg per foreskin) present for the extraction of RNA, DNA, or protein. In addition, primary fibroblasts can be isolated from the dermal layer and will allow for the creation of an *in vitro* system for living cell experiments that are representative of the neonatal environment (or *in utero* exposures). In fact, other laboratories are already utilizing skin-derived fibroblasts in the study of aging (Harper et al., 2007; Salmon et al., 2005). The purpose of this study is to introduce the foreskin as a novel model to examine developmental programming in human neonates, particularly in regards to adipogenesis and insulin receptor signaling, major contributors to age-associated disease.

2.0 METHODS

Approximately 58% of male infants in the United States are circumcised (Owings, 2013). Neonatal foreskins from the Birthing Center at the University of Kentucky Chandler Hospital were collected immediately following planned circumcision into phosphate buffered saline (PBS) and transported on ice to the laboratory. Foreskins were collected from male infants undergoing routine circumcision within 96 hours of birth. Study protocols were either approved by or considered exempt by the Institutional Review Board of the University of Kentucky.

2.1 Experimental Design

2.1.1 Experiment 1: Confirm that foreskin primary dermal fibroblasts differentiate into lipid droplets and markers regulating adipogenesis appear to be regulated epigenetically—

Markers of increased and decreased adiposity such as fatty acid binding protein 4 (FABP4) (mRNA) (Krusinova and Pelikanova, 2008; Terra et al., 2011) and Ras and Rab Interactor 3 (RIN3) (mRNA and DNA methylation), respectively,

(Padilla et al., 2014; Wens et al., 2013) were measured from foreskin primary dermal fibroblasts.

2.1.2 Dissection and plating of foreskin dermal primary fibroblasts—Fat and loose fascia (dartos/hypodermal layer) were grossly dissected away from the epidermal/dermal layers. Dermal cells were isolated and plated according to standardized procedures from Life Technologies with slight modifications. In brief, tissue was treated with 5 mL dispase II solution (25 U/mL) (Life Technologies, catalogue #17105-041) for 16–20 hours in order to separate the dermis from the epidermis. Using forceps, the epidermis was peeled from the dermis and discarded. The dermal layer was put in a culture dish with 10 mL of media (Gibco, catalogue #10569-010) supplemented with 10% fetal bovine serum and gentamicin/amphotericin (Life Technologies, catalogue #R01510) and cut into small pieces. Media and dermal pieces were then placed in a 50 mL conical tube with 10 mL of collagenase type IV solution (1,500 U/mL) (Life Technologies, catalogue #17104-019) with an additional 10 mL of media (total of 30 mL) for 1 hour at 37°C. Every 15 min the tube was shaken for 30 seconds to agitate and breakdown dermal pieces. Following digestion, the conical tube was spun at 180g for 7 min to pellet tissue and cells. Media and collagenase were aspirated off and cells were re-suspended in 5 mL of media. The media was then filtered through a 0.22 μm filter (BD Transduction, catalogue #352340) onto a 100 × 20 mm round plate. Another 5 mL of media was rinsed through the filter and added onto the plate. Cells were grown in a 37°C incubator with 5% CO_2. Media was changed 24 hours following plating and every 48 hours following until cells were at 80% confluency (generally 3–4 days). Cells were passaged onto a 12 well plate for adipocyte differentiation experiments.

2.1.3 Adipocyte Differentiation—Two days following confluency, adipocyte differentiation induction medium [cocktail of media (Gibco, catalogue #10569-010), 10% fetal bovine serum, gentamicin/amphotericin, 1μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 10μg/mL insulin and 1.0 μM rosiglitazone] was added to half of the wells for 72 hours to induce adipocyte differentiation. The remaining wells served as controls and only contained cell media (Gibco, catalogue #10569-010), 10% fetal bovine serum and amphotericin. Insulin (10 μg/mL), rosiglitazone (1.0 μM), and cell media were replaced every 48 hours for an additional 11 days. Media was refreshed on the control cells at the same time. Cells were stained for lipid droplets (described below) and DNA, RNA, and protein were collected.

2.1.4 DNA methylation—Foreskins were collected and dermal primary fibroblasts grown in culture on 12-well plates. Cells were isolated for the examination of DNA methylation of an array of genes assessed 48 hours post-confluency and 14 days following adipogenesis (Section 2.1.3) (Green et al., 2016). DNA was extracted from cell pellets using the Qiagen DNease mini kit via standard manufacturer procedures, and the quality and quantity of DNA was assessed via NanoDrop spectrophotometry. Isolated DNA underwent sodium bisulfite modification using the EZ Methylation kit (Zymo Research, Irvine, CA), were plated randomly across 12 sample batches, and assessed for genome-wide DNA methylation using the Infinium MethylationEPIC Bead Chip (Illumina, San Diego, CA) profiling methylation status for approximately 800,000 CpG loci. The microarrays were processed at the

Biomedical Genomics Center at the University of Minnesota (Minneapolis, MN) following manufacturer's protocol. Data was assembled using the BeadStudio methylation software package (Illumina) and then processed using the 'minfi' package in R (R Core Team 2013). Data were normalized using the functional normalization (funNorm) protocol within the 'minfi' package as specified by the software authors (Guide available at: http://bioconductor.org/packages/release/bioc/html/minfi.html). Following normalization, all X and Y probes were removed. Differentially methylated loci were identified with a 2 × 2 factorial linear regression where individual subjects were included as a random effect. Within the linear model, contrasts were made between cultures before and after adipogenic differentiation. Bonferroni correction was used when appropriate.

2.1.5 RNA quantification—RNA was collected and isolated from a different set of primary dermal fibroblasts than those included in the DNA methylation experiments using standard procedures from the Qiagen RNeasy kit. mRNA of RIN3 and FABP4 were assessed via quantitative real-time PCR using the Step One Plus Real-Time PCR System (Applied Biosystems, Life Technologies). 20 ng cDNA per reaction was used with RIN3 or FABP4 TaqMan Probes (Applied Biosystems, Life Technologies). Tubulin, beta class I (TUBB) was selected as the housekeeping gene.

2.1.6 Protein quantification—Protein was collected from primary fibroblasts following 14 days of incubation in the adipogenic cocktail as described above. Briefly, 50 μL of RIPA buffer (ThermoFisher Scientific, catalogue # 89900) was added to each well for 5 min. Cells were collected after scraping with a cell scraper and immediately frozen at −80°C until analysis for FABP4 protein via ELISA (R&D Systems, catalogue # DFBP40). A Bradford protein assay was used to assess protein concentration of each sample.

2.1.7 Oil Red O staining—Oil Red O staining was performed with modifications as previously described (Zhang et al., 2013). 0.25 g of Oil Red O (Sigma, catalogue #O-0625) was mixed with 50 mL of isopropanol. 15 mL of this stock Oil Red O solution was mixed with 10 mL of diH_2O and sat at room temperature for at least 5 min prior to filtration (Millipore, catalogue #8CGP00525) to make the working solution of Oil Red O. Cells were placed in 4% paraformaldehyde (Sigma, catalogue #441244) for 15 min prior to being washed once for 5 min in phosphate buffer saline (PBS). The cells were incubated in PBS for 5 min and then 60% isopropanol for an additional 5 min. Isopropanol was removed and Oil Red O working solution was added to each well for 30 min. Following removal of Oil Red O, cells were washed 4 times for 5 min in diH_2O and imaged. After imaging, cells were treated with 100% isopropanol and Oil Red O was extracted out of each well. The isopropanol/Oil Red O solution (250 uL) was loaded in triplicate on a 96 well plate and read on a plate reader at 500 nm for quantification of Oil Red O staining in each well.

2.2.1 Experiment 2: Examine whether primary dermal fibroblasts respond to insulin stimulation—Foreskins were collected and plated as described in section 2.1.2 to assess insulin signaling proteins following insulin stimulation. Cells were stimulated with insulin for 15 min and protein collected to assess phosphorylation of Protein Kinase B (pAKT) and AKT signaling.

2.2.2 Insulin Signaling—Primary fibroblasts from each of the 10 samples were plated onto 2, 60 mm × 15 mm style cell culture dishes. 24 hours post confluency media was removed from cells and Krebs-Ringer-Phosphate (KRP) buffer plus 1% BSA was added and the cells incubated for 1 hr at 37°C. Then half of the plates were treated with 100 nM insulin (Sigma, catalogue #I0908) for 30 min and half served as controls with KRP buffer only. Cells were washed twice in cold KRP buffer and protein collected as described above. Protein from each sample was mixed with cell lysis buffer and 5× Laemmli to dilute each sample to 2 ug/uL. 20 ug of sample was loaded onto a 10% gel (Bio-Rad, California, USA) and run at 100 volts for 10 min, then 200 volts for 45 min. Protein in the gel was then transferred to membrane at 100 volts for 45 min and incubated for 1 hour at room temperature in 5% milk. Total AKT (Cell Signaling, catalogue #4685s) and pAKT ser 473 (Cell Signaling, catalogue #9271S) primary antibodies were added to each respective membrane overnight at 1:1000 dilution at 4°C. Membranes were washed 3 times for 5 min in Tris-Buffered Saline and Tween 20 buffer (TBST) and secondary antibody (Life Technologies, catalogue #A10547) was added at 1:2000 dilution for 1 hour at room temperature. Membranes were washed again (5 times for 5 min each in TBST) and imaged using femto max (Life Technologies, catalogue #34095). Bands were quantified using Quantity One software (Bio-Rad).

2.3.1 Statistics—A one tailed, paired, student's t-test was used to analyze the difference in pAKT and AKT in non-stimulated and insulin stimulated cells and change in adipogenesis (mRNA, protein, and Oil Red O staining) in basal and differentiated cells. Natural log transformation was performed where data were not normally distributed (FABP4 mRNA and protein). Significance was set at $p < 0.05$ and individual data points are shown on figures with median bars.

3.0 RESULTS

3.1 Primary Dermal Fibroblasts

3.1.1 Experiment 1: Confirm that foreskin primary dermal fibroblasts differentiate into lipid droplets and markers regulating adipogenesis appear to be regulated epigenetically—Primary neonatal fibroblasts incubated in the adipogenesis media for 14 days showed a significantly greater abundance of vacuoles staining positive with Oil Red O (Fig. 1A) compared to basal conditions ($p=0.002$). FABP4 mRNA (Figure 1B) and protein (Figure 1C) were significantly increased in the differentiated cells compared to basal conditions ($p < 0.001$).

Genome-wide DNA methylation analysis indicated that 459 CpG sites demonstrated differential methylation between differentiated and basal cells (Bonferroni, $p < 0.05$), with probe ch.14.1488981R, in the promoter region of the RIN3 gene exhibiting the greatest differential methylation of an identified gene following adipogenesis. The DNA methylation was significantly higher at this site in cells following adipogenesis (Figure 2A) compared to the basal state ($p < 0.001$).

To then assess the functional importance of this methylation change, we examined expression of the gene via qPCR, and demonstrated a significantly reduced expression of

RIN3 mRNA following adipogenesis compared to basal ($p < 0.05$, Figure 2B), suggesting that methylation may be functionally reducing the gene expression.

3.1.2 Experiment 2: Examine whether primary dermal fibroblasts respond to insulin stimulation—AKT phosphorylation at serine 473 occurs downstream of insulin binding to the insulin receptor and is considered one of the main markers for insulin signaling (Bae et al., 2003). Primary dermal foreskin fibroblasts demonstrated increased pAKT (Figure 3A) in response to insulin stimulation ($p < 0.001$); while no significant changes in total AKT (Figure 3B) were observed ($p > 0.05$).

4.0 DISCUSSION

We demonstrate that foreskin primary fibroblasts can be plated and grown from foreskin tissue collected following circumcision of human infants. These dermal primary foreskin fibroblasts can differentiate into adipocyte-like cells expressing increased and decreased mRNA levels of FABP4 and RIN3, respectively, as well as increased RIN3 DNA methylation. These cells also display increased phosphorylation of AKT when stimulated with insulin. Thus, this tissue appears to be a good surrogate for examining the influences of birth weight or environmental parameters during pregnancy on metabolic activity in offspring utilizing a tissue that comes directly from the neonate after birth.

We recognize that gene expression in the foreskin is not likely the driving factor for later life onset of obesity or diabetes; however, cells of foreskin are representative of the types of programming that can occur. Further, one study demonstrates that skin glucose content correlates to venous blood glucose levels in individuals with type 1 diabetes undergoing a three stage glucose clamp (Jensen et al., 1995); indicating that a relationship between skin glucose and whole body plasma glucose exists. Whether or not these findings would be demonstrated in non-diabetic individuals is not known. However, it is likely that skin glucose uptake is mediated through insulin receptor substrate 2 (IRS2), as previous studies indicate that dermal fibroblasts lacking IRS2 have reduced cellular glucose uptake (Sadagurski et al., 2005). Thus, it appears that mechanisms of glucose uptake are similar between skin and other tissues more classically believed to be involved with regulating whole body blood glucose control. While earlier studies by others were not directly performed in foreskin dermal tissue, we demonstrate that this tissue maintains the basic features of insulin signaling similar to other tissues. Therefore, experiments in foreskin tissue and foreskin dermal primary fibroblasts are a critical step along the way to examining how environmental exposures during pregnancy may impact offspring glucose regulation and will serve to guide upcoming studies in humans. Future steps in our lab will be to examine radiolabeled glucose uptake in whole tissue and primary fibroblasts grown in culture, and these experiments will provide a functional measure of glucose homeostasis following a wide variety of environmental exposures *in utero*.

Thus, we demonstrate the capabilities of using foreskin dermal primary fibroblasts to examine adipogenic potential of cells from human infants. These findings confirm what others have shown in adult dermal primary fibroblasts (Jaager and Neuman, 2011). Collectively, these data support the use of foreskin from infants as a metabolically active

tissue to examine insulin signaling and adipogenesis. Obesity and diabetes are associated with a shortened lifespan (Kitahara et al., 2014; Leal et al., 2009). Thus, identifying mechanisms which are altered in early life may identify individuals who are at a greater risk of dying early and provide a targeted approach for therapeutic interventions to increase healthy aging.

Other laboratories are currently utilizing skin derived fibroblasts to study aging. The ability to respond to environmental and cellular stress plays a role in healthy aging. Salmon *et al.* (Salmon et al., 2005) demonstrate that dermal fibroblasts from a long lived mice strain have developed a resistance to cellular oxidative and non-oxidative stress which may be a mechanism of their increased lifespan. These results provide exciting support for the use of primary dermal foreskin fibroblasts in stress response experiments to examine the role of developmental programming and aging. While the foreskin tissue can only be collected at birth from neonates following circumcision, we can collect skin biopsy samples from adults later in life which would allow us to make multiple comparisons over time of how genes adapt and change following various *in utero* stimuli with aging.

In summary, the proposed studies introduce a novel approach to examine insulin signaling and adipogenesis in neonatal tissue. Using skin as a means to study mechanistic alterations in infants is the only viable neonatal tissue that can be easily collected following birth (circumcision) as well as the individual ages into adulthood (skin biopsy). The foreskin should be considered a tissue of interest for fetal programming studies in order to assess mechanisms of dysfunction in insulin signaling and adipocyte differentiation in response to various maternal exposures during pregnancy. Utilizing this model provides the foundation for future studies to predict pediatric and adulthood obesity, diabetes, and ultimately early death risk. Maternal factors which may impact early aging in offspring, such as maternal smoking, obesity, and exercise, can be examined via this model and will provide direct evidence of those factors which promote early aging in offspring. Thus, future studies should work towards long-term follow-up outcomes, examining the foreskin tissue shortly after birth and skin tissue into adulthood following specific maternal exposures to examine mechanisms of healthy (or diseased) aging, as well as the predictive potential of this tissue prospectively in children's growth and metabolic related outcomes.

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Highlights

- Foreskin primary fibroblasts accumulate lipid in response to an adipogenic cocktail
- Insulin stimulation increases insulin signaling in foreskin primary fibroblasts
- Foreskin is a useful tissue to study the developmental programming of aging

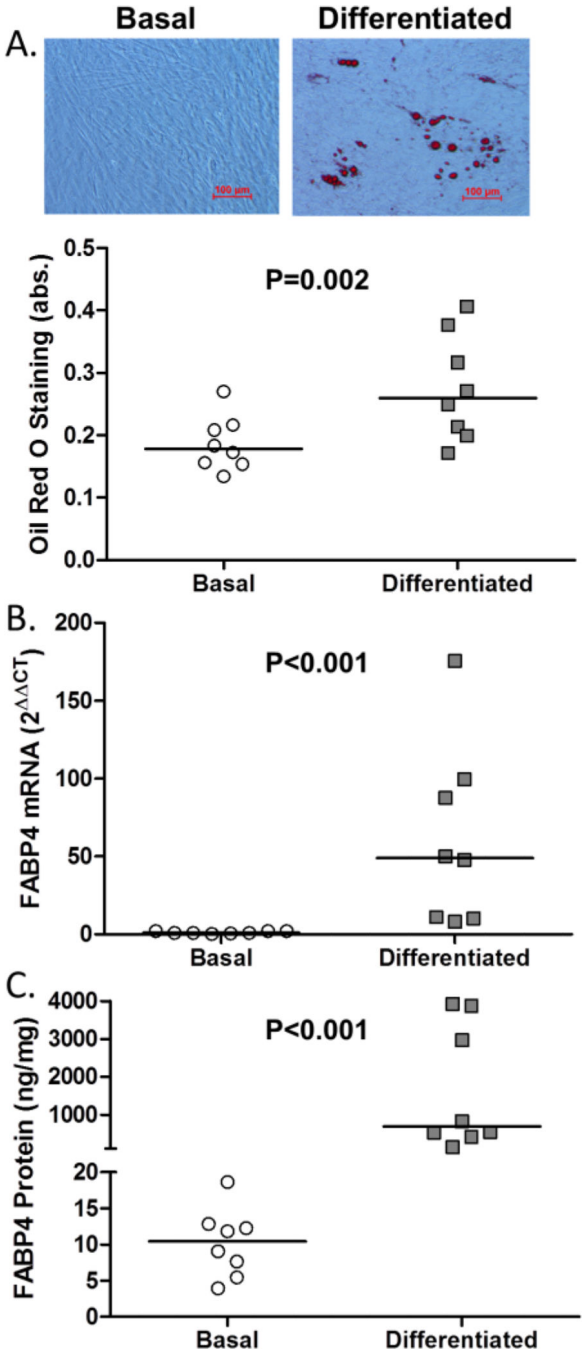

Figure 1.

Primary foreskin dermal fibroblasts following 14 days of basal media and adipocyte differentiation media. The cells were stained with Oil Red O in order to quantify lipid content *(A)*. Each image was obtained at a power of 20×. Fatty Acid Binding Protein 4 (FABP4) mRNA (*B*) and protein *(C)* increased following adipocyte differentiation.

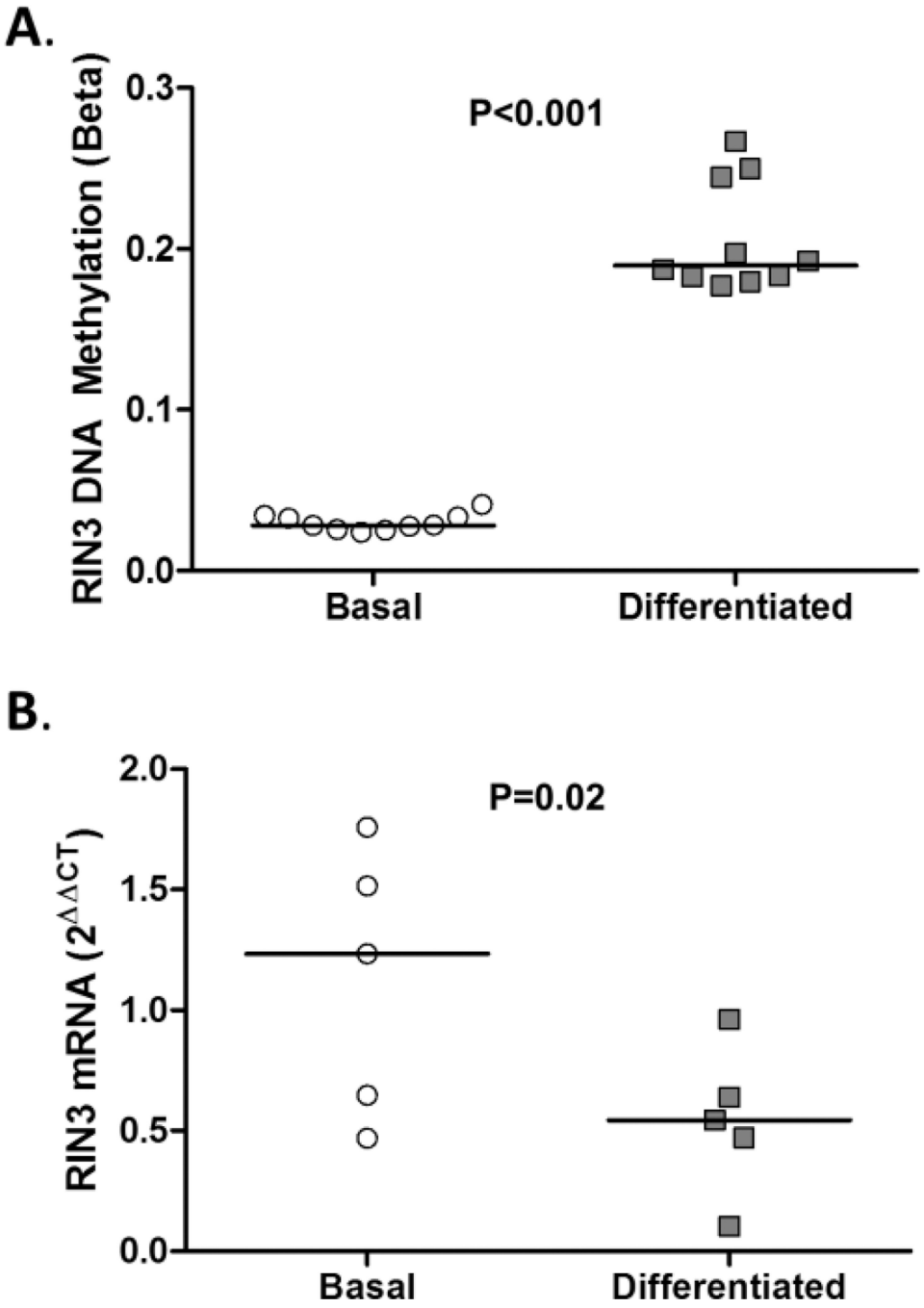

Figure 2.

Primary foreskin dermal fibroblasts DNA methylation at position 93077342 on chromosome 14 *(A)* and mRNA expression *(B)* of RIN3 48 hours after plating and following 14 days of adipocyte differentiation media.

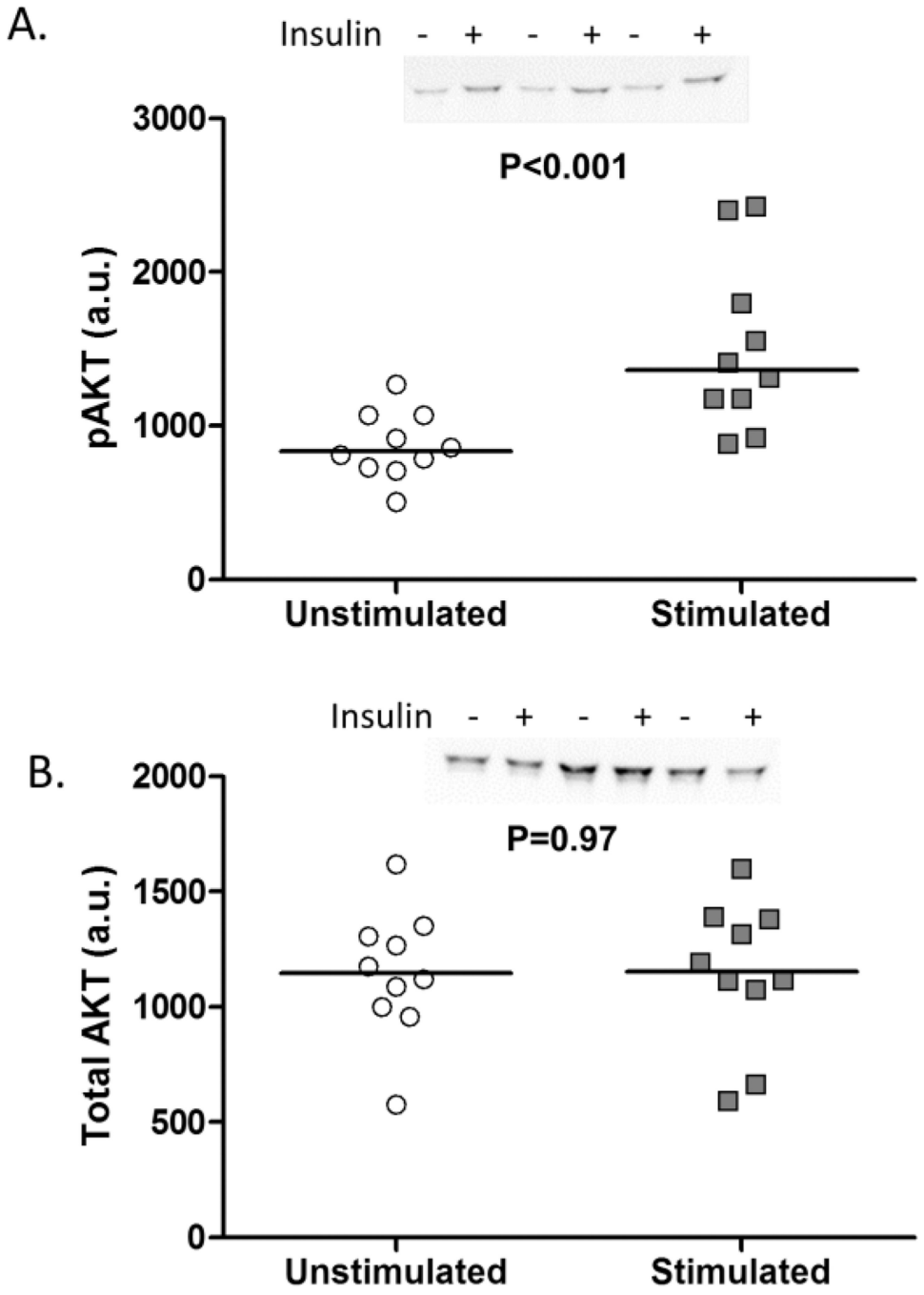

Figure 3.

Primary foreskin dermal fibroblasts with or without insulin had significantly increased AKT phosphorylation *(A)* but showed no change in total AKT *(B)*. Three representative samples out of 10 different primary foreskin dermal fibroblasts are shown.