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Maternal Protein Restriction Permanently Programs Adipocyte Growth and Development in Adult Male Rat Offspring

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Abstract We previously demonstrated that maternal protein restriction (MPR) during pregnancy and lactation led to fetal growth restriction and development of increased visceral adiposity in adult male rat offspring. Here we studied the rate of proliferation and differentiation of adipocyte precursors (preadipocytes) in vitro to investigate whether MPR may permanently program adipocyte growth and development in adult male offspring. Preadipocytes were isolated from visceral adipose tissue of control and MPR offspring at 130 days of age, and cultured under standard conditions. The rate of proliferation was studied by [^{3}H]-thymidine incorporation, and the rate of differentiation assessed with the use of biochemical and morphological markers. Although it did not affect the rate of differentiation, MPR increased the rate of preadipocyte proliferation by almost twofold. To ascertain if the increased proliferation was due to persisting in vivo influences or aberrations inherent in the precursor cells, we studied the rate of preadipocyte proliferation in subcultures. We found that the increased rate of proliferation of MPR preadipocytes persisted throughout the first two subcultures, indicative of an inherent abnormality. In addition, we examined the rate of preadipocyte proliferation under reduced serum conditions. We showed that MPR reduced the rate of preadipocyte proliferation to 56 and 35% of the control in the presence of 5 and 2.5% serum, respectively. Taken together, these results demonstrate that MPR permanently programs adipocyte growth and development such that adipocyte precursors derived from MPR offspring replicate excessively under standard culture conditions but exhibit markedly attenuated growth rate under reduced serum conditions. J. Cell. Biochem. 101: 381–388, 2007.

Key words: maternal nutrition; visceral adiposity; adipogenesis; preadipocytes; proliferation and differentiation

Obesity is a serious medical problem not only because it substantially impairs quality of life and but also because it increases the risk of hypertension, type 2 diabetes, coronary heart disease, sleeping disorders, and cancers [Mokdad et al., 2003]. There is strong evidence for a genetic component to human obesity [Hofbauer, 2002]. Multiple systems regulate energy homeostasis [Montague et al., 1997; Krysiak et al., 2001] and a number of genes associated with human obesity have been identified [Froguel and Boutin, 2001], yet the genetic component of this condition cannot explain the dramatic increase in the prevalence of obesity in recent years.

A large number of epidemiological studies have revealed a strong statistical association between poor fetal growth and the subsequent development of type 2 diabetes, hypertension,

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and obesity, visceral obesity in particular [Osmond and Barker, 2000]. These observations were made initially by Barker and colleagues in England, but have now been reproduced in a large number of populations worldwide [Byrne and Phillips, 2000]. These findings have led to the "fetal origins" hypothesis, which states that an adverse intrauterine environment programs or imprints the development of fetal tissues, permanently determining physiological responses, and ultimately producing dysfunction and disease [Purdy and Metzger, 1996]. However, the molecular mechanisms that underpin this relationship remain elusive.

Since the world-wide maternal malnutrition is the most common cause for poor early growth, and amino acids play a critical role in fetal growth [Petry et al., 2001], the maternal protein restriction (MPR) rat model has become one of the most extensively studied models of fetal growth restriction [Ozanne, 2001]. In this model, rat dams are subjected to a low protein diet (8% protein) instead of control diet (20% protein) throughout pregnancy and lactation. As a consequence, the resulting offspring exhibit low-birth weight and become diabetic, insulin resistant, and hypertensive [Hales et al., 1996; Petry et al., 1997]. It has been reported that MPR had a long-term influence on the structure and function of certain organs, such as pancreas [Snoeck et al., 1990; Dahri et al., 1991; Petrick et al., 1999; Holness et al., 2000; Joanette et al., 2004], liver [Ozanne et al., 1996a; Burns et al., 1997] and muscle [Ozanne et al., 1996b].

Recently we have demonstrated that protein restriction during pregnancy and lactation leads to fetal growth restriction and development of increased visceral adiposity in adult male rat offspring [Guan et al., 2005]. Furthermore, we have also obtained evidence suggesting that visceral adiposity in our rat model is characterized by adipocyte hyperplasia because there were no apparent differences in the size of adipocytes between control and MPR offspring. This contention is consistent with the DNA microarray results showing that levels of a number of genes involved in cellular proliferation and differentiation were up-regulated in MPR adipose tissue while the expression of genes involved in apoptosis did not change [Guan et al., 2005]. Since adipocyte hyperplasia results from the recruitment of new adipocytes from precursor cells in adipose tissue and involves the proliferation and differentiation of preadipocytes, the present study was designed to test the hypothesis that preadipocytes from MPR offspring possess a greater potential for proliferation and differentiation.

MATERIALS AND METHODS

Experimental Animals and Dietary Manipulations

The MPR rat model was established as described previously [Guan et al., 2005]. In brief, virgin female Wistar rats (Charles River Laboratories, Wilmington, MA) weighting 240–260 g were housed individually and maintained at 22°C on a 12:12-h light-dark cycle. They were mated, and day 0 of gestation was set as the day on which vaginal plugs were expelled. The pregnant dams were fed either a diet containing 20% protein (control diet) or an isocaloric diet containing 8% protein (low-protein diet) throughout pregnancy and lactation. At 3 days of age, litters were randomly reduced to 8 pups, thus ensuring a standard litter size per mother. At 21 days of age, all offspring were weaned onto a 20% protein diet. At 130 days of age, male offspring were sacrificed and their visceral fat pads (composed of mesenteric, omental, and retroperitoneal fat masses) were isolated. For simplicity, the two groups of offspring will be termed control and MPR rats.

Isolation and Culture of Preadipocytes

Preadipocytes were isolated from control and MPR rats as described previously [Aoki et al., 2004]. Briefly, the visceral fat pads were dissected from visible blood vessels and connective tissue, weighed, finely minced and digested in digestion buffer (3 ml/g tissue) consisting of Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Life Technologies, Burlington, Ontario, Canada), 0.5 mg/ml collagenase class IV (Sigma, Oakville, Ontario, Canada) and 1.5% bovine serum albumin (BSA) (Sigma) for 45 min at 37°C, under mild controlled agitation. The resultant digest material was filtered through 250 μm nylon mesh and centrifuged at 600*g* for 5 min to separate the floating adipocytes. The cell pellet was resuspended, washed with dulbecco's phosphate-buffered saline (DPBS) containing 10% newborn calf serum (NCS) (Invitrogen Life Technologies), filtered through 25 μm nylon mesh and then centrifuged. The pelleted preadipocytes were resuspended in standard

culture medium (DMEM/F-12 medium (Invitrogen Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Sigma), 50 U/ml penicillin and 50 μg/ml streptomycin (Invitrogen Life Technologies)). The cell number was determined with a hemocytometer. Preadipocytes were seeded in 24-well plates and 100-mm dishes and cultured in a humidified incubator at 37°C, in the presence of 5% CO_2. For simplicity, preadipocytes derived from control and MPR rats will be termed control and MPR preadipocytes.

For subculture, primary preadipocytes were seeded in 100-mm dishes and cultured in standard culture medium for 3–4 days until they reached 60–70% confluence. Cells were then washed, detached by incubation with Trypsin-EDTA and centrifuged at 600*g* for 5 min. The resulting pellets were used for subculture. The first two subcultures were used for proliferation study.

Proliferation Assay-[^{3}H]-Thymidine Incorporation

Proliferation capacity of preadipocytes was assessed by measuring the rate of [^{3}H]-thymidine incorporation. Primary preadipocytes were seeded in 24-well plates at a density of 1×10^4 cells/cm^2 (day 0) and incubated in standard culture medium for 24 h. Subsequently, the medium was changed to DMEM/F-12 containing with 10, 5, or 2.5% FBS. [^{3}H]-thymidine (0.5 μCi/well) (75.2 Ci/mmol, PerkinElmer Life and Analytical Sciences, Woodbridge, Ontario, Canada) was added at 24-h intervals until day 4. At days 2, 3 and 4, the medium was removed, and cells were washed twice with ice-cold phosphate-buffered saline (PBS), once with 5% trichloroacetic acid (TCA) and once with 95% ethanol. Cells were then solubilized by the addition of 200 μl of 0.5 M NaOH. The solubilized cell lysate (100 μl) was added to 4 ml of scintillation fluid and the incorporation of [^{3}H]-thymidine into DNA was determined by scintillation counting. Protein concentration in the cellular lysate was quantified by the Bradford technique. The incorporation of thymidine was normalized by protein content.

Preadipocytes in the first and second subcultures were plated in 24-well plates at a density of 7,500 cells/cm^2, and incubated in standard culture medium for 24 h. Thereafter, medium was changed, and cells were pulse labeled with [^{3}H]-thymidine (0.5 μCi/well) for 24 h. The rate of [^{3}H]-thymidine incorporation was determined as described above.

Adipocyte Differentiation

Primary preadipocytes were seeded in 24-well plates at a density of 5×10^4 cells/cm^2 and cultured in standard culture medium until they reached confluence. Two days after confluence (day 0), cells were induced to differentiate by the addition of a differentiation cocktail containing 1 μM dexamethasone (Dex) (Alpharma, Boucherville, Quebec, Canada), 0.5 mM methyl-3-isobutylxanthine (IBMX) (Sigma), and 5 μg/ml insulin (Eli Lilly Canada Inc., Toronto, Ontario, Canada) and 10% FBS in DMEM. After 48 h, medium was replaced with standard culture medium plus insulin only, and cells were then fed every 2 days. Differentiated primary preadipocytes were harvested at days 3, 6, and 9 of differentiation. The rate of differentiation was scored by analysis of the expression of differentiation marker genes (peroxisome proliferator-activated receptor γ (PPARγ) and lipoprotein lipase (LPL)), and by lipid accumulation (oil red O staining).

Real-time Quantitative RT-PCR (qRT-PCR)

To determine if MPR altered the rate of preadipocyte differentiation, levels of PPARγ mRNA and LPL mRNA were assessed by a two-step real-time quantitative RT-PCR (qRT-PCR), as described previously [Guan et al., 2005]. Briefly, 1 μg of total RNA was reverse transcribed in a volume of 20 μl with the High Capacity Complementary Deoxybribonucleic Acid (cDNA) Archive Kit (Applied Biosystems, Foster City, CA), following the manufacturer's instructions. For every RT reaction, one RNA sample was set up without reverse transcriptase enzyme to provide a negative control against possible genomic DNA contamination. Gene-specific primers were designed by using Primer Express software (Applied Biosystems), and the optimal concentrations for each gene were determined empirically. All primers were purchased from Sigma Genosys: PPARγ, 5′-TTGGCCATATTTATAGCTGTCATTATT-3′ and 5′-TGTCCTCGATGGGCTTCA-3′; LPL, 5′-GGGTCGCCTGGTCGAAGT-3′ and 5′-AAAGTGCCTCCATTGGGATAAA-3′; 28S, 5′-GAATCCGCTAGGAGTGTGTAACAA-3′ and 5′-GCTCCAGCGCCATCCAT-3′. The SYBR Green I assay was performed with the SYBP Green PCR Master Mix (Applied Biosystems) and a modified universal thermal

cycling condition (2 min at 50°C and 10 min at 95°C, following by 40 cycles of 10 s each at 95, 60, and 72°C) with the standard disassociation/melting parameters (15 s each at 95, 60, and 95°C) on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The specificity of the SYBR Green I assay was verified by performing a melting curve analysis and by subsequent sequencing of the PCR products.

Levels of 28S rRNA (housekeeping gene) and target mRNAs in each RNA sample were quantified by the relative standard curve method (Applied Biosystems). Briefly, standard curves for 28S rRNA and each target gene were generated by performing a dilution series of a mixed cDNA pool. For each RNA sample, the amount of target mRNA relative to that of 28S rRNA was obtained. For each target gene, fold changes in the MPR group compared with the control were then calculated, and expressed as mean ± SEM.

Oil Red O Staining

Adipocyte monolayers (at days 3, 6, and 9) were washed with DPBS, fixed for 1 h with 4% paraformaldehyde at room temperature and incubated in 60% isopropanol for 5 min. Oil red O (3 g/L) (Sigma) in 99% isopropanol was diluted with water, filtered and added to the fixed cell monolayers for 5 min and then nuclei were stained with hematoxylin for 30 s. Cell monolayers were then washed with water and the stained triglyceride droplets were visualized and photographed.

Statistical Analyses

Results are presented as mean ± SEM of four to eight independent experiments (i.e., individual rats), as indicated. Data were analyzed by a standard Student *t*-test. Significance was set at $P < 0.05$. Calculations were performed using SPSS software version 9.0 (Chicago, IL).

RESULTS

Effects of MPR on DNA Synthesis in Preadipocytes in Primary Culture

To determine if preadipocytes from MPR rats exhibited a greater potential for proliferation, [^{3}H]-thymidine incorporation was used to measure the rate of DNA synthesis in preadipocytes isolated from control and MPR visceral adipose tissue under standard culture conditions at several time points (days 2, 3 and 4) before cells reached confluence. As shown in Figure 1, preadipocytes from both groups exhibited the highest DNA synthesis rate on day 2. With increasing time in culture, DNA synthesis rate decreased. However, the rate of DNA synthesis in preadipocytes from MPR offspring was consistently higher than that in control preadipocytes at all three time points, with the greatest difference at day 2 (1.8-fold increase; Fig. 1). As a result, all the subsequent proliferation experiments were carried out on day 2.

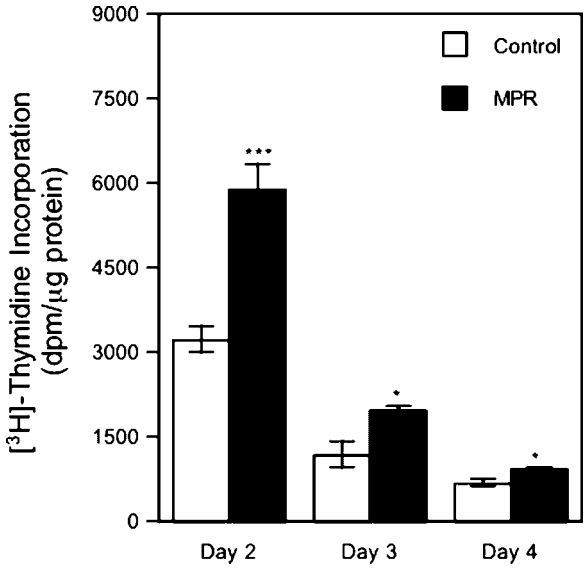

Fig. 1. Effects of MPR on the rate of preadipocyte proliferation in primary culture. Preadipocytes were isolated from visceral adipose tissue of control and MPR male offspring at 130 days of age. Cells were plated on 24-well plates at a density of 1×10^4 cells/cm^2 (day 0), incubated in standard culture medium (10% FBS) for 24 h, and then pulse labeled with [^{3}H]-thymidine (0.5 μCi/well) for 24 h at 24-h intervals. The rate of [^{3}H]-thymidine incorporation was determined at days 2, 3, and 4. Data are presented as mean ± SEM of 4–7 independent experiments (individual rats) each performed in triplicate (*$P < 0.05$, ***$P < 0.001$ vs. control).

Effects of MPR on DNA Synthesis in Preadipocytes in Subculture

Data showed in Figure 1 indicated that preadipocytes from MPR offspring had greater proliferation capacity in primary culture. To ascertain whether this characteristic was caused by persisting in vivo influences or inherent aberrations in the precursor cells, the rate of DNA synthesis in the first two subcultures were studied and compared between control and MPR offspring. Our rationale was that if excessive proliferation persisted in

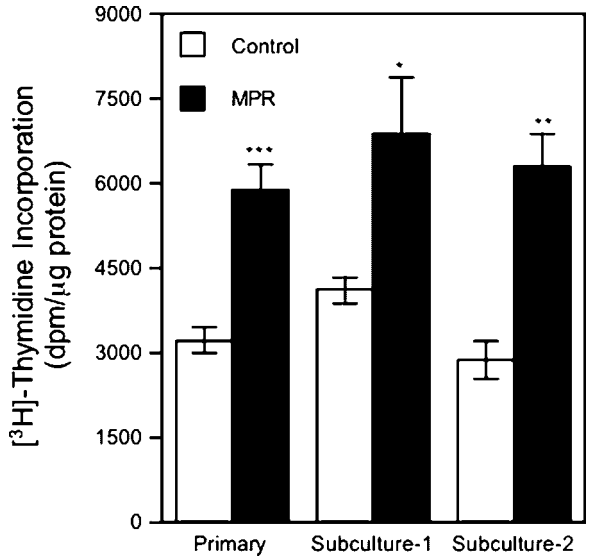

Fig. 2. Effects of MPR on the rate of preadipocyte proliferation in subculture. Preadipocytes were isolated from visceral adipose tissue of control and MPR male offspring at 130 days of age. Cells were cultured, and passaged under standard culture conditions. Cells from the first two subcultures were plated on 24-well plates at a density of 7,500 cells/cm^2 (day 0), incubated in standard culture medium (10% FBS) for 24 h, and then pulse labeled with [^{3}H]-thymidine (0.5 μCi/well) for 24 h. The rate of [^{3}H]-thymidine incorporation was determined. Data are presented as mean ± SEM of four independent experiments (individual rats) each performed in triplicate (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control).

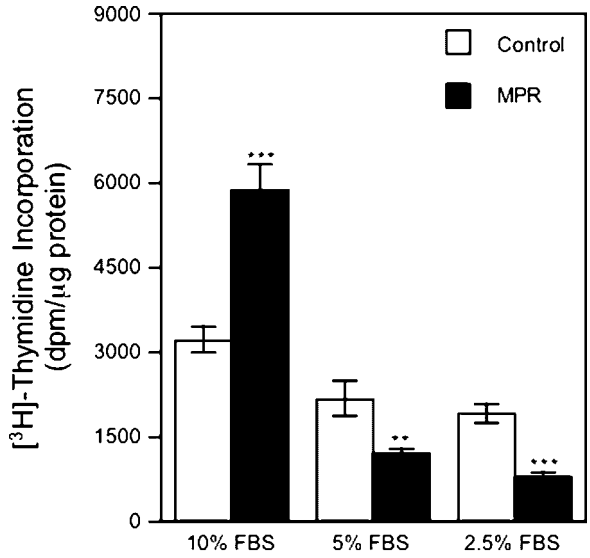

Fig. 3. Effects of MPR on preadipocyte proliferation under reduced serum conditions. Preadipocytes were isolated from visceral adipose tissue of control and MPR male offspring at 130 days of age. Cells were plated on 24-well plates at a density of 1×10^4 cells/cm^2, incubated in standard culture medium (10% FBS), or medium containing reduced serum (5 and 2.5% FBS) for 24 h, and then pulse labeled with [^{3}H]-thymidine (0.5 μCi/well) for 24 h. The rate of [^{3}H]-thymidine incorporation was determined. Data are presented as means ± SEM of four independent experiments (individual rats) each performed in triplicate (*$P < 0.05$, ***$P < 0.001$ vs. control).

subculture, it would be indicative of an inherent abnormality, because cells were removed from mitogenic factors that might circulate at elevated levels in MPR offspring. Results showed that the increased rate of DNA synthesis persisted throughout the first two subcultures (Fig. 2). In addition, fold changes in primary and subcultures were similar (1.8-, 1.7- and 2.2-fold increases in primary, the first and second subculture, respectively; Fig. 2). Taken together, these data suggested that the higher proliferation rate of MPR preadipocytes was likely due to inherent abnormalities in these precursor cells rather than persisting in vivo influences.

Effects of MPR on DNA Synthesis in Preadipocytes Under Reduced Serum Conditions

To determine if the enhanced proliferation of MPR preadipocytes could be sustained in a decreased trophic factor environment, we studied the rate of preadipocyte proliferation under reduced serum conditions. As expected, rate of DNA synthesis of preadipocytes from both groups was suppressed by reduced serum in a concentration-dependent manner (Fig. 3). However, in contrast to higher proliferation rate observed in the presence of 10% serum, MPR preadipocytes showed attenuated DNA synthesis rate under reduced serum conditions in a concentration-dependent manner (56 and 35% of control in the presence of 5 and 2.5% serum, respectively; Fig. 3), suggesting that MPR preadipocytes were permanently programmed such that they exhibited enhanced growth rate under standard culture conditions but attenuated growth rate under reduced serum conditions.

Effects of MPR on Preadipocyte Differentiation

To determine if MPR altered adipocyte differentiation, preadipocytes isolated from control and MPR offspring were subjected to a standard in vitro differentiation protocol. The rate of differentiation was assessed at discrete times (days 3, 6, and 9) with the use of biochemical (PPARγ, LPL) and morphological (oil red O staining) markers. PPARγ is a master

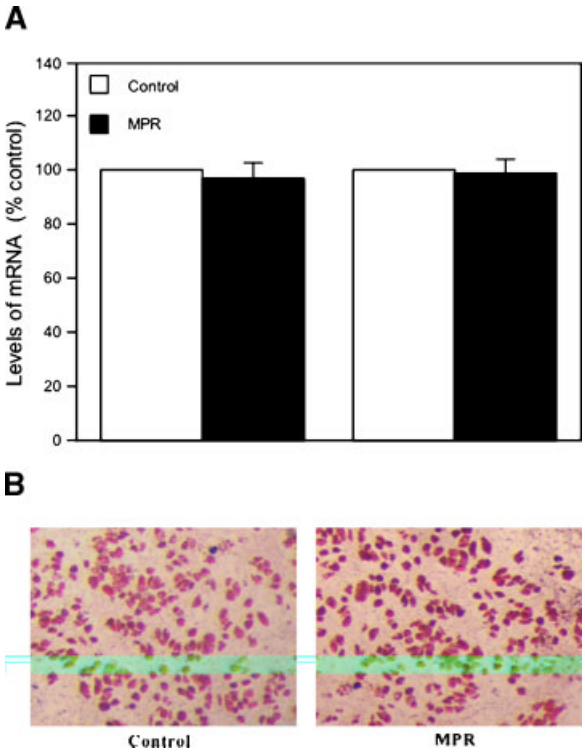

Fig. 4. Effects of MPR on the rate of adipocyte differentiation. Preadipocytes were isolated from visceral adipose tissue of control and MPR male offspring at 130 days of age. Cells were plated on 24-well plates at a density of 5×10^4 cells/cm^2, and incubated in standard culture medium (10% FBS). At 2 days post-confluence (day 0), preadipocytes were subjected to a standard differentiation protocol. The rate of preadipocyte differentiation was assessed at day 6 during differentiation with the use of biochemical and morphological markers, as described in the Materials and Methods. **A**: Expression of differentiation marker genes. Levels of PPARγ mRNA and LPL mRNA were quantified by real-time quantitative RT-PCR. Data are expressed as the mean ± SEM of four independent experiments. **B**: Oil red O staining of differentiated adipocytes on day 6 (×10). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

transcription factor essential for adipocyte differentiation [Rosen et al., 2000]. LPL is a critical enzyme involved in lipogenesis [Goldberg, 1996]. The expression of these two genes has been used extensively as an indicator of preadipocyte differentiation [Gregoire et al., 1998]. Figure 4A indicated that no differences in the level of PPARγ mRNA and LPL mRNA between control and MPR preadipocytes were observed at day 6. Furthermore, these PCR data were corroborated by oil red O staining which showed that lipid accumulation did not differ between the two groups (Fig. 4B). Similar results were obtained at days 3 and 9 of differentiation (data not shown).

DISCUSSION

In the present study, we studied the long-term effects of MPR on adipose tissue growth and development using an in vitro cell culture approach. We demonstrated that preadipocytes from adult male MPR offspring exhibited a greater potential for proliferation, which persisted in subcultures. In marked contrast, these preadipocytes displayed an attenuated rate of proliferation under reduced serum conditions, indicative of an increased vulnerability to a reduction in trophic factor support. However, the rate of preadipocyte differentiation was similar between control and MPR offspring. Taken together, our present findings suggest that MPR permanently alters the growth potential of adipocyte precursor cells in adult male offspring such that they proliferate excessively when trophic factors are abundant but poorly under reduced trophic factor environment.

The first finding of this study was that in primary culture, DNA synthesis rate in preadipocytes from 130-day-old male MPR offspring was consistently higher than that in control preadipocytes at all three time points studied. This observation suggested that preadipocytes isolated from male MPR offspring exhibited a greater potential for growth. The difference between these two groups was initially high but decreased with increasing time in culture, which may be due to the occurrence of contact inhibition [Van Harmelen et al., 2004]. However, one previous study reported that moderate protein restriction, imposed during either gestation and/or lactation, did not affect the proliferation capacity of preadipocytes derived from fetuses, neonates and weaning offspring [Bieswal et al., 2004]. The discrepancy between the current and previous studies may be explained by the age of animals studied, and/or the composition of diets. In the present study, male offspring were used at 130 days of age when they were found to exhibit visceral obesity and insulin resistance for the first time [Guan et al., 2005]. It is interesting to note that the enhanced preadipocyte proliferation was also reported in massively obese humans [Roncari et al., 1981], and ob/ob mice [Black and Begin-Heick, 1995], suggesting that this phenomenon may be common in different obese models.

It has long been recognized that some trophic factors in obese subjects (such as TGFα)

circulate at inappropriate levels and stimulate inordinate replication of adipocyte precursors [Roncari et al., 1981; Djian et al., 1983]. This influence may continue in primary culture. Therefore, one of the reasons for the excessive growth of MPR preadipocytes in primary culture may be persisting in vivo influences. Since we have demonstrated altered patterns of gene expression in MPR visceral adipose tissue [Guan et al., 2005], another reason for the increased MPR preadipocyte proliferation may be inherent aberrations in gene expression. To distinguish these two possibilities, the rate of preadipocyte proliferation in subculture was compared. Our rationale was that in subculture, if excessive proliferation persisted, it would be indicative of an inherent abnormality, because cells are removed from mitogenic factors that might circulate at elevated levels in MPR offspring. Our results showed that the rate of DNA synthesis in MPR preadipocytes was continuously higher in the first two subcultures and the magnitude of increase was similar to that in primary culture, suggesting that the exaggerated replication of adipocyte precursors from MPR rats was an intrinsic characteristic, and likely a consequence of aberrations in gene expression. A similar finding was reported by Roncari et al. [1981] in massively obese humans.

Aberrant adipose tissue gene expression could have several consequences. First, it may lead to altered responsiveness of adipocytes and/or preadipocytes to hormones and growth factors [Djian et al., 1983]. For example, adipocytes from MPR rats displayed a selective resistance to the antilipolytic action of insulin [Ozanne et al., 1997; Shepherd et al., 1997]. A previous study also reported that the growth of preadipocytes from ob/ob mice was more sensitive to serum, when compared with lean mice [Black and Begin-Heick, 1995]. To determine if MPR preadipocytes exhibited aberrant growth rate under reduced trophic factor environment, serum concentrations in culture medium were decreased from 10 to 5 and 2.5%. It is interesting to note that DNA synthesis rate in preadipocytes from MPR offspring was progressively lower than that in preadipocytes from control offspring under reduced serum conditions, despite the fact that these cells exhibited higher proliferation rate in standard culture conditions. This result suggested that MPR preadipocytes were more vulnerable to reductions in trophic factor support.

Abnormal adipose tissue gene expression may also lead to increased levels of known adipogenic factors [Djian et al., 1983]. For example, it has been reported that exaggerated release of basic fibroblast growth factor from preadipocytes might act as an autocrine factor contributing to adipocyte hyperplasia in obese human and Zucker rats [Lau et al., 1987; Marques et al., 1998]. The expression of genes encoding TGFα and IGF-II, both of which are known to stimulate adipogenesis, was up-regulated in our rat model [Guan et al., 2005]. Another potential consequence of aberrant gene expression in MPR adipose tissue could be elevated levels of novel adipogenic factors. Indeed, we have identified a number of candidate genes whose expression is uniquely up-regulated in our rat model [Guan et al., 2005]. We are currently studying the role of these candidate genes in regulating adipogenesis.

Having established an effect of MPR in preadipocyte proliferation, we also studied the effect of MPR on preadipocyte differentiation. We observed no apparent differences in levels of both biochemical (PPARγ and LPL) and morphology (lipid accumulation) markers between control and MPR groups at the early, mid and late stages of differentiation. These findings suggested that MPR had no effect on adipocyte differentiation. Similar results were reported in preadipocytes derived from fetuses, neonates and weaning MPR offspring [Bieswal et al., 2004]. It is noteworthy that the rate of differentiation was first assessed 5 days after the addition of a differentiation cocktail, which corresponded to 7 days post-confluence. Consequently, this experimental paradigm allowed us to separate the effects of MPR on preadipocyte differentiation from those on preadipocyte proliferation.

In conclusion, the present study demonstrates that MPR permanently programs adipocyte growth and development such that adipocyte precursors derived from adult MPR offspring replicate excessively under standard culture conditions but exhibit markedly attenuated growth rate under reduced trophic factor environment. This in vitro phenomenon likely results from inherent abnormalities in the precursor cells, which may contribute to the pathogenesis of adipocyte hyperplasia characteristic of visceral adiposity in this early-life programmed rat model.

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