Maternal Obesity Programs Senescence Signaling and Glucose Metabolism in Osteo-Progenitors From Rat and Human

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Nutritional status during intrauterine and early postnatal life impacts the risk of chronic diseases, presumably via epigenetic mechanisms. However, evidence on the impact of gestational events on regulation of embryonic bone cell fate is sparse. We investigated the effects of maternal obesity on fetal osteoblast development in both rodents and humans. Female rats were fed control or an obesogenic high-fat diet (HFD) for 12 weeks and mated with male rats fed control diets, and respective maternal diets were continued during pregnancy. Embryonic rat osteogenic calvarial cells (EOCCs) were taken from gestational day 18.5 fetuses from control and HFD dams. EOCCs from HFD obese dams showed increases in p53/p21-mediated cell senescence signaling but decreased glucose metabolism. Decreased aerobic glycolysis in HFD-EOCCs was associated with decreased osteoblastic cell differentiation and proliferation. Umbilical cord human mesenchymal stem cells (MSCs) from 24 pregnant women (12 obese and 12 lean) along with placentas were collected upon delivery. The umbilical cord MSCs of obese mothers displayed less potential toward osteoblastogenesis and more towards adipogenesis. Human MSCs and placenta from obese mothers also exhibited increased cell senescence signaling, whereas MSCs showed decreased glucose metabolism and insulin resistance. Finally, we showed that overexpression of p53 linked increased cell senescence signaling and decreased glucose metabolism in fetal osteo-progenitors from obese rats and humans. These findings suggest programming of fetal preosteoblastic cell senescence signaling and glucose metabolism by maternal obesity. (***Endocrinology*** **157: 4172–4183, 2016)**

Interactions between genes and dietary factors essentially influence tissue development and remodeling during both pre- and postnatal life. Obesity involves altered development and remodeling of adipose tissue, and largely results from over nutrition or excessive consumption of a high caloric, high-fat diet (HFD). Obesity during pregnancy increases risk of obesity in offspring and other long-term health outcomes in both the mothers and their offspring (1). Evidence from experimental animal models suggest that maternal obesity (or obesogenic HFD), independent of birth weight, leads to developmental programming of adiposity gain in the offspring (2–5), supporting the concept of developmental origins of health and diseases (6). Although bone mineralization within the skeletal envelope is strongly influenced by adult lifestyle such as the amount of physical activity, intrauterine programming of another chronic disorder, osteoporosis, has only been recently suggested (7).

Results from clinical studies determining the effect of obesity on postnatal bone quantity are inconsistent (8–11); however, feeding a HFD rich in saturated fat and cholesterol to experimental rodents has consistently been

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Abbreviations: Akt, protein kinase B; α-MEM, α-minimum essential media; ECAR, extracellular acidification rate; EOCC, embryonic rat osteogenic calvarial cell; FBS, fetal bovine serum; HFD, high-fat diet; MMP, matrix metalloproteinase; MSC, mesenchymal stem cell; OB, osteoblast; OCR, oxygen consumption rate; p, phosphorylated; PPAR, peroxisome proliferator-activated receptor; SAβG, senescence-associated β-galactosidase; SASP, senescence-associated secretory phenotype; shRNA, small hairpin RNA; t, total; UC, umbilical cord.

shown to inhibit bone formation (12, 13). Although it is largely unknown in human subjects, previous animal data indicate that inhibition of perinatal skeletal formation by HFD consumption during gestation (14, 15) is associated with DNA hypermethylation, suggesting intrauterine skeletal programming (15). In such maternal obesity-associated events of intrauterine fetal skeletal programming, specific blood-borne bioactive signals, such as nonesterified free fatty acids, in both maternal and fetal circulation, maybe play a causative role (15, 16).

As in other tissues, intrauterine skeletal programming is complex, and may undergo postnatal reprogramming (17). Nonetheless, the mechanisms by maternal obesity and HFD lead to lower differentiation potential of fetal mesenchymal stem cell (MSC) towards osteoblasts (OBs) are currently unknown. We have previously observed in a postnatal rat study that HFD-induced obesity promoted osteoblastic cell senescence (18). Cellular senescence was also recently identified during mammalian embryonic development (19, 20), leading to the present report investigating links between gestational obesity and fetal osteoblastic cell senescence. Cellular senescence is one general antiproliferative process and is thought to occur in most cells of organisms during aging and most notably within tumors (21, 22). In noncancer cells, how cell senescence signaling plays a pathophysiologic role has not been well studied. Although many senescence mediators have been characterized, in most rodent cells, induction of tumor suppressor genes p53, p21, and p16 are critical to the induction of senescence. Senescent cells increase the expression and secretion of numerous cytokines, chemokines, matrix metalloproteinases (MMPs), and other proteins that can alter local tissue environments. This feature has been termed the senescence-associated secretory phenotype (SASP) (23, 24). Previous reports also indicate that senescence-associated β-galactosidase (SAβG) activity is the most widely used marker to detect senescence both in vitro and in vivo (25, 26).

It is unknown how cell senescence signaling is controlled during the embryonic stage and what a role cell senescence plays in maintaining cellular homeostasis and metabolism at the molecular and biochemical level. p53 is well known as a senescence mediator in many cancer cells, it also coordinates how nontumor cells use nutrients to preserve its new role in their cellular metabolism. p53 has been shown to have a profound effect on increasing aerobic metabolism and inhibiting glycolysis (27). In our current report, using these cellular senescence markers, we set out to explore whether senescence signaling in fetal pre-OBs was programmed by maternal HFD and whether p53-mediated cellular signals regulate glucose metabolism in fetal pre-OBs.

Materials and Methods

Animals and diets

Female Sprague-Dawley rats were purchased from Harlan. Subsequently, these rats were divided into 2 groups, one group of rats received control AIN-93G diet (18.8% kcal from protein, 63.9% kcal from carbohydrate, and 17.2% kcal from fat, kcal density 3.8 kcal/g), and the other group of rats received a HFD (15.2% protein, 42.7% carbohydrate, and 42.2% fat, as %calories, kcal density 4.5 kcal/g) (see Supplemental Table 1 for detailed diet composition). After 12 weeks of diets, rats were time impregnated (n = 6 per group) by control male rats as described previously (15). Pregnant rats were individually housed in an Association for Assessment and Accreditation of Laboratory Animal Care-approved animal facility at the Arkansas Children's Research Institute with constant humidity and lights on from 6 AM to 6 PM at 22°C. All animal procedures were approved by the Institutional Animal Care and Use Committee at University of Arkansas for Medical Sciences. At gestational day 18, embryos were taken, and fetal calvarial cells were isolated for experimental analysis (see experiment design in Supplemental Figure 1). Body composition of dams immediately before mating was presented previously (15).

Pregnant lean and obese women and isolation of human umbilical cord (UC)-MSCs

Samples for this study were collected as part of a longitudinal trial of mothers and infants (Glowing study) which included collection of placenta and cord-tissue for UC-MSC derivation. Samples from 24 pregnant women were used in the present report: 12 obese (prepregnancy body mass index $\geq$ 30 kg/m^2) and 12 lean (prepregnancy body mass index between 19 and 25 kg/m^2). All participants provided written informed consent during the first trimester of pregnancy. Placentas and UC tissue were collected within 30 minutes after delivery. The protocol was approved by the Institutional Review Board at the University of Arkansas for Medical Sciences. As described previously (28), cells from the UC matrix (UC-MSCs) were isolated from the UCs, pooled, and expanded. UC-MSCs were counted and plated in growth media in a single well of a 6-well plate. Quality and purity UC-MSCs for cultures were described (28), and cells from passage 2 were used for all experiments described herein.

Isolation of fetal calvarial cells

Pregnant female rats were sacrificed on day 18 postcoitum (with 0 considered the day of the positive vaginal smear) and fetuses collected. For the isolation of fetal calvarial cells, sequential collagenase digestion of fetal calvarial tissue was performed, and cells from second and third digestion were collected and pooled. These embryonic osteogenic cells contain a mixture of differentiating osteoblastic cells, and cells from passage 2 were used in all experiments described in the result section. The procedure was similar to isolation of neonatal mouse and rat calvarial osteoblastic cells as previously published (15).

Cell cultures, SAβG activity, and nonradioactive cell proliferation assay

Isolated fetal/embryonic rat osteogenic calvarial cells (EOCCs) or human UC-MSCs were cultured in α-MEM, α-minimum essential media (Invitrogen) supplemented with 10% fetal

bovine serum (FBS) (HyClone), penicillin (100 U/mL), streptomycin (100 μg/mL), and glutamine (4mM). α-MEM supplemented with 10% FBS, 1mM ascorbyl-2-phosphate (Sigma-Aldrich), and 4mM L-glutamine was used as OB differentiation medium, whereas α-MEM supplemented with 10% FBS was used as without OB medium. UC-MSCs in 6-well plates were treated with rosiglitazone (5μM), insulin (10μM) and IGF-1 (10μM) in the presence or absence of adipogenic medium (Lonza, Poietics human MSCs instructions for use) for appropriate times. Protein SAβG activity assay was performed according to a method described previously (29). A nonradioactive cell proliferation assay (TB169; Promega Corp) was performed according to the protocol provided by the manufacturer. This assay measures absorbance at 490 nm, and there is a linear correlation (the correlation coefficient of the line is 0.997) between the number of cells and absorbance at 490 nm; results were converted absorbance to number of cells.

Rat embryo and human placenta histology and immunostaining

SAβG staining was performed on cryosections of whole-mount rat embryos either from HFD obese rats or control rats, and placentas from either obese or lean mothers. The SAβG staining procedure was described previously (30, 31). Standard antibody staining for p53 and p21 (Cell Signaling) was performed on paraffin embedded sections (32) of rat embryos or human placentas.

RNA and protein isolations, real-time RT-PCR

Total RNA from isolated in vitro cultured cells were extracted using TRI Reagent (MRC, Inc) according to the manufacturer's recommendation followed by deoxyribonuclease digestion and column cleanup using QIAGEN mini columns (33). Reverse transcription was carried out using an iScript cDNA synthesis kit from Bio-Rad. All primers for real-time PCR analysis used in this report were designed using Primer Express software 2.0.0 (Applied Biosystems), and they were listed in Supplemental Table 2.

Western blotting, p53 overexpression, shRNA, and inflammation antibody array

Standard Western blottings were performed using total protein isolated from cultured cells. The following antibodies were used for Western blottings: β-actin (dilution 1:5000, A1978; Sigma), MMP9 (dilution 1:2000, AB19016; Millipore), phosphorylated (p)-protein kinase B (Akt) (dilution 1:2000, sc-135650; Santa Cruz Biotechnology), total (t)-Akt (dilution 1:2000, 9272; Cell Signaling), and total-p53, rabbit polyclonal (dilution 1:2000, 9282; Cell Signaling). SuperSignal West Pico chemiluminescent substrate (Pierce) was used for developing blots. Standard p53 overexpression (p53-GFP, plasmid 11770 from Addgene) and p53 small hairpin RNA (shRNA) (TRP53, SKU TG500002 from OriGene) were performed using Amaxa Cell Line Nucleofector kit V (from Lonza). Mouse (AAM-INF-1–8; RayBiotech, Inc) and human (AAH-INF-2–8; RayBiotech, Inc) inflammation antibody arrays were performed according to protocols provided by the company.

XF96 analyzer and glucose and seahorse bioscience

Standard protocol provided by manufacturer was used at the optimal cell density (20 000 cells per well) for the different cells (rat EOCCs and human UC-MSCs) to study oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) using Seahorse XF96 analyzer (catalog number 101022–100; Seahorse Bioscience). Oligomycin and carbonyl cyanide p-trifluoromethoxy-phenylhydrazone (Seahorse stress kit) were prepared in XF assay medium with final concentration of 5μM and 1μM, respectively. Cell culture medium glucose measurement was performed according to method provided by Sigma (Glucose Assay kit, product code GAHK-20; Sigma), and lactate measurement was carried out using protocol from Eton Bioscience (L-Lactate Assay kit I, SKU number 1200012002; Eton Bioscience).

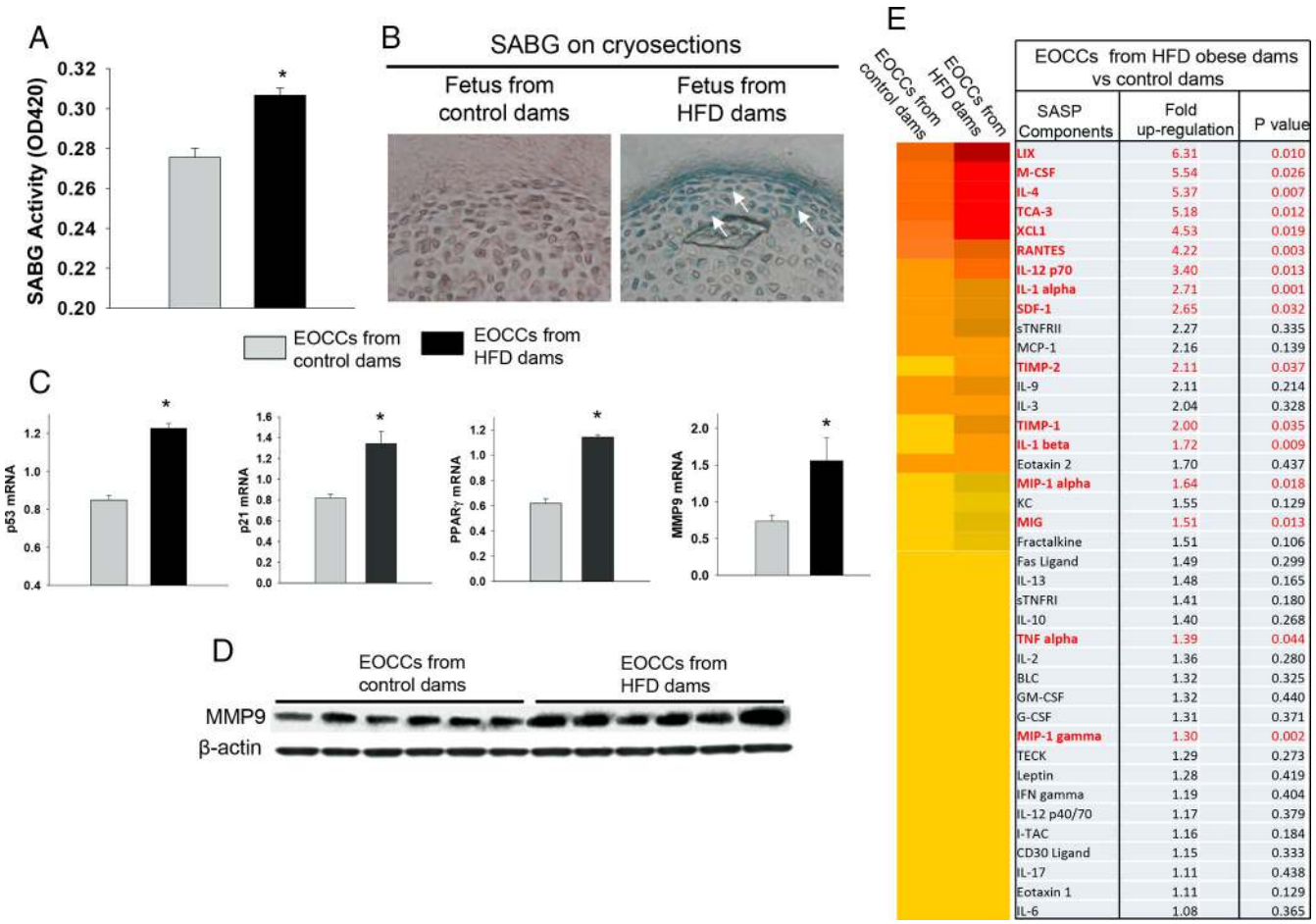
Statistical analyses

Before inferential analyses, we checked data for potential outliers and aberrant measurements. Data were also checked and verified that they were normally distributed. Numerical variables were expressed as mean ± SDM. One-way ANOVA followed by Tukey-Kramer's test as the post hoc test was employed to identify groups whose means differ significantly while retaining the family-wise error rate at 5%. Comparisons between groups were also performed with the nonparametric Kruskall-Wallis test, followed with Dunn's test for not normally distributed human data. The critical P value for statistical significance was $P = .05$.

Results

Increased senescence signaling in fetal osteoblastic cells from HFD obese rat dams

Recently identified cellular senescence signaling during mammalian embryonic development (19, 20), and our previous observation from a postnatal rat study that HFD-induced obesity promoted osteoblastic cell senescence (18), triggered us to investigate links between gestational obesity and fetal osteoblastic cell senescence programming. HFD maternal obesity-associated increasing of osteoblastic cell senescence signaling may be one of the persistent mechanisms involved in regulation of fetal bone development. We began by performing SAβG activity measurements in proteins isolated from EOCCs. The SAβG activity was highly and significantly increased in EOCCs from HFD obese dams compared with EOCCs from control diet dams (Figure 1A). Exploratory SAβG staining on cryosections of whole-mount rat embryos was performed. Strong SAβG staining was found in embryonic skeletal tissue of sagittal sliced femur heads (Figure 1B). Additional SAβG staining data on different fetal skeletal tissues and p53 and p21 antibody staining were presented in Supplemental Figure 2. Increased SAβG activity was accompanied by increased gene expression of p53 and p21 (Figure 1C), 2 well-known mediators associated with cel-



EOCCs from HFD obese dams vs control dams

SASP Components	Fold up-regulation	P value
LIX	6.31	0.010
M-CSF	5.54	0.026
IL-4	5.37	0.007
TCA-3	5.18	0.012
XCL1	4.53	0.019
RANTES	4.22	0.003
IL-12 p70	3.40	0.013
IL-1 alpha	2.71	0.001
SDF-1	2.65	0.032
sTNFRII	2.27	0.335
MCP-1	2.16	0.139
TIMP-2	2.11	0.037
IL-9	2.11	0.214
IL-3	2.04	0.328
TIMP-1	2.00	0.035
IL-1 beta	1.72	0.009
Eotaxin 2	1.70	0.437
MIP-1 alpha	1.64	0.018
KC	1.55	0.129
MIG	1.51	0.013
Fractalkine	1.51	0.106
Fas Ligand	1.49	0.299
IL-13	1.48	0.165
sTNFRI	1.41	0.180
IL-10	1.40	0.268
TNF alpha	1.39	0.044
IL-2	1.36	0.280
BLC	1.32	0.325
GM-CSF	1.32	0.440
G-CSF	1.31	0.371
MIP-1 gamma	1.30	0.002
TECK	1.29	0.273
Leptin	1.28	0.419
IFN gamma	1.19	0.404
IL-12 p40/70	1.17	0.379
I-TAC	1.16	0.184
CD30 Ligand	1.15	0.333
IL-17	1.11	0.438
Eotaxin 1	1.11	0.129
IL-6	1.08	0.365

Figure 1. Increased senescence signaling in EOCCs and fetal skeletal tissues from HFD obese dams. A, SAβG activity measurement in EOCCs from control or HFD obese dams. B, SAβG staining on cryosection of whole-mount rat embryos; white arrows are pointing blue color positively SAβG-stained cells from embryonic rat femurs (see more data in Supplemental Figure 1). C, Real-time PCR for p53, p21, and PPARγ, and MMP9 mRNA expression in EOCCs from control or HFD obese dams. D, Western blotting for MMP9 protein expression in EOCCs from control or HFD obese dams. E, Increased SASP in EOCCs from HFD obese dams. Proteins isolated from EOCCs either from control or HFD obese dams were analyzed by antibody arrays. Shown is the heat map analysis for comparison of all factors in EOCCs from control and HFD obese dams, color changes from yellow to red means expression from lower to higher. $P < .05$ means factors are significantly increased in EOCCs from HFD obese dams compared with those from control diet dams. *, $P < .05$ means significant changes of EOCCs from control vs EOCCs from HFD obese dams.

lular senescence, in EOCCs from HFD obese dams. Additionally, peroxisome proliferator-activated receptor (PPAR)γ, which is also known to be involved in cell senescence (18), was significantly increased in EOCCs from HFD obese dams (Figure 1C). One of the features of cellular senescence is increased expression and secretion of proteinases involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling. We previously determined that one such proteinase is MMP9 (15). Both mRNA and protein expression of MMP9 were found to be significantly higher in EOCCs from HFD obese dams compared with EOCCs from control dams (Figure 1, C and D). Senescent cells are characterized by a SASP (22, 23). We evaluated SASP in EOCCs from HFD-induced obese dams compared with EOCCs from control dams using an inflammation antibody array (Figure 1E). Surprisingly, many of these SASP molecules were significantly up-regulated in EOCCs from HFD-induced obese dams compared with EOCCs from control dams (Figure 1E).

Increased senescence signaling in fetal osteoblastic cells from HFD obese rat dams is associated with decreased glucose metabolism

Currently the biological importance of senescent or prematurely senescent noncancer somatic cells is not fully understood, but it is reasonable to consider that senescence could slow cell proliferation and alter metabolism. Indeed, the proliferative potential of EOCCs from HFD obese dams was significantly decreased compared with EOCCs from control dams (Figure 2A). EOCCs were cultured in the same concentration and amount of glucose containing medium for up to 72 hours. Glucose consump-

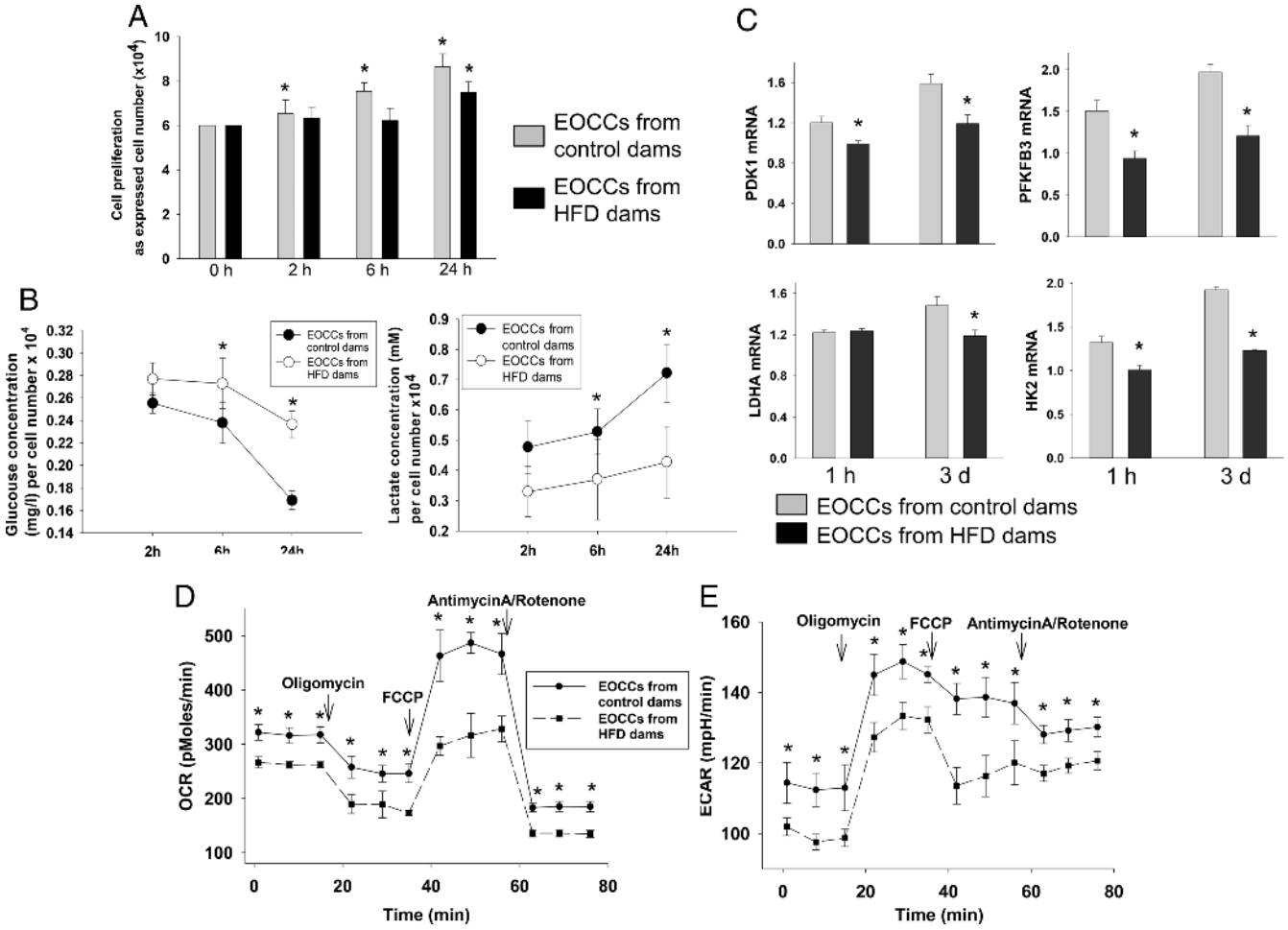


Figure 2. Decreased glucose metabolism in EOCCs from HFD obese rats. A, Cell proliferation analysis at 3 different culture times. *, $P < .05$ means significant changes vs 0 hours for both EOCCs from control and HFD obese dams. B, Decrease in glucose consumption by EOCCs from HFD obese rats (left panel), decrease in media lactate levels in EOCCs culture from HFD obese rats (right panel). C, mRNA expression of glycolytic regulators in EOCCs either from control or HFD obese rats after 2 different culture times. D and E, OCR and ECAR in EOCCs either from control or HFD obese rats. *, $P < .05$ means significant changes vs control.

tion by EOCCs from HFD-induced obese dams was significantly lower, consequently lactate production was lower compared with EOCCs from control diet dams (Figure 2B).The decreased glucose consumption could be due to decreased expression of genes involved in regulation of glucose and fatty acid metabolism and homeostasis, as suggested by decreased pyruvate dehydrogenase kinase 1, 6-phosphofructo-2-kinase/fructose2, 6-bisphosphatase 3, and hexokinase II mRNA expression (Figure 2C). It is interesting that mRNA expression of these genes in EOCCs from HFD-fed dams had little or no change between 1 hour and 3 days of culture; however, expression of these was increased in EOCCs from control dams after 3-day culture compared to those from HFD dams (Figure 2C). All the differences we observed above between EOCCs from HFD-induced obese dams and control dams prompted us to compare the metabolic profiles of EOCCs from HFD-induced obese dams and control dams. To study the EOCCs metabolic profiles, we used the Seahorse XF-24 extracellular flux and metabolic analyzer to measure ECARs and OCRs. ECAR is predominately the result of glycolysis, and OCR is an indicator of mitochondrial respiration. We found that both ECAR (Figure 2E) and OCR (Figure 2D) were clearly lower in EOCCs from HFD obese dams compared with those from control dams.

Decreased osteogenic potential of human MSCs from UC of infants born to obese mothers

Given the remarkable metabolic changes in EOCCs from HFD-induced obese rodent dams, we next considered whether such events may occur in human subjects. Considering that EOCCs are not feasible to study in pregnant mothers, we studied senescence and metabolic phenotypes of isolated MSCs from human UC tissue to evaluate osteoblastogenic potential. UC-MSCs were cultured in the osteogenic medium for 25 days. Von Kossa staining

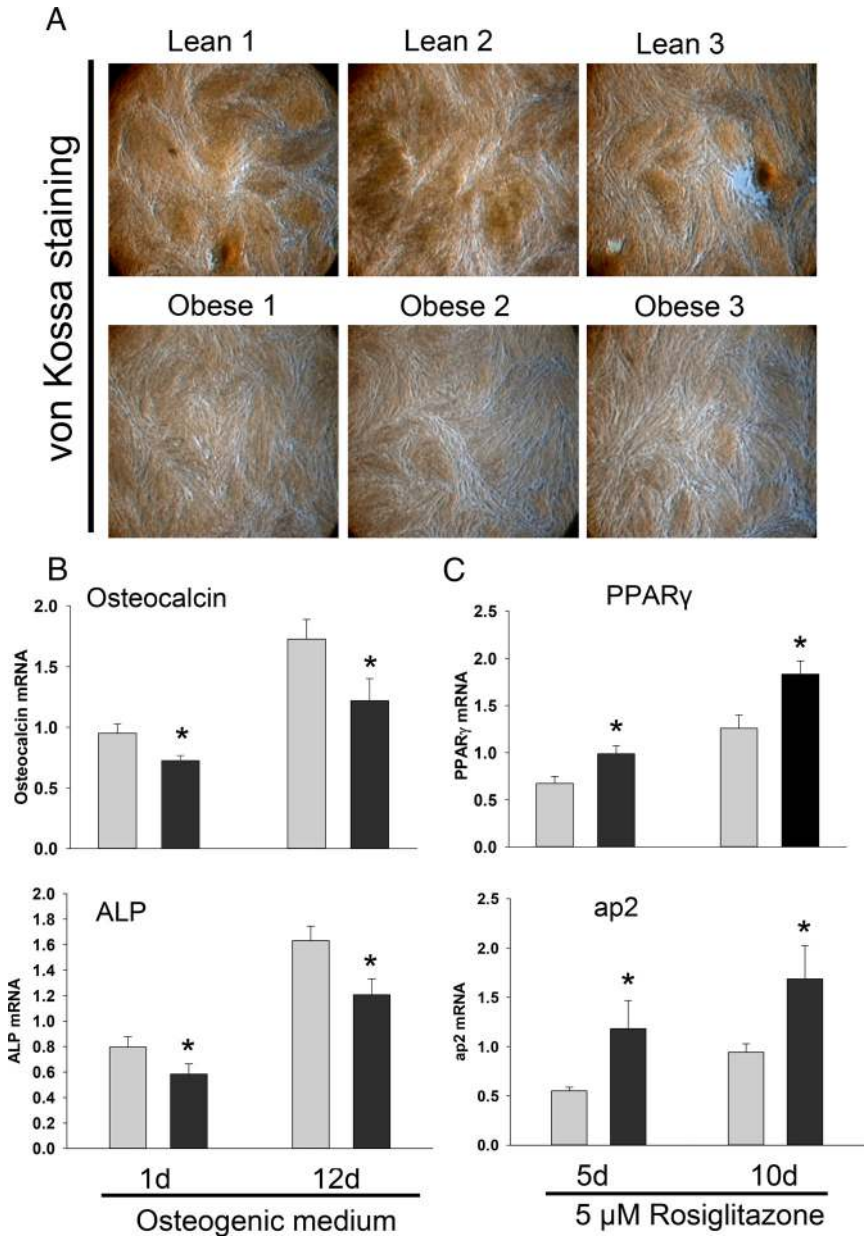


Figure 3. Decrease in osteoblastogenic potential of human UC-MSCs from obese mothers. A, UC-MSCs from either lean or obese mothers were cultured in the presence of osteogenic medium for 25 days; pictures are representing calcification von Kossa-stained 3 subjects per group. B, Osteogenic gene (osteocalcin and alkaline phosphatase [ALP]) mRNA expression by real-time PCR in total RNA isolated from human UC-MSCs from either lean or obese mothers after 1 or 12 days of culture in the presence of osteogenic medium. C, Adipogenic gene (PPARγ and ap2) mRNA expression by real-time PCR in total RNA isolated from human UC-MSCs from either lean or obese mothers after 5 or 10 days of culture in the presence of 5μM rosiglitazone. *, $P < .05$ means significant changes vs human UC-MSCs from lean mothers.

for bone mineralization indicated that UC-MSCs from obese mothers have less activity to mineralize materials compared with cells from lean mothers (Figure 3A). Moreover, we collected mRNA from cells cultured in osteogenic medium for 1 and 12 days. Real-time PCR analysis showed significantly decreased osteocalcin and alkaline phosphatase mRNA expression in UC-MSCs from obese mothers compared with those from lean mothers (Figure 3B). Alternatively, in order to confirm whether these UC-MSCs have increased potential to differentiate toward adipocytes, cells were treated with rosiglitazone for 5 and 10 days, and expression of PPARγ and adipocyte protein 2, 2 adipogenic markers, was measured. Both PPARγ and adipocyte protein 2 mRNA expression were significantly higher in UC-MSCs from obese mothers compared with those from lean mothers (Figure 3C).

Increased cell senescence, decreased glucose metabolism, and insulin resistance in human UC-MSCs of obese mothers

To examine senescence signaling in UC-MSCs, we measured SAβG activity in human UC-MSCs from obese and lean mothers. SAβG activity was significantly higher in UC-MSCs from obese mothers compared with those from lean mothers, with very small SDs (Figure 4A). Strong SAβG signals were found in placenta collected from obese mothers (one typical area is shown in Figure 4B, and additional data are presented in Supplemental Figure 3). In addition, mRNA expression of cell senescence mediators p53, p21, and PPARγ were increased in UC-MSCs from obese mothers compared with those from lean mothers (Figure 4C). We next examined the SASP in human UC-MSCs from obese mothers using inflammation-related antibody arrays and compared with to those from lean mothers (Figure 4D). Although SASP components in the human kit are a little different than those we measured in the rat, SASP molecules in UC-MSCs from obese mothers were generally increased, similar to findings in rats.

In our previous postnatal rodent study, we showed insulin resistance in OBs and bone in a HFD-driven obesity model (13). Here, we asked whether UC-MSCs from pregnant obese mothers have altered profiles of insulin sensi-

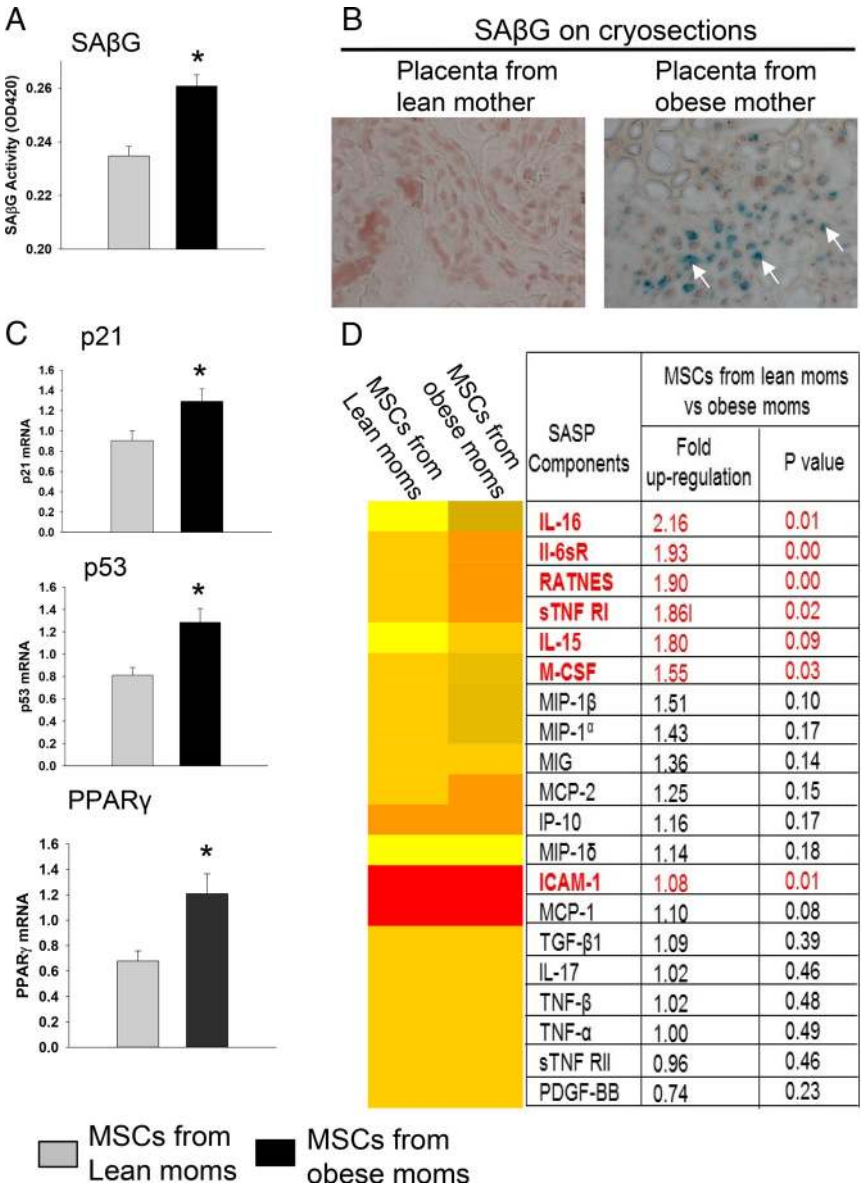


Figure 4. Increased senescence signaling in UC-MSCs and placentas from pregnant obese mothers. A, SAβG activity measurement in total proteins isolated from UC-MSCs either from lean or obese mothers. B, SAβG staining on cryosection of whole-mount placentas; white arrows are pointing blue color positively SAβG-stained cells from a typical area of placenta (see more data in Supplemental Figure 2). C, p53, p21, and PPARγ mRNA expression in total RNA isolated from human UC-MSCs either from lean or obese mothers. *, $P < .05$ means significant changes vs control. D, Increased SASP in UC-MSCs from pregnant obese mothers. Proteins isolated from UC-MSCs either from lean or obese mothers were analyzed by antibody arrays. Shown is the heat map analysis for comparison of all factors in UC-MSCs from lean and obese mothers, color changes from yellow to red means expression from lower to higher. $P < .05$ means factors are significantly increased in UC-MSCs from obese mothers compared with those from lean mothers.

tivity or signaling and glucose metabolism. We found increased basal phosphorylation of AKT in UC-MSCs from obese mothers compared with those from lean mothers (Figure 5A, basal level without insulin). However, when these cells were treated with insulin or IGF-1 for 2 and 24 hours, significantly increased AKT phosphorylation was only found in UC-MSCs from lean mothers. UC-MSCs from obese mothers showed diminished response to insulin or IGF-1 stimulation on AKT phosphorylation (Figure 5A). Similar to previous results in rodents, cell proliferative potential of UC-MSCs from obese mothers was significantly below control (Figure 5B). Likewise, we found significantly lower glucose consumption by UC-MSCs from obese mothers (cells were cultured in same concentration and amount of glucose containing culture medium for up to 24 h). Lower lactate production was also found in UC-MSCs from obese mothers compared with UC-MSCs from lean mothers (Figure 5C). Decreased mRNA expressions of genes involved in regulation of glucose and fatty acid metabolism and homeostasis as 6-phosphofructo-2-kinase/fructose2, 6-bisphosphatase 3; lactate dehydrogenase A; and hexokinase II were found in UC-MSCs from obese mothers compared with UC-MSCs from lean mothers especially after 24 hours of culture (Figure 5D). Using a similar approach to our studies in rats, we examined metabolic profiles of UC-MSCs from obese mothers and lean mothers. ECARs and OCRs were measured using the Seahorse XF-24 extracellular flux and metabolic analyzer. Both ECAR and OCR were significantly decreased in UC-MSCs from obese mothers compared with those from lean mothers (Figure 5E). These results indicated that UC-MSCs from pregnant obese mothers have impaired glucose metabolism and decreased insulin sensitivity.

To better understand the mechanism linking maternal obesity-associated increase of senescence signaling and altered glucose metabolism in osteo-progenitors, we lastly performed p53 gene knock-in and knock-down experiments using rat EOCCs and human UC-MSCs. As expected, overexpression of p53 gene (Figure 6, A and B) in rat EOCCs and human UC-MSCs led to increases in SAβG activity (Figure 6C) regardless of whether the fetal cells were from HFD or control diet rat dams (data in rat

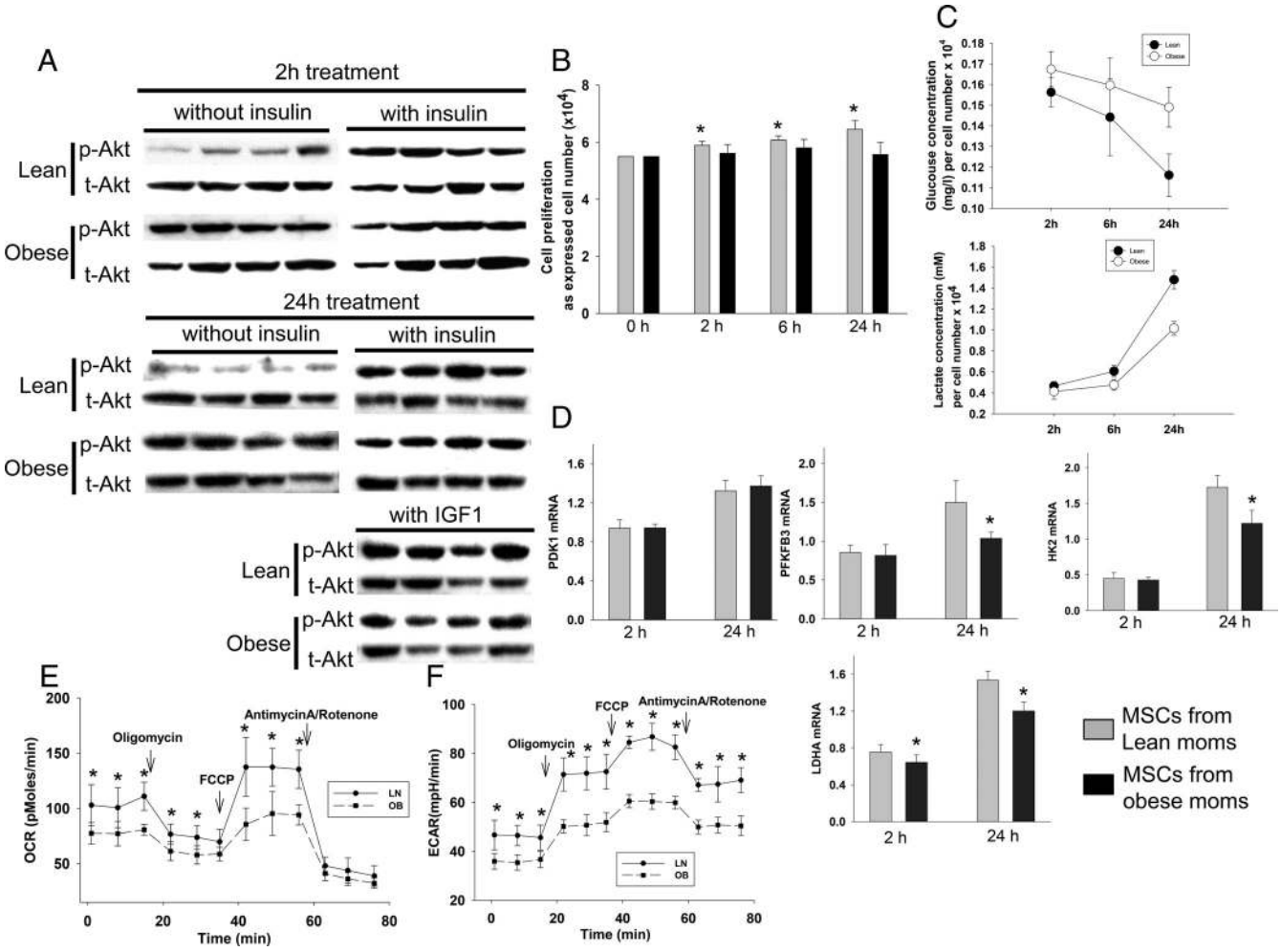


Figure 5. Insulin resistance and decreased glucose metabolism in human UC-MSCs from obese mothers. A, Western blottings for p-Akt and t-Akt expression in human UC-MSCs with or without insulin or IGF-1 treatment. B, Cell proliferation analysis at 3 different culture times. *, $P < .05$ means significant changes vs 0 hours. C, Decrease in glucose consumption by UC-MSCs from obese mothers (upper panel), decrease in media lactate levels in UC-MSCs culture from obese mothers (lower panel). D, mRNA expression of glycolytic regulators in UC-MSCs either from lean or obese mothers after 2 different culture times. E and F, OCR and ECAR in UC-MSCs either from lean or obese mothers. *, $P < .05$ means significant changes vs lean.

EOCCs were presented in Supplemental Figure 4), or lean or obese mothers. On the other hand, knock-down ofp53 (Figure 6, A and B) in rat EOCCs and human UC-MSCs led to decreases in SAβG activity (Figure 6C and Supplemental Figure 4) regardless of fetal cell origin. After transfection, cells were cultured in same concentration of glucose containing culture medium for up to 24 hours. Significantly lower glucose consumption and lower lactate production by p53-overexpressed rat EOCCs (Supplemental Figure 4) and human UC-MSCs were found (Figure 6D). In sharp contrast, when p53 was knocked down in rat EOCCs and human UC-MSCs, these cells showed increased glucose consumption and lactate production compared with those cells transfected with control vector (Figure 6D). These data suggest that regulation of glucose metabolism by cellular senescence signaling in osteo-progenitors is, at least in part, due to p53-mediated actions.

Discussion

The maternal environment, in particular, the nutritional status and dietary composition during pregnancy, has been shown to be important in the developmental trajectory of the fetus and influences the risk for chronic diseases such as cardiovascular disease, obesity and diabetes in the offspring (34, 35). Maternal obesity is an important factor predisposing offspring to obesity and metabolic diseases. However, fetal skeletal development associated with maternal obesity is understudied. We hypothesized that the fetal skeleton may represent another target for developmental programming

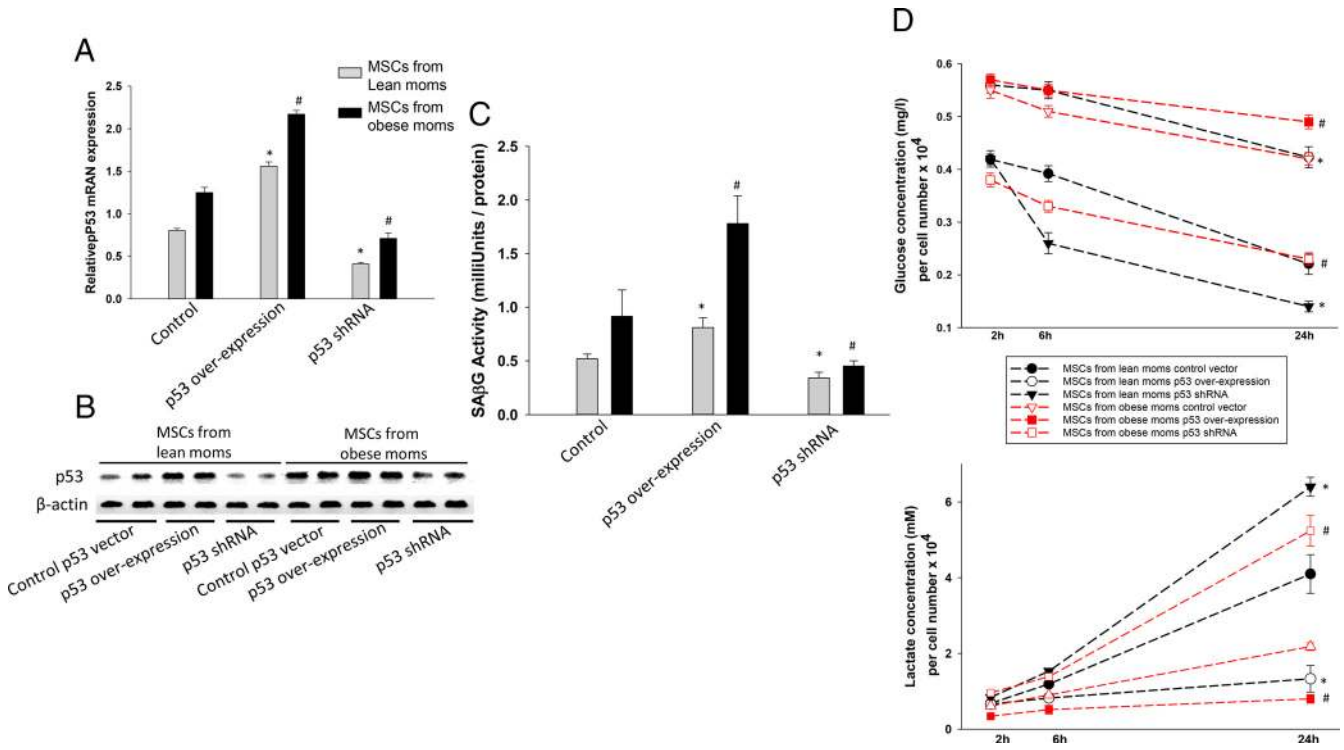


Figure 6. p53 is a linker between increased cellular senescence and decreased glucose metabolism in fetal osteo-progenitors. A, p53 mRNA expression in human MSCs from either lean or obese moms after p53 overexpression or shRNA. Control, cells are transfected with control vector (scrambled shRNA cassette in pGFP-V-RS); p53 overexpression, cells are transfected with p53 overexpression plasmid (p53-pEGFP-N1); p53 shRNA, cells are transfected with p53 shRNA (p53 shRNA-pGFP-V-RS). B, Western blotting to confirm p53 overexpression or silencing in MSCs from lean or obese moms. C, SAβG activity in MSCs from either lean or obese moms after p53 overexpression or shRNA. D, Glucose and lactate concentration in culture media of MSCs from either lean or obese moms after p53 overexpression or shRNA. *, $P < .05$ vs control vector-transfected MSCs from lean moms; #, $P < .05$ vs control vector-transfected MSCs from obese moms. In D, data were analyzed at 24-hour time point; n = 3 from 3 different moms.

and might lead to changes in the ability to attain peak bone mass and thus alter the risk of osteoporosis in later life (36). In the current study, we provide evidence that maternal obesity regulates cellular senescence signaling genes in EOCCs from obese dams and human MSCs from obese mothers. Taken together with our previous findings (15, 18), our current report suggests that in fetal cells from both obese mothers of rodents and humans, increased cell senescence signaling in embryonic osteoblastic cells leads to a lower potential of cell differentiation toward OBs and, conversely, favors differentiation towards adipocytes (28). Although our data suggest a novel phenomenon of maternal programming of preosteoblastic cell senescence signaling and glucose metabolism, they also implicate a novel potential mechanism for poorly defined maternal programming in other tissue systems.

Mechanistic processes of normal development of bone and adipose tissue during early life are actively being characterized. It is known, however, that maternal obesity enhances differentiation of stromal vascular cells and MSCs to adipocytes both in human subjects and animal models (37, 38). Consistent with this evidence and our previous report in rats (15), the present work showed less potential of cells from obese mothers to differentiate toward OBs. Previous evidence presented by us also suggested an epigenetic basis of such unbalanced differentiation potential between adipogenesis and osteoblastogenesis. Although more studies are needed, our data suggest that altered senescence signaling in precursors may interfere with adipogenic and osteogenic signals and, therefore, determine alternate cell fates (33).

Obesity in general is considered to be associated with an increase in cellular senescence and low-grade chronic inflammation (39), although much less is known about how maternal obesity effects fetal cell senescence pathways. Interestingly, during preparation of our current report, Kim et al described that gestational diabetes may affect fetal cell senescence (40). Chronic low-grade inflammation is a hallmark of obesity, and it is possible that low-grade inflammation may occur in fetal tissues from obese mothers. Cellular senescence has been proposed to promote chronic inflammation through the SASP (39). This is consistent with results presented in the current report (Figures 1 and 4). Especially, in maternal obesity-associated

embryonic osteogenic cells, increased p53 expression may collude with other cell senescence mediators such as p21, p16, and SASP to orchestrate senescence signaling in these cells. It is unclear whether maternal obesity epigenetically regulate PPARγ gene expression in osteo-progenitors; however, we believe that increased PPARγ expression plays a role on linking maternal obesity and fetal cell senescence. Supporting such a scenario, we have shown that HFD-induced obesity increases PPARγ expression to promote p53/p21-mediated cell senescence signaling in bone and osteoblastic cells (18), in a previous postnatal study. However, to untangle these questions in vivo, more studies are required, using cell type-specific conditional genetic loss of function approaches.

Decreased glucose metabolism and insulin resistance in embryonic osteogenic cells are likely to represent end results of increased cell senescence signaling. It has also been recently demonstrated that lactate-producing glycolysis may in turn play an important role in OB differentiation and function (41). OB differentiation is regulated by numerous developmental signals and energy use by OBs to synthesize matrix is critical for the developing the skeleton. The idea that leads to engage increased cell senescence signaling with regulation of embryonic osteogenic cellular metabolism to influence cell fate and function may be exciting. Although little is known about glucose transporters in OBs, active glycolysis has been demonstrated a long time ago (42), and calvarial osteoblastic cells were recently shown to use both aerobic glycolysis and oxidative phosphorylation during differentiation in vitro (43). We did not find differences in the mRNA expression of glucose transporters between fetal calvarial cells from control and obese dams (similarly, MSCs from lean and obese pregnant mothers); however, down-regulation of key glycolytic enzyme expression in fetal calvarial cells from obese dams resulted in obviously reduced aerobic glycolysis. Furthermore, down-regulation of both OCRs and ECARs was observed in fetal osteogenic cells from obese dams from both rodents and humans. These findings are interesting, because they are associated with decreased OB differentiation: differentiating OBs need to increase glycolysis in order to fuel collagen synthesis and matrix mineralization. More interestingly, we provided evidence that maternal obesity-associated increased p53 expression may be a key regulator or linker between increased cellular senescence and decreased glucose metabolism. Indeed, p53 is not only well known as a tumor suppressive gene, an additional role of p53 has been recently established on regulation of aging via its transcriptional control of cellular metabolism. p53 has also been found to play an important role on regulation of glucose metabolism (44). Our results are consistent with the previously demonstrated role of p53 on controlling glucose metabolism in different tissue cell types.

Insulin is a well-known critical hormone controlling whole-body glucose homeostasis, and OBs can respond to insulin and IGF-1 by up-regulating glucose uptake through glucose transporter. We did not expect the basal level of phosphorylated AKT to be higher in MSCs from obese pregnant mothers; however, AKT's phosphorylation status failed to respond to either insulin or IGF-1 treatment in these cells. These data indicated that MSCs from obese pregnant mothers are resistant to insulin and IGF-1. It has been demonstrated that both insulin and IGF-1 have bone anabolic effects and induce glucose uptake in osteoblastic cells (45).

In summary, we have presented evidence suggesting regulation of embryonic osteogenic cell differentiation by maternal obesity both in rats and humans. We demonstrated that p53/p21-mediated increasing of cell senescence signaling and decreasing of aerobic glycolysis were imprinted in both rodent EOCCs and human MSCs. Human MSCs from pregnant obese mothers were insulin resistant. We speculate that maternal obesity-induced increasing of senescence signaling in fetal pre-OBs may result in increased chronic inflammation, insulin resistance, decreased glucose metabolism, and cell differentiation.

Appendix

Table 1. Antibody Table

Peptide/ Protein Target	Antigen Sequence (if Known)	Name of Antibody	Manufacturer, Catalog Number, and/or Name of Individual Providing the Antibody	Species Raised in; Monoclonal or Polyclonal	Dilution Used
MMP9		Anti-MMP9 antibody	Millipore, AB19016	Monoclonal	1:2000
β-Actin		Anti-β-actin antibody	Sigma, A1978	Monoclonal	1:5000
p53		Anti-total-p53	Cell Signaling, 9282	Polyclonal	1:2000
p-Akt		Anti-phospho-Akt	Santa Cruz Biotechnology, sc-135650	Polyclonal	1:2000
t-Akt		Anti-total-Akt	Cell Signaling, 9272	Polyclonal	1:2000

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Author contributions: J.-R.C., A.A., K.S., and T.M.B. conceived and planned the study. J.-R.C. designed the experiments, wrote the article, and conducted the experiments. O.P.L., M.L.B., and S.R. conducted the experiments. R.E.F. discussed and designed the experiments. A.A. helped to provide human samples. T.M.B. and K.S. discussed the experimental design. All authors discussed the results and commented on the manuscript, and J.-R.C. supervised the entire project.

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