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# High Glucose Induces Adipogenic Differentiation of Muscle-Derived Stem Cells

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**Dottorando**
Dott.ssa Aguiari Paola

**Tutore**
Prof. Rizzuto Rosario

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# Abstract

Regeneration of mesenchymal tissues depends on a resident stem cell population, which in most cases remains elusive in terms of cellular identity and differentiation signals. We here show that primary cell cultures derived from adipose tissue or skeletal muscle differentiate into adipocytes when cultured in high glucose. High glucose induces ROS production and PKCβ activation. These two events appear crucial steps in this differentiation process that can be directly induced by oxidizing agents and inhibited by PKCβ siRNA silencing. The differentiated adipocytes, when implanted in vivo, form viable and vascularized adipose tissue.

Overall, the data highlight a previously uncharacterized differentiation route triggered by high glucose that drives not only resident stem cells of the adipose tissue but also uncommitted precursors present in muscle cells to form adipose depots. This process may represent a feed-forward cycle between the regional increase in adiposity and insulin resistance that plays a key role in the pathogenesis of diabetes mellitus.

# Introduction

## Adipose Tissue

Adipose tissue is a specialized connective tissue and represents, in humans, the body's largest energy store. When energy intake exceeds energy expenditure, energy is stored in the form of triglycerides, which during starvation are mobilized through lipolysis process to provide fuel to other organs and to deliver glycerol to liver for gluconeogenesis and free fatty acids to lipoprotein synthesis.

Adipose tissue is a heterogeneous organ constituted by many fat depots differently located in the body. Mechanical support is given by fat pads located in the heels, in the fingers, in the toes and in the periorbital region. Adipocytes are found also in the subcutaneous layers between the muscle and dermis, commonly known as subcutaneous adipose tissue (SAT), while some adipose depots are within the body cavity, around the heart, kidneys, and other internal organs, associated with the mesentery or in the retroperitoneum and represent the visceral fat (VAT) [1]. VAT comprises omental and mesenteric deposits and represents 10–20% of total body fat in thin and obese men, and 5–10% of total body fat in women, while SAT represents 80% of a healthy individual's total fat mass. In addition, a further distinction can be made between brown and white adipocytes, constituting functional and morphologically different tissues, Brown Adipose Tissue (BAT) and White Adipose Tissue (WAT), respectively. Brown adipocytes are found only in mammals, and are multilocular and contain less overall lipid than white adipocytes, and are particularly rich in mitochondria. Furthermore, brown adipocytes differ from white adipocytes because they express uncoupling protein-1 (UCP1), which dissipates the proton gradient across the inner mitochondrial membrane, to generate heat.

While rodents have a distinct brown fat pad, which lies in the interscapular region, in humans brown adipose tissue surrounds the heart and great vessels in infancy but disappears during growth until only scattered cells can be found within white fat pads.

Adipose tissue has been considered an energy storage depot with few interesting attributes. The past two decades, however, have seen an increasing scientific interest in this tissue, triggered by evidences about its strong correlation with Obesity and Metabolic Syndrome, and also by the recognition that, further than the key role in controlling the energy metabolism homeostasis, adipose tissue integrates many homeostatic processes, such as immune response, blood pressure, bone mass, haemostasis, and reproductive function [2]. These processes are coordinated mainly through the synthesis and release of peptide hormones by adipocytes.

Obesity is now recognized as a major health problem, since it represents the most important independent risk factor for insulin resistance, Type II Diabetes, dyslipidaemia, high blood pressure and thrombosis. Increased fat storage in fully differentiated adipocytes, resulting in enlarged fat cells, is well documented and is thought to be the most important mechanism whereby fat depots increase in adults [3]. Nevertheless, the development of Obesity is prevalently due to an increase in the number of adipocytes. Thus, actually the most int
riguing subject for research in Obesity is how adipose tissue originates and grows.

# Adipocyte Differentiation

The adipose cells arise from mesoderm, and white adipose tissue formation begins before birth and proceeds after birth, because of increased size of existing fat cells and proliferation of pre-adipocyte cells. It has been demonstrated that fat cell number can increase when rats are fed a high-carbohydrate or high-fat diet [4, 5], or in the obese state; thus the potential to generate new fat cells is maintained throughout life, and is in function of environmental factors, such as nutritional status [6]. Although the complete knowledge about the developmental origin of fat cells still lacks, it is now clarified that the adipocyte lineage derives from a mesenchymal multipotent precursor, able to differentiate in adipocyte, chondrocyte, osteoblast, fibroblast and myocyte. Although the attempts to identify the cellular intermediates between the mesenchymal stem cell and the mature adipocyte, such intermediates have been difficult to characterize at the molecular level. Nonetheless, adipogenesis can be described in two phases, as described in *figure 1*.

The first phase is known as determination, and results in the conversion of the stem cell to a mesenchymal multipotent intermediate, which can originate a pre-adipocyte, that is morphologically indiscernible from its precursor but has lost the potential to differentiate into other cell types. In the second phase, the so-called terminal differentiation, the pre-adipocyte acquires the machinery necessary for lipid transport and synthesis, insulin sensitivity and secretion of adipokines.

*In vitro* model of differentiation and studies in transgenic mice are widely used to get some insight in the molecular mechanisms and signals transduction pathways involved, and terminal differentiation is more extensively characterized than determination, because most studies used cell lines with a differentiation potential restricted to adipogenic lineage. The most employed preadipose cell lines are 3T3-L1 and 3T3-F442A, cloned from heterogeneous Swiss 3T3 cells, that had been derived from dissociated near term mouse

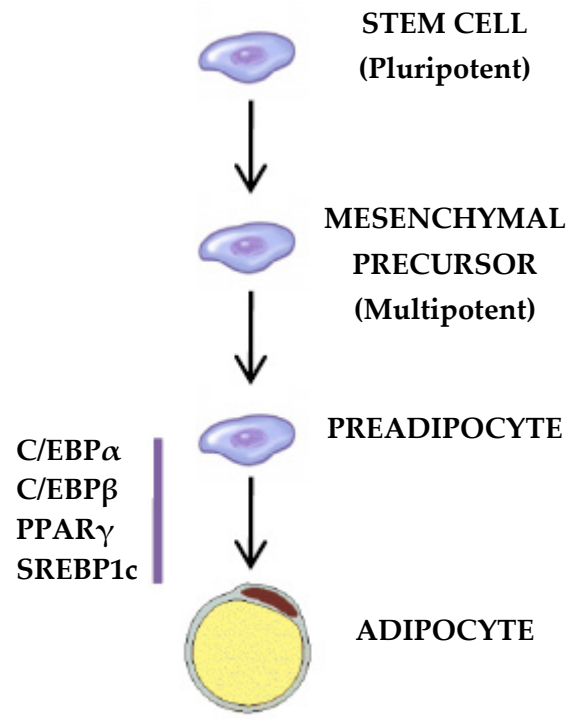


*Figure 1 Adipocyte Differentiation A pluripotent stem cell precursor gives rise to a mesenchymal precursor cell with the potential to differentiate along mesodermal lineages of myoblast, chondroblast, osteoblast, and adipocyte. Given appropriate environmental stimuli, preadipocytes undergo clonal expansion and subsequent terminal differentiation [7]. For more details about the molecular factors involved see the next section.*

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embryos [8, 9], and the Ob17 cell line, derived from epididymal fat pads of genetically obese (ob/ob) adult mice [10]. Following seeding, cells display an exponential growth phase until reaching confluence. In preadipose cell lines as well as in primary preadipocytes, growth arrest and not cell confluence or cell-cell contact per se appears to be required for adipocyte differentiation.

During the growth phase, cells of preadipocyte lines as well as primary preadipocytes are morphologically similar to fibroblasts. Confluent 3T3-L1 preadipocytes differentiate upon exposure to the adipogenic inducers fetal bovine serum (FBS), dexamethasone, isobutylmethylxanthine (IBMX), and insulin. This cocktail activates an adipogenic program, which occurs in two well-defined phases. The stimulated cells immediately

reenter the cell cycle and progress through at least two cell-cycle divisions, a phase often referred to as clonal expansion. During this time, the cells express specific adipogenic transcription factors as well as cell cycle regulators that together facilitate expression of PPARγ and C/EBPα. Following this event, the committed cells undergo terminal differentiation manifested by production of lipid droplets as well as expression of multiple metabolic programs characteristic of mature fat cells. The validity of this 3T3-L1 system as an appropriate model of adipocyte formation in the animal has been supported by many studies performed in both mouse and human tissue.

# Transcriptional regulation of adipocyte differentiation

Adipogenesis in vitro follows a highly ordered and well characterized temporal sequence. The first step is the growth arrest of proliferating preadipocytes, achieved in cultured cell lines after contact inhibition, although cell–cell contact is *not* absolutely required for growth arrest to occur [7, 11]. In cultured preadipocyte cell lines, treatment with adipogenic hormone induces growth arrest and is followed by one or two additional rounds of cell division, known as clonal expansion.

Below is presented an overview on factors and pathways involved in transcriptional regulation of adipocyte differentiation, as extensively reviewed in [7, 12-17].

Adipocyte differentiation is regulated by a network of transcriptional factors managing the expression of hundreds of proteins responsible for establishing the mature fat-cell phenotype. This process is mainly regulated by three principal adipogenic factors, the key transcription factors peroxisome proliferator- activated receptor γ (PPARγ), the CCAAT/enhancer binding protein α(C/EBPα) and the sterol regulatory element binding protein 1(SREBP1).

PPARγ is a member of the peroxisome proliferator activated receptors (PPAR) subfamily of nuclear hormone receptors and was cloned independently by several groups. Three groups found two homologues to PPARα (then simply called PPAR) in *Xenopus* and mice, shown to activate the acyl CoA oxidase promoter in response to a variety of xenobiotic and hypolipidemic agents, and called them PPARβ (also called PPARδ, NUC-1, and FAAR) and PPARγ. Looking for transcription factors promoting fat cell development, Spiegelman identified an adipose cell-selective fatty acid binding protein aP2 enhancer and discovered that this enhancer is sufficient to direct gene expression and differentiation to fat cells in culture and in transgenic mice. Furthermore, Spiegelman was the first to highlight the key role of an adipocyte-specific transcription factor primarily called ARF6,

and then discovered to be a heterodimeric complex of PPARγ and the retinoid X receptor (RXR).

PPARγ is the master regulator of adipogenesis; without it, precursor cells are incapable of developing the adipocyte phenotype, and alone it can initiate the entire adipogenic program. In fact, most pro-adipogenic factors seem to function at least in part by activating PPARγ expression or activity. PPARγ exists as two isoforms, PPARγ1 and PPARγ2, generated by alternative promoter usage of the same gene, and the two forms differ in that PPARγ2 has an NH2-terminal extension of 30 amino acids. Both isoforms are induced during adipogenesis, but PPARγ2 is found selectively in adipocytes, whereas γ1 is expressed at low level in many tissues, such as colonic epithelium, macrophages, liver and heart.

Nuclear hormone receptor superfamily members are ligand-activated transcription factors, and PPARγ is not an exception. PPARγ can be activated by synthetic compounds called thiazolidinediones (TZDs), which are used clinically as antidiabetic agents treating hyperlipidemia and insulin resistance. Treatment of preadipocytes with TZDs increases both the extent and the rate of adipogenesis and this occurs *in vivo* as well as *in vitro*, although it is not clear if the adipogenic potential of these agents is related to their antidiabetic actions. Despite the utility of TZDs as investigative and therapeutic tools, no endogenous PPARγ ligand has been fully elucidated yet. The search for such a compound has identified some natural ligands, including 15 deoxy-D12,14 prostaglandin J2, 15dPGJ2 are able to bind to and activate PPARγ, and eventually promote adipogenesis when added to cultured fibroblasts. Moreover, some findings indicate that ligand activation of PPARγ is required to induce adipogenesis but not to maintain PPARγ -dependent gene expression in mature adipocytes.

Since PPARγ is involved in formation of the trophoblast, attempts to develop PPARγ gene knockout result in embryonic lethality; thus, evidences about PPARγ involvement in adipogenic transcriptional program were given by experiments on chimeric mice derived from homozygote embryonic stem cells. The experiments demonstrated that, while wild-

type cells can differentiate in mature adipocytes, knockout cells were unable to develop into adipocytes. Consequently, it was difficult to assess what impact the absence of PPARγ has on adipose tissue function, but other experiments demonstrated that effectively PPARγ deficiency resulted in failure to form adipose tissue, and the establishment of white adipose tissue (WAT)-hypomorphic PPARγ knockdown mice resulted in animals that were severely lipodystrophic; these data authenticate PPARγ as the master regulator of adipogenesis.

PPARγ interacts with other transcription factors, such as C/EBP$\alpha$. The C/EBPs belong to the basic-leucine zipper class of transcription factors. Six isoforms have been described, all acting as homo- and/or heterodimers formed via a highly conserved bZIP domain, and some of them, C/EBP$\alpha$, C/EBPβ, C/EBPγ, C/EBPδ and CHOP (transcription factor homologous to CCAT-enhancer binding protein) are expressed in adipocytes. Their tissue distribution is not restricted to fat cells, since C/EBP proteins are also involved in the terminal differentiation of granulocytes and hepatocytes; in resistance to infection and the tissue response to injury in addition to transactivating a wide variety of target genes. Regulated expression is seen for several C/EBP family members during adipogenesis, and recent gain- and loss of-function studies indicate that these proteins have a profound impact on fat cell development. In particular, C/EBPα is induced in adipogenesis and experiments of C/EBPα expression in fibroblasts at levels equivalent to those seen in fat demonstrate that it can cooperate powerfully with PPARγ triggering adipogenesis even if in absence of PPARγ ligands. This ability of PPARγ and C/EBPα to promote differentiation is not limited to fibroblasts, but can cause also the *in vitro* "transdifferentiation" of myoblasts to adipocytes. Moreover, C/EBPα has itself adipogenic action when expressed at high level since its overexpression in 3T3-L1 preadipocytes induces differentiation.

Thus, C/EBPα and PPARγ participate in a common pathway of adipogenesis, in which PPARγ is the dominant factor, since experiments in C /EBPα-deficient MEFs PPARγ can induce alone adipogenesis, whereas C/EBPα is incapable of driving the adipogenic program in the absence of PPARγ.

Studies about the temporal expression of the C/EBP family members indicate a cascade whereby early induction of C/EBPβ and C/EBPδ are expressed earlier than C/EBPα during adipogenesis leads to induction of C/EBPα. These factors appear to be very important in the induction of PPARγ in adipocyte differentiation, and some data show that conditional expression of C/EBPβ and C/EBPδ yield expression levels of PPARγ equivalent to those seen in adipocytes, indicating that the expression of PPARγ is depend on C/EBPβ and δ expression.

Another factor that can co-work with PPARγ is the adipocyte determination and differentiation factor 1/sterol response element binding protein 1(ADD1/SREBP1), a member of the basic helix-loop–helix (bHLH) family of transcription factors. This family has been implicated in tissue-specific gene regulation, particularly in muscle, and SREBP1 was independently identified as a potent regulator of adipogenesis and fatty acid metabolism and as a key factor in cholesterol homeostasis. The human SREBP-1c isoform, which predominates in animal tissues, is the most studied, and co-expression of SREBP-1c with PPARγ increases the transcriptional activity of PPARγ, even in absence of its ligands. Since SREBP-1c is involved in the expression of key genes in fatty acid metabolism, such as Fatty acid Synthetase (FAS) or Lipoprotein Lipase (LPL), and SREBP1c alone had little effect, one interpretation is that SREBP1c is responsible for generating some factor that enhances PPARγ activity, i.e. its endogenous ligand.

The body of knowledge concerning all the events occurring during adipocyte differentiation has led to a model for a transcriptional network (described in *figure 2*) but the scheme have to be completed by a group of protein, called nuclear cofactors, such as the p160/CBP/p300 complex, the DRIP/TRAP complex and PPARγ coactivator-1 and -2 (PGC1 and PGC2), operates to open up tightly wound chromatin structures and recruit elements of the basal transcription apparatus, in order for transcription factors to activate gene expression. The interaction of multiple coactivators with transcription factors in different temporal and spatial contexts provides another possible level of regulation to gene expression.

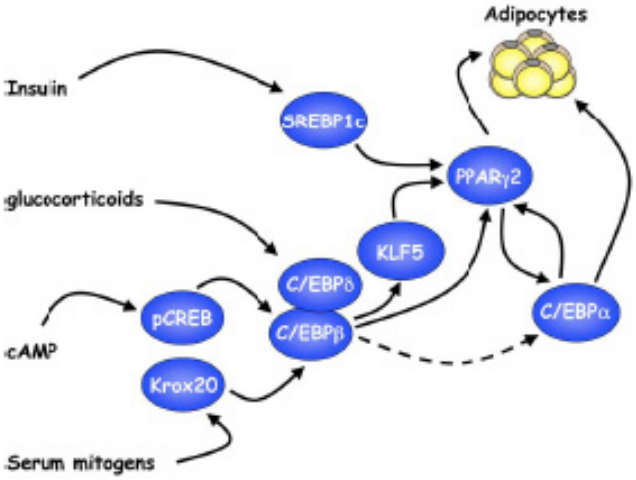


*Figure 2 Transcriptional Regulation of Adipocyte Differentiation PPARγ and C/EBP family interact to control adipose differentiation; one major function of C/EBPβ and C/EBPδ is to induce the expression of PPARγ, which in turn activates C/EBPα. C/EBPα exerts positive feedback on PPARγ to maintain the differentiated state. SREBP1 can activate PPARγ by inducing its expression as well as by promoting the production of an endogenous PPARγ ligand. Activation of the transcription factors involved ca be achieved by exposure of preadipocytes to a cocktail of adipogenic inducers comprised of insulin, glucocorticoids, IBMX, and fetal bovine serum [13].*

# Obesity

Obesity occurs when caloric intake exceeds caloric expenditure, defined in medicine as an excess of body fat and it is now considered a public health problem in most economically advanced countries. The prevalence of the disorder in the United States comprises ~1 in 3 adults [18]. The Obesity epidemic has developed with considerable rapidity over the past 2 decades, with the incidence rising 3-fold in the U.K., for example, since the early 1980s [19]and the problem is not limited to adults, a marked increase in Obesity is found among children [20]. Obesity is associated with a reduction in life span of ~8 years, as well as with an increased risk of several major diseases, including Type II Diabetes, coronary heart disease, and certain cancers (such as breast and colon). In the case of Type II Diabetes, the risk is particularly marked because being obese increases the likelihood of developing the disease by ~10-fold once a BMI of 30 $kg/m^2$ is reached. Furthermore, the greater is the degree of Obesity, the higher is the relative risk.

The human classification of Obesity is based on the Body Mass Index (BMI): a BMI between 20 to 30 $kg/(m^2)$ represents an overweight state, while Obesity is defined as a BMI value above 30 $kg/(m^2)$.

In many countries Obesity has reached epidemic proportions and it is more prevalent in certain ethnic groups, but in general Obesity increases with age, it is higher in women and those from lower socio-economic strata in Western countries; the distribution of fat is sexually dimorphic: in general more subcutaneous fat is found in women (gynecoid distribution), while an increase in intra-abdominal fat (android distribution) is found in men. These two types of adipose tissue present metabolically differently behaviors, since android Obesity is strongly associated with insulin resistance and increased cardiovascular morbidity and mortality (i.e. risk of heart attacks).

The primary role of adipose tissue is the control of energy intake through fatty acid deposition (as triacylglycerols) and release, and the apparent simplicity of both white

adipocytes and of WAT itself, histologically and metabolically, are the key reasons why the organ has been relatively ignored until recently. However, this has changed radically over the past few years with the tissue becoming a focus of intense research activity, given that Obesity is defined by the expansion of the tissue.

White adipose tissue (WAT) is a now recognized as major secretory organ, particularly through the release of free fatty acids (FFA) during fasting.

In addition to FFA, adipose tissue also releases other lipid moieties, such as cholesterol, retinol, steroid hormones, and prostaglandins [21]. Cholesterol and retinol are not synthesized by WAT, but rather are taken up and stored within the tissue, and steroid hormone conversions can take place in white adipocytes [22]. Moreover, the enzyme lipoprotein lipase (LPL) is released from adipocytes for the breakdown of circulating triacylglycerols to FFA, which are subsequently stored within fat cells.

Moreover, WAT is the primary site of the production of key hormones involved not only in energy balance, but also involved in a range of metabolic and physiological processes; some of these factors, termed adipokines, are implicated in the pathologies associated with Obesity, particularly insulin resistance and the Metabolic Syndrome [21, 23, 24].

## Etiology of Obesity

Obesity takes place when food intake exceeds energy expenditure. In a sedentary adult with as standard average daily intake, total energy expenditure is given by basal (resting) metabolic rate (60–70%) plus obligatory thermogenesis (5–15%), and physical activity (spontaneous activity: 20–30%, physical work and exercise will need additional energy). The factors that control food intake are complex and not only involve physiological control mechanisms but also social, cultural aspects about meals as well as about physical activity. While Western industrialized nations become progressively more sedentary, food

industries and food technology provide cheap and highly palatable food, which is rich in fat as this is one of the mechanisms generating palatability, but fat is also energy dense at 9 kcal/g compared with carbohydrate or protein at 4 kcal/g.

About basal metabolic rate, there is no evidence that the obese have a low resting metabolic rate; indeed an obese person has a higher rate than a lean person of the same height, since as the total body mass increases, resting metabolic rate increases.

It is now well recognized that, for about 80% of the effect, the development of Obesity is given by a genetic component, described first by experimental rodent models of Obesity and defects found in human, including: the loss of function mutation in leptin gene (*ob*/*ob* mouse); the loss of function mutation in leptin receptor *(db*/*db* mouse); the melanocortin 4 receptor defects; Pro-opiomelanocortin cleavage defect leading to loss of the Melanocyte Stimulating Hormone **(**MSH). In addition, there are a number of other rare disorders associated with Obesity such as Prader-Willi or Bardet-Biedl syndromes. For the most part, the exact genes involved in development of Obesity remain to be elucidated.

# Adipose Tissue as a secretory organ

The pivotal change in perspective on the role of WAT as a secretory organ came with the identification of the hormone leptin in 1994, which followed the search for the Ob gene, a mutation in which is responsible for the Obesity of the *ob*/*ob* mouse [25]. Leptin, a 16 kDa cytokine-like protein, is a critical hormonal signal from adipocytes in the regulation of appetite and energy balance [26, 27], interacting with several hypothalamic orexigenic and anorexigenic pathways.

The diversity of the adipokines (described below) secreted by the adipose tissue in addition to leptin, is considerable, in terms of both protein structure and function. The adipokines *(figure 3)* include classical cytokines (e.g., TNFα, IL6), chemokines (monocyte chemoattractant protein-1 (MCP1)), proteins of the alternative complement system (adipsin), and proteins involved in vascular hemostasis (plasminogen activator inhibitor-1 (PAI1)), the regulation of blood pressure (angiotensinogen), lipid metabolism (cholesteryl ester transfer protein, retinol binding protein-4), glucose homeostasis (adiponectin), and angiogenesis (vascular endothelial growth factor (VEGF)).

From the wide range of adipokines identified over the past few years, it is apparent that white fat is a secretory organ of considerable complexity communicating extensively with other organs and closely integrated into overall physiological and metabolic control.

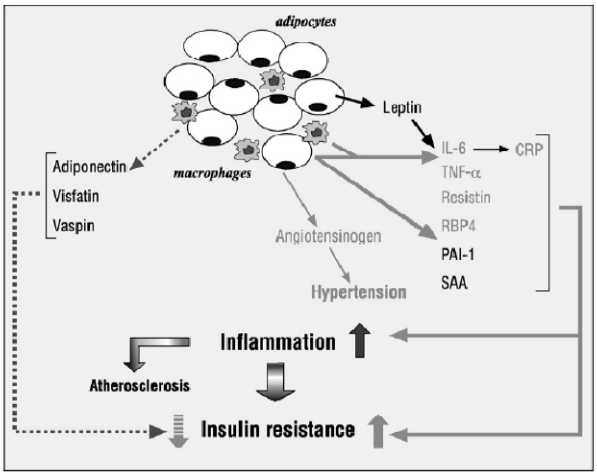


*Figure 3 Adipokines Adipokines produced and secreted by adipocytes and their role in Type II Diabetes and in the Metabolic Syndrome [28].*

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# Leptin

An endocrine role for WAT in the regulation of energy balance and other physiological processes has been established through the identification of leptin, the hormone being secreted principally from adipocytes, with actions both centrally (particularly in the hypothalamus) and in peripheral organs.

Leptin (called the Adipocyte Hormone) is the most important protein factor secreted by differentiated mature white adipocytes [29] discovered in 1994 by Friedman and colleagues [25], with the identification of the mutant gene which underlies the development of the Obesity of the *ob*/*ob* mouse.

The leptin gene (ob) encodes an 18 kDa protein containing a signal sequence cleaved to produce the mature hormone of molecular weight 16 kDa [25]. Since initial studies indicates white adipose tissue as the unique site of leptin synthesis, it is now recognized that it can be secreted also by brown adipose tissue, the stomach, placenta, mammary gland, ovarian follicles and fetal heart and bone or cartilage, and perhaps even the brain [21, 30]. Anyway, WAT is the main site of leptin production and the major determinant of the level of the circulating hormone; as the amount of body fat increases, the plasma leptin

does and both circulating levels and adipose tissue mRNA expression of leptin [31] are strongly associated with BMI and fat mass in Obesity; conversely, transgenic mice have very low circulating leptin levels [32]. Thus the circulating hormone level is a marker of total body fat mass [33, 34], where the subcutaneous fraction represents about 80% of total fat.

Leptin production in adipose tissue presents differences given by species and developmental stage; in rodents the levels of *ob* gene expression are highest in the gonadal and perirenal adipose tissue of the mature animal, and lowest in the subcutaneous depots [35], while in humans the subcutaneous tissue exhibits higher levels of *ob* mRNA than omental fat [36]. Nutritional state influences leptin synthesis in adipose tissue [30]: in fact, *ob* gene expression is suppressed by fasting (effect reversed on refeeding), such as acute exposure to cold [37] and neurotransmitters as catecholamines, both noradrenaline and adrenaline [30]. On the contrary, several hormones stimulate leptin production: insulin, glucocorticoids and oestrogens and cytokines as TNFα [30].

The hormone has an autocrine and paracrine function in adipose tissue because of the presence of one or more leptin receptor isoforms are found in WAT and in many other tissues [38].

The most important receptor isoform in leptin signaling is found in regions of the brain that represent the major target for leptin: the arcuate nucleus and paraventricular nucleus of the hypothalamus [39, 40] which are associated with the central control of food intake and energy balance.

The main role of leptin is the regulation of energy homeostasis [41] through its interaction with several central neuroendocrine systems, including suppression of neuropeptide Y secretion [42], leading to the inhibition of food intake [43, 44]. Other key effects of the hormone are involved in energy expenditure, in angiogenesis, in the immune system and in sexual maturation in females [45-48], or in many metabolic processes, such as inhibition of insulin secretion by pancreatic β-cells, stimulation of glucose transport or coagulation [49-51].

In addition to its well-described role in energy balance, the anti-hyperglycemic actions of leptin are mediated through several different organs. In muscle, leptin improves insulin sensitivity and reduces intra-myocellular lipid levels through a combination of direct activation of AMP-activated protein kinase (AMPK) and indirect actions mediated through central neural pathways [52]. As in muscle, leptin also improves insulin sensitivity in the liver, and reduces hepatic intracellular triacylglycerol levels [53].

## Adiponectin

Adiponectin is a 30-kDa protein highly expressed in adipose tissue, but in contrast to other adipose- derived factors, adiponectin acts as an insulin sensitizer hormone toward insulin sensitive tissues. It was identified by several different groups and given various names (apM1, GBP28, AdipoQ and ACRP30) [54] and circulates at great high concentrations (5–10 μg/ml), accounting for 0.01% of all plasma protein, and its blood levels are inversely correlated with body mass. In fact, adiponectin is underexpressed in obese patients with insulin resistance, Type II Diabetes, and in patients with coronary heart disease, and presents an insulin-sensitizing effect like leptin, stimulating fatty acid oxidation in an AMPK and PPAR$\alpha$ -dependent manner [55, 56]; adiponectin is involved in the regulation of glucose homeostasis since it ameliorates hyperglycemia reducing hepatic glucose production, by decreasing the expression of two essential gluconeogenesis enzymes: phosphoenolpyruvate carboxykinase (PEPK); and glucose-6-phosphatase (G6P) [57].

In addition, adiponectin has a vascular-protective effect in the atherogenesis process, and could reduce the inflammatory response induced by TNFα [29, 58, 59].

## Retinol Binding Protein 4

The Retinol Binding Protein 4 (RBP4) is the only known specific transporter protein for retinol (vitamin A) in the circulation, and its only known function was to deliver retinol to tissues, but Yang et al. in 2005 reported RBP4 is the link between Obesity and insulin resistance and defined its role in impairing insulin sensitivity in insulin-sensible tissues, such as skeletal muscle and liver [60]. Yang and colleagues generated mice with an adipose-specific reduction of GLUT4 (adipose GLUT4$^{-/-}$ knockout mice), which develop liver and skeletal muscle insulin resistance. In this model, plasma levels of insulin and adipokines known to influence insulin sensitivity remain normal, thus suggesting that a circulating factor secreted by adipocytes causes insulin resistance in these mice. RBP4 serum levels are elevated in insulin-resistant rodents, and in obese or type II diabetic humans; this confirmed that RBP4 was the factor secreted by adipocytes which reduces phosphatidylinositol-3-OH kinase (PI3K) signaling in muscle and enhances expression of the gluconeogenic enzyme PEPCK in the liver through a retinol-dependent mechanism, and thus participating in Type II Diabetes pathophysiology.

## Free fatty acids

The most important adipose secreted products are the FFA, the nutrient source released during fasting for the rest of the body. To this aim, the whole effect of circulating FFA is to promote lipid burning as an energy source in most tissues sparing carbohydrate for neurons and red blood cells, which depend on glucose, and thus they are able to regulate glucose homeostasis, reducing adipocyte and muscle glucose uptake and promoting hepatic glucose production [61].

# Inflammation and Obesity

A recent and striking discovery is that Obesity is associated with a chronic inflammatory response in adipose tissue, characterized by abnormal adipokine production and activation of some pro-inflammatory signaling pathways, resulting in the induction of several biological markers of inflammation [62]. Conversely, a reduction in body weight is accompanied by a decrease or even a normalization of these biological parameters [63]. The role of fat cells in metabolic dysfunctions has long been considered, but their potential role in an inflammatory process is a new concept and several animal models suggest that these inflammatory processes have a causal relationship with Obesity and insulin resistance, Type II Diabetes and cardio vascular diseases. Several findings have converged to indicate that adipocytes share with immune cells certain properties such as complement activation [64] and pro-inflammatory cytokine production [65]. Moreover, preadipocytes have the capacity for phagocytosis in response to several stimuli [66] and numerous genes that code for transcription factors, cytokines, inflammatory signaling molecules, and fatty acid transporters essential for adipocyte biology, are also expressed and functional in macrophages [67]. In Obesity, the inflammatory state involves not only adipose tissue, but altered levels of several circulating factors such as an increase in the plasma levels of C-reactive protein (CRP), TNF$\alpha$, IL-6, and other biological markers of inflammation, suggest an overall inflammatory state [68].

Other adipose-specific molecules that are involved in the control of energy metabolism also regulate immune responses. Several studies have shown that adipokine production is altered in Obesity, type II Diabetes and Metabolic Syndrome. This is observed for leptin, TNF$\alpha$, IL-6, adiponectin and resistin, angiotensinogen, PAI-1 or visfatin.

## Tumor Necrosis Factor α

A major step forward in the recognition of the secretory role of WAT occurred in the early 1990s with the discovery that the proinflammatory cytokine tumor necrosis factor-α (TNFα) is synthesized and released by adipocytes in rodents and in humans [65]. TNFα expression increases in Obesity, and this cytokine plays an important role in the pathophysiology of insulin resistance (see Introduction-Insulin Resistance and Type II Diabetes). Moreover, TNFα was shown to have extensive metabolic effects in adipose tissue, including the stimulation of lipolysis and apoptosis [69, 70].

## Interleukin 6

Another classical cytokine expressed by adipose tissue is Interleukin-6 (IL6), produced by fibroblasts, endothelial cells, monocytes and adipocytes. In Obesity, its production by adipocytes is increased [24] even if in the absence of an acute inflammatory process [71]. Secretion of IL6 from adipose tissue is higher in visceral than in subcutaneous depots (three times more), underlining the fundamental role of central Obesity in cardiovascular diseases. Moreover, IL6 production derives mainly from the stromal vascular fraction (endothelial cells, monocytes/macrophages) rather than from adipocytes themselves [72].

Deleterious effects given by IL6 secretion are due to its diverse target tissues and function. First of all, IL6 controls the hepatic production of inflammatory proteins such as CRP, important cardiovascular risk factor [73]. Moreover, IL6 produced by visceral adipose tissue directly contributes to hypertriglyceridemia by stimulating hepatic secretion of very low-density lipoproteins (VLDL) [74].

## Resistin

Resistin (FIZZ3) is an inflammatory molecule with hyperglycemic action belonging to the family of cysteinerich resistin-like molecules (RELMs); it was discovered as a secreted product of mouse adipocytes that was repressed by thiazolidinediones [75].

While levels of resistin are elevated in many murine models of Obesity, the role of resistin in humans remain controversial; data suggest that it could be the product of macrophages or other stromal cells within the fat pad [76] and might be involved in reducing glucose uptake by muscles and fat, but poor is known about its effect on the liver.

Leptin, in addition to its key role in food intake and energy expenditure also regulates immune processes; although leptin acts mainly at the level of the central nervous system regulating food intake and energy expenditure, there is a relationship between leptin and the lowgrade inflammatory state in Obesity, suggesting that leptin could exert peripheral biological effects [77]. Indeed, leptin is able to control TNF$\alpha$ production and activation by macrophages [78], but the underlying mechanisms have not been clearly identified, and leptin-deficient mice or humans display an altered immune status [79].

# Obesity-induced Insulin Resistance and Type II Diabetes

Obesity has been identified as a major causative factor for the insulin resistance and hyperglycemia associated with Diabetes [80], and Obesity-induced Diabetes is emerging as a global health-care problem reaching epidemic proportions in the industrialized countries and also increasing in developing nations. Along with Diabetes, there has been a concomitant increase of the incidence of an Obesity-linked condition, called the Metabolic Syndrome. The Metabolic Syndrome is defined as a clustering of diseases, such as abdominal Obesity, insulin resistance, dyslipidemia, and elevated blood pressure, reflecting overnutrition and sedentary lifestyles [81], associated with a doubling of cardiovascular disease risk and a 5-fold increased risk for incident Type II Diabetes. Many conditions are directly associated with the Metabolic Syndrome, mainly because of the excess in adiposity and the insulin resistance: Nonalcoholic fatty liver disease, Polycystic ovarian syndrome, Obstructive sleep apnea, Hypogonadism, Lipodystrophy and Microvascular disease.

The most critical factor in the emergence of metabolic diseases is Obesity, since it is responsible for the risk factor common to all the pathologies of the Metabolic Syndrome: the insulin resistance [81].

## Insulin Resistance and Type II Diabetes

Insulin is the principal hormone of glucose homeostasis; it stimulates glucose influx into muscle, glycogen synthesis in the liver and muscle, and fat deposition in adipocytes [82]. Other important actions of insulin include the enhancement of protein synthesis, cell survival and growth, prevention of protein catabolism, and anti-inflammatory effects [83-86].

The intracellular insulin signal transduction pathway is summarized in *figure 4*. The binding of insulin to the α-subunit of the insulin receptor (IR) molecule induces rapid autophosphorylation of the β subunit, which turns on its tyrosine kinase activity [82]. This gives IR the ability to phosphorylate various tyrosine residues of other cytosolic moieties, including insulin receptor substrates (IRS) 1 and 2. The tyrosine phosphorylation of IRS proteins leads to the second intracellular step of insulin action, the association of phosphorylated IRS1 or IRS2 with the enzyme phosphoinositide-3-kinase (PI3K). The IRS-activated PI3K in turn affects several downstream signaling pathways through the generation of a lipid second messenger, phosphatidyl-inositol-3,4,5-triphosphosphate, and through the phosphorylation of its critical target, the serine–threonine kinase B (Akt/PKB) [87]. Akt/PKB is the major effector of the IR–IRS-1–PI3K pathway and drives the metabolic actions of insulin, including glucose transport, glycogen synthesis, fat deposition, and protein synthesis, and also cell growth and cell survival [88].

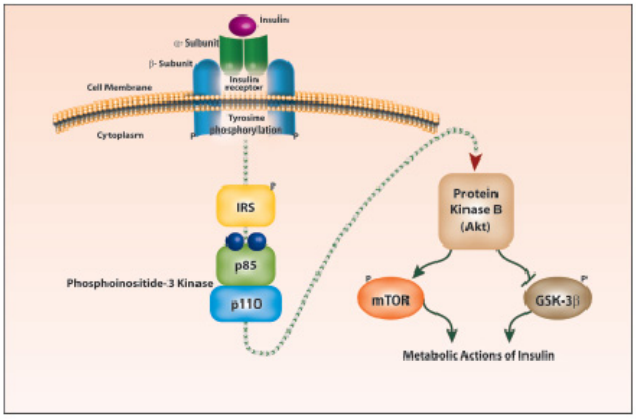


*Fig. 4 Intracellular Insulin Signal Transduction. Insulin binding to the insulin receptor triggers its autophosphorylation and catalyzes the tyrosine phosphorylation of IRS1 and 2. These IRS proteins interact with diverse signaling molecules, including PI3K which in turn activate Akt/PKB. The downstream proteins controlled by Akt/PKB include mTOR) and GSK-3. The actions of insulin include glucose metabolism, glycogen–lipid–protein synthesis, cell growth and survival [89].*

A defect in insulin release by the β-cell is crucial to the pathogenesis of Type II Diabetes and how insulin resistance causes the onset Type I Diabetes through β-cell dysfunction and glucotoxicity is actually mainly known. β-cells are responsible for sensing and secreting the appropriate amount of insulin in response to a glucose stimulus. In healthy individuals, there is a feedback loop between the insulin-sensitive tissues and the β-cells, with β-cells increasing insulin supply in response to demand by the liver, muscles and adipose tissue [90]. In the early stages of Type II Diabetes, euglycemia is maintained and insulin resistance is countered by the continuous insulin production in the pancreas. Progressively, β-cells fail to respond adequately to blood glucose stimulation, and this importantly contributes to reduce insulin release. As a result of β-cell dysfunction, decreased insulin secretion determinates disordered regulation of glucose levels by decreasing suppression of hepatic glucose production and reducing the efficiency of glucose uptake in insulin-sensitive tissues. Decreased insulin output could also impair adipocyte metabolism, resulting in increased lipolysis and elevated FFA levels. Chronic exposure to FFA causes a decrease in insulin secretion and this effect is mediated mainly by lipotoxicity induced apoptosis of islet cells [91]. Moreover, the extremely elevated blood glucose levels observed in diabetes contribute to further disease progression through glucotoxic effects on the β-cell. Impairments of insulin release may have central effects on metabolic homeostasis: insulin acts in the hypothalamus to regulate body weight, and impaired insulin signaling is associated with changes in food intake and body weight [92]. Thus, β-cell dysfunction resulting in a relative reduction in insulin release would be expected to result in decreased insulin action in this crucial brain region and be associated with weight gain and an aggravation of insulin resistance.

# Pathogenesis of Insulin Resistance

Although many is known about the onset of Type II Diabetes starting from an insulin resistant condition, the pathogenesis of Obesity-induced insulin resistance itself has not been fully elucidated.

As a person gains weight, adipose tissue becomes morphologically and metabolically dysfunctional, with visceral adipocytes particularly affected [93-95]. These changes contribute to aberrant endocrine function, with multiple potential consequences in terms of metabolic dysfunction, insulin resistance, and cardiovascular disease risk [96]. Central appear to be some of the derangements in insulin signaling caused by the adipose tissue release of FFA, hormones and proinflammatory cytokines [80, 97] which are overproducted in Obesity, but a further fundamental role is attributed to the fat depots distribution, to polymorphism of some key genes and to the marked infiltration of adipose tissue by macrophages associated with Obesity [98, 99].

In an expanded adipose tissue the turnover of FFA is increased, as the process of FFA mobilization (lipolysis) from stores is accelerated [100]. Under normal conditions, insulin inhibits adipose tissue lipolysis; however, in the setting of insulin resistance, insulin is unable to properly suppress lipolysis, resulting in increased circulating FFA levels [101]. Not only insulin resistance appears to cause circulating FFA increase, but the release of FFA may be a critical factor in modulating insulin sensitivity, and many evidences suggest that the visceral adipose depot mainly contributes to increased FFA turnover and insulin resistance [102].

Increased intracellular FFA in peripheral tissues might result in competition with glucose for substrate oxidation, leading to the serial inhibition of three key enzymes of the glucose fatty-acids cycle[103], and in an increase in the intracellular content of fatty acid metabolites such as diacylglycerol (DAG), fatty acyl-coenzyme A (fatty acyl-CoA), and ceramides, which cause serine/threonine phosphorylation of IRS1and IRS-2, and a reduced

ability of these molecules to activate PI3K [104]. Moreover, the increase in circulating levels of FFA enhances skeletal muscle lipid accumulation, and this has been implicated in the induction of Obesity-related insulin resistance [105]. In fact, increased lipid accumulation results in activation of protein kinase C θ and the JNK/SAPK pathway, in part through the elevated production of ceramide, which mediates impaired insulin signal transduction in muscle [106, 107].

Among the various adipokines secreted by adipose tissue influencing the insulin signaling, the most important seems to be the RBP4; it induces insulin resistance interfering with the intracellular insulin signaling pathway in muscle, through reduced PI3K signaling, and enhanced expression of the gluconeogenic enzyme Phosphoenolpyruvate Carboxykinase (PEPCK) in the liver through a retinol-dependent mechanism [60].

Another manner in which adipose tissue contributes to the pathophysiology of insulin resistance is through the excessive release of proinflammatory cytokines. In Obesity, the classic features of acute inflammation tumor, rubor, dolor, calor (swelling, redness, pain, and fever are absent, but chronic inflammation and release of inflammatory cytokines, including TNFα, IL6 and interleukin-1β (IL1β), is observed. The source of these cytokines in adipose tissue is debated. A report by Ferrante and colleagues [98] suggested the primary role of macrophages infiltration in adipose tissue; adipocyte cell death typical of Obesity is the primary event underlying insulin insensitivity, because it triggers the release of macrophage chemoattractant protein-1(MCP1) by the adipocyte and recruitment of macrophages in the tissue for clearance purposes; the subsequent macrophages infiltration appears to explain the presence of chronic inflammation [108]. The infiltrating macrophages are implicated in cytokine production, but the role of adipocytes in adipose tissue inflammation development in Obesity has been revalued, since it is now clear that larger fat cells also produce more cytokines [109].

Not only are circulating cytokines from adipose tissue important to insulin action in other tissues such as the liver or skeletal muscle, but paracrine effects of the cytokines may also modify insulin action locally in adipose tissue [110].

In general, factors that negatively influence the intracellular insulin signal transduction act through serine/threonine phosphorylation of IRS1, and this process has two major effects. First, serine/threonine phosphorylated IRS molecules are less able to associate with the insulin receptor and downstream target molecules, especially PI3K *(Evans et al. 2005),* resulting in impaired insulin action and Akt/PKB activation. Secondly, the serine/threonine phosphorylated forms of IRS molecules are more susceptible to proteasome mediated degradation [111]. Inflammatory cytokines work at the same level. TNFα, through the serine-phosphorylation of the IRS1, prevents its interaction with the insulin receptor β subunit, and stops the insulin signaling pathway [65]. Subsequently, events downstream of insulin-receptor signaling are diminished. Moreover, TNF-$\alpha$ and IL-6 act through classical receptor-mediated processes to stimulate both the c-Jun aminoterminal kinase (JNK) and the IκB kinase-β (IKK-β)/nuclear factor-κB (NF-κB) pathways, resulting in upregulation of potential mediators of inflammation that can lead to insulin resistance.

The anatomical distribution of fat depots is a critical determinant of insulin. Abdominal obese patients, which have a predominant accumulation of fat in the upper part of the body have also more extensive visceral adipose depots (VAT), and this raises the risk of insulin resistance, Type II Diabetes, and further risk factors of metabolic and cardiovascular diseases [112], given that visceral abdominal adipocytes are more endocrinologically active than subcutaneous adipocytes [95]. Excess of VAT is characterized by anomalies of blood-glucose homoeostasis, elevated plasma triglycerides and low levels of high-density lipoprotein (HDL) cholesterol. These disorders are often associated with a prothrombotic and proinflammatory state that contributes to the later appearance of cardiovascular complications.

Two hypotheses have been proposed to explain the glucose intolerance, insulin resistance and lipid abnormalities associated with visceral Obesity. The first and older explanation is the "portal hypothesis" advanced by Björntorp [113]. It has been shown that VAT releases a large amount of FFA and hormones/cytokines in the portal vein that are then directly delivered to the liver; VAT-derived FFA alters several liver functions: reduce hepatic glucose uptake in the postprandial period, positive regulate gluconeogenesis, reduce insulin suppression of gluconeogenesis, and induce insulin resistance, mainly through PKC activation. The second hypothesis concerns the delivery of the adipokines involved in insulin sensitivity (TNFα, IL6, interleukin, leptin, resistin, adiponectin) released within VAT, into the portal vein.

Moreover, also in lean individuals insulin resistance is strongly associated with differences in body fat distribution: lean individuals with a more peripheral distribution of fat are more insulin sensitive than lean subjects who have their fat distributed predominantly centrally [114].

Differences in the characteristics of adipose tissue from visceral and subcutaneous depots explain in part the different metabolic effects of the two tissues. For example, intra-abdominal fat expresses more genes encoding secretory proteins and proteins responsible for energy production [115], and the amount of protein released per adipocyte also differs according to their location [72]. Although the secretion of the insulin sensitizing cytokine adiponectin by visceral adipocytes is greater than that of subcutaneous adipocytes, the subcutaneous depot represents a greater proportion of total body fat, and thus its contribution to total adiponectin levels is greater.

Many genes interact with the environment to produce insulin resistance. The gene variant most commonly associated with insulin sensitivity is the P12A polymorphism in *PPARγ*, which is associated with an increased risk of developing diabetes [116]. A number of genes associated with β-cell dysfunction have been identified, such as the hepatocyte nuclear factor-4*α* and 1*α* genes, but many other candidates (calpain 10, adiponectin,

PPARγ coactivator 1 (*PGC1*) and the glucose transporter *GLUT2)* are object of research [116].

# Reactive Oxygen Species

Reactive Oxygen Species (ROS) are a group of highly reactive molecules including oxygen anions and radicals, or milder oxidants such as hydrogen peroxide. In the cells, ROS are prevalently generated at the mitochondrial level in a non-enzymatic way, because ROS are normal products of the electron transport chain (ETC), where oxygen is reduced to produce water. In few, but constant cases, a proximal and incomplete reduction of molecular oxygen occurs, primarily at two discrete points, namely at complex I (NADH dehydrogenase) complex III (ubiquinone–cytochrome *c* reductase), leading to the formation of the superoxide radical ($O_2^-$) [117].

Superoxide anions can react with nitric oxide produces peroxynitrite, or be enzymatically converted by a manganese Superoxide Dismutase (MnSOD) to hydrogen peroxide, that together with superoxide anions and peroxynitrite represent the mitochondrial ROS, very powerful chemical oxidants (Turrens, 2003). Cytosolic enzyme systems also contribute to oxidative stress, among others, the plasma membrane/phagosome associated enzyme complex, termed NADPH oxidase, a superoxide-generating system that was first described in phagocytic cells of the immune systems, as macrophages and neutrophils, which uses the superoxides to damage and kill pathogenic organisms. However, it has now become clear that NADPH oxidase is not restricted to the immune system but alternative isoforms may be active in many other cell types as an essential component of redox signaling mechanisms.

Cells arrange antioxidant systems to neutralize ROS, to prevent their accumulation inside the cell and thus the so-called oxidative damage, and they are represented by the enzymatic scavengers SOD, Catalase and Glutathione (GSH) Peroxidase. In mitochondria, the MnSOD provides the enzymatical conversion of superoxide anions to hydrogen peroxide, while hydrogen peroxide is rapidly removed by the GSH Peroxidase. Another defense mechanism is the antioxidant enzyme Catalase, which acts detoxifying hydrogen

peroxide to molecular oxygen and water, and is found exclusively in peroxisomes (Turrens, 2003). Mutations in genes involved in the cellular scavenging system are associated with neurological diseases, such as the Parkinson's disease (mutation in the mitochondrial targeting sequence (MTS) of MnSOD gene) [118], and Amyotrophic lateral sclerosis (ALS) (mutation in the gene coding for the Copper Zinc Superoxide Dismutase (CuZnSOD), the cytosolic isoform of the scavenging enzyme) [119]. Moreover, non-enzymatic molecules are involved in scavenging ROS: ascorbate, pyruvate, flavonoids, carotenoids and the vitamin E, located in inner mitochondrial membrane, is a powerful antioxidant which accepts unpaired electrons to produce a stable product.

Although the cellular defense mechanisms, it is still possible for ROS to accumulate within the cell, to lead to oxidative damage, that has been implicated in several pathological conditions, such as inflammation, type II Diabetes, aging and tumorigenesis [120], Oxidative damage causes protein damage, lipid peroxidation and several types of DNA lesions: single- and double- strand brakes, adducts and crosslinks, and accumulation of these damages inside the cell is responsible for cell death or acceleration in ageing and age-related diseases. Both the mitochondrion and nucleus contain a variety of DNA repair enzymes to correct damage to DNA or mutations induced by oxidants [117, 121], but mitochondrial DNA is generally more sensitive then nuclear DNA to oxidative damage, probably because of its limited DNA repair system, or its proximity to the main source of oxidants within the cell. Damaged mitochondria are replaced by autophagy and mitochondrial biogenesis.

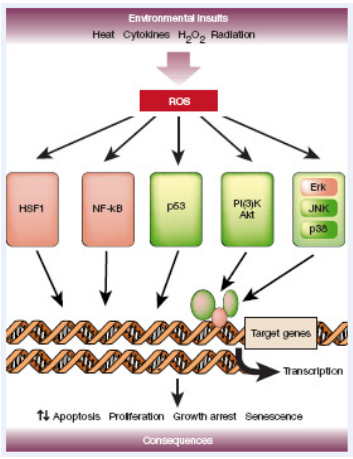


*Figure 5 Major signaling pathways activated in response to oxidative stress. ROS can originate outside or inside the cell and activate several distinct pathway, depending on the nature and duration of the stress, as well as the cell type. The consequences of the response ranges from cell proliferation, growth arrest, senescence and cell death (HSF1: Heat-shock transcription factor 1) [122] .*

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In addition to these effects, a rise in ROS levels may constitute a stress signal that activates specific redox-sensitive signaling pathways (summarized in *figure 5*). This will lead to cell dysfunction and possibly death resulting in oxidative stress-related diseases.

Apoptosis (or programmed cell death) is the mechanism used by mammals, plants and other organisms to eliminate redundant or damaged cells [123], and can be triggered by extracellular signals (extrinsic pathway) or by intracellular processes (intrinsic pathway). An increased mitochondrial formation of ROS triggers the intrinsic pathway, by increasing the permeability of the outer mitochondrial membrane through the opening of transition pores. As a result, cytochrome *c* moves from the intermembrane space into the cytoplasm where it joins another factor (Apaf-1), and this in turn activates the death program. As cytochrome *c* is an $O_2$ scavenger and at its release the respiratory chain becomes more reduced because electron flow between Complex III and Complex IV slows down [124], permeability transition pore opening favors the mitochondrial generation ROS, augmenting the intracellular oxidative stress and leading to further damages.

Furthermore, the continuous formation of ROS in mitochondria and the slow accumulation of DNA damages, results in the aging process [125]. Thus, factors that increase resistance to stress should have anti-ageing benefits and lead to enhanced life span. Many mutations have revealed links between longevity and stress resistance in multicellular organisms: loss-of-function mutations of the *age-1* (the human PI3K homologue) gene in *C. elegans* is associated with a 65% increase in lifespan [126], while in Drosophila, mutations in the *methuselah* (*mth*) gene not only enhances longevity but also increases resistance to heat stress and intracellular ROS [127].

Another study found an unexpected correlation between life span and control of the oxidative stress response: targeted mutation of the *shc* gene locus, which caused ablation of a splice variant of 66 kDa (p66shc), resulted in selective resistance to oxidative stress and extended (+30%) life span in mice [128]. p66shc is one of three splice variants derived from the *shc* locus: p52shc and p46shc are important adaptor proteins regulated by tyrosine phosphorylation and participating in growth-factor- and stress-induced ERK activation. p66shc also undergoes tyrosine phosphorylation in response to extracellular signals, however the p66shc is unique in that it also becomes phosphorylated on serine residues in response to certain extracellular signals and, in the case of oxidative stress, this leads to apoptosis. Serine phosphorylation of p66shc is also stimulated by insulin [129].

Caloric restriction has been shown to extend life span in a wide range of species, and the favored hypothesis proposes that it acts by decreasing oxidative stress [130], since the rate of oxidant generation of mitochondria from calorically restricted mice is significantly lower than from their ad libitum-fed counterparts [131].

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# ROS in insulin resistance and Type II Diabetes

It is well established that chronic elevation of plasma glucose causes many of the major complications of diabetes, including nephropathy, retinopathy, neuropathy, and macro- and microvascular damage [132]. Furthermore, in a hyperglycemic state, elevated FFA levels are positively correlated with both insulin resistance and the deterioration of β-cell function [133]. These effects may result from oxidative stress.

Many studies have shown that diabetes and hyperglycemia increase oxidative stress, a concept reviewed by Brownlee: hyperglycemia causes diabetic complications through increased polyol pathway flux, increased advanced glycation end-product (AGE) formation, activation of protein kinase C (PKC) isoforms and increased hexosamine pathway flux, and the four different pathogenic mechanisms reflects a single hyperglycemia-induced process: overproduction of superoxide by the mitochondrial electron-transport chain (*figure 6*).

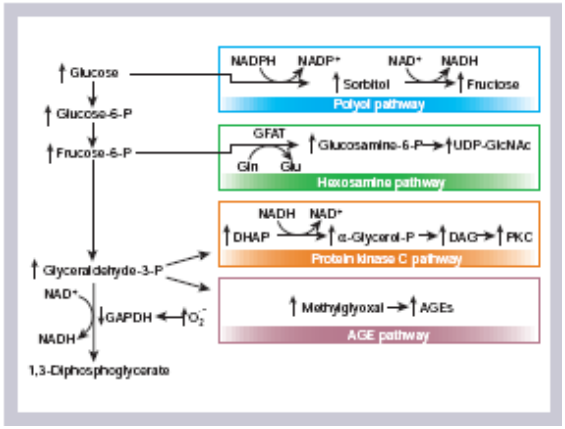


*Figure 6 Hyperglycemia activates four intracellular pathways through mitochondrial superoxide overproduction. Excess superoxide partially inhibits the glycolytic enzyme GAPDH, thereby diverting upstream metabolites from glycolysis into pathways of glucose overutilization. This results in increased flux of dihydroxyacetone phosphate (DHAP) to DAG, an activator of PKC, and of triose phosphates to methylglyoxal, the main intracellular AGE precursor. Increased flux of fructose-6-phosphate to UDP-N-acetylglucosamine increases modification of proteins by O-linked N-acetylglucosamine (GlcNAc) and increased glucose flux through the polyol pathway consumes NADPH and depletes GSH [134].*

Moreover, increased FFA metabolism may also lead to increased ROS production. It has been reported that glucose or FFA initiate the formation of ROS in muscle, adipocytes, pancreatic β-cells and other cells. In fact, fatty acids compete with glucose for substrate oxidation in isolated cardiac muscles, and increased oxidation of fatty acids would cause an increase in the intramitochondrial NADH/NAD+ ratio. In this way, increased FFA may also lead to an increased ROS production [134]. An additional target of oxidative stress is the β-cell, since many studies have suggested that β cell dysfunction is the result of prolonged exposure to high glucose, elevated FFA levels, or a combination of the two [135].

As previously discussed, insulin resistance plays a central role in the development of several metabolic disorders and diseases, such as Obesity, Type II Diabetes and the Metabolic Syndrome. Oxidative stress has been postulated to play an important role in diminishing insulin sensitivity [136], and actually the cellular effectors linking the oxidative damage to the onset of an insulin resistant state are object of intense research. The oxidative stress-induced insulin resistance hypothesis was finally confirmed in a paper by Bloch-Damti *et al*., [137]: the authors found that oxidative stress condition (treatment with $H_2O_2$) caused an impairment of the intracellular insulin pathway, thus leading to insulin resistance in 3T3-L1 adipocytes. At this level, insulin resistance could represent a defensive system of the cell: as it inhibits any insulin action, should result in a decreased cellular ROS production. Fridlyand *et al*. suggest that, because of cellular incapacity to modify glucose, FFA or insulin concentrations in the blood, the restriction of glucose entry prevents ROS overproduction, as to say insulin resistance could be a physiological mechanism of prevention of oxidative stress, activated by the cell in response to conditions stimulating ROS production [138].

Thus, concomitant with the failure of antioxidant defense, ROS contribute to the onset of Type II Diabetes and its complications [134], acting not only through the induction of a macromolecular damage, involving oxidation of DNA, lipid and protein; in addition, ROS can work as signaling molecules, activating cellular stress-sensitive kinases. In fact,

exposure of different cell lines to hydrogen peroxide leads to the activation of stress kinases, such as c-JunN-terminal kinase, p38-MAPK, IκB kinase, and extracellular receptor kinase 1/2, and this activation determines insulin resistance achieved through the serine/threonine phosphorylation of IRS1 [137]. Additional oxidative stress-sensitive kinases are reported to be involved in IRS-mediated insulin resistance and include the mammalian target of rapamycin (mTOR) [139], several isozymes of PKC, including PKCβ and PKCε [140, 141], and the IKKβ–NFκB signaling cascades [142], and acting through phosphorylation of the insulin receptor or IRS proteins.

Looking for cellular intermediates between oxidative stress and insulin resistance, evidence for ceramide as a candidate linking nutrient excess to the induction of insulin resistance in rodents has recently been published [143]. In addition, diet-induced Obesity in mice increases the expression of inducible nitric oxide synthase (iNOS) in skeletal muscle which may provoke S-nitrosylation an consequently, degradation of the insulin receptor and impaired insulin signaling in rat muscle [144].

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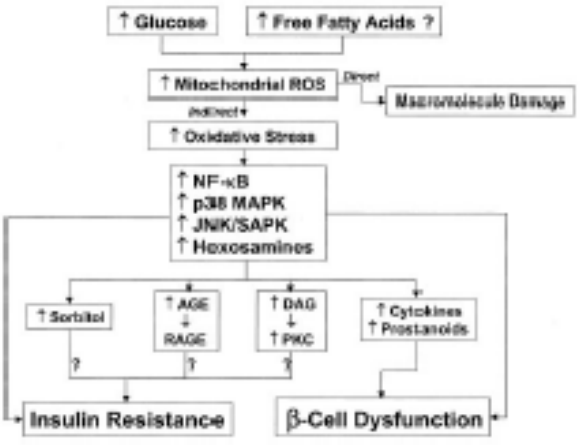



*Figure 7 How elevated glucose and FFA levels contribute to the ROS-induced pathophysiology of diabetes via the activation of numerous stress sensitive pathways. ROS (and Reactive Nitrate Species RNS) function as signaling molecules to activate several stress sensitive pathways in Type II Diabetes development. Activation of these pathways, such as NF-kB, p38 MAPK, JNK/SAPK, and hexosamine, leads to both insulin resistance and impaired insulin secretion by elevations in glucose and FFA levels. Other stress pathways: the increased production of AGE, sorbitol, cytokines, and prostanoids along with PKC activation [145].*

# Protein Kinase C

Protein kinase C (PKC) are a family of serine/threonine-specific kinases implicated in intracellular signal transduction of many key cellular events, as cell proliferation, apoptosis, smooth muscle contraction, and secretion [146], and also involved in the pathogenesis of Obesity and insulin resistance [147]. PKC consists of several isoforms subdivided into three classes: (1) the classical or conventional PKCs (α, βI, βII and γ) activated by $Ca^{2+}$ and DAG; (2) the new or novel PKCs (δ, ε, η and θ) activated by DAG but $Ca^{2+}$ independent; and (3) the atypical PKCs (λ and ζ), which are $Ca^{2+}$ and DAG insensitive [148]. Although most cells express more than one type of PKC, differences among the isoforms with respect to activation conditions and subcellular locations, suggest that individual PKCs mediate distinct biological processes [149]; however, the exact role of any PKC isoform has to be elucidated.

Nine of eleven PKC isoforms are activated by the lipid second messenger DAG and it is now well established that, in diabetic animals, hyperglycemia increases the amount of DAG in many tissues exposed to Type II Diabetes complications, as microvascular cells, retina and renal glomeruli. Hyperglycemia achieves this primarily by increasing de novo DAG synthesis, and this, in turn, increases activates PKC (primarily β and δ isoforms) both in cultured vascular cells [150] and in retina and glomeruli of diabetic animals [151]. Activation of PKC in cells exposed to hyperglycemia can be achieved through alternative routes, including intermediates from metabolic pathways, the AGE-RAGE (advanced glycation end products and their receptors) pathway activation and oxidative stress (*figure 8*). Moreover, Type II Diabetes is often associated with derangement of metabolism such as dyslipidemia. Increased circulating FFA in Type II Diabetes has been shown to activate PKC in vascular cells [152]. Altered polyol and hexosamine pathways are involved in development of diabetic complications through activation of PKC [153, 154]. Moreover,

aberrant expression of growth factor and cytokine contributes to the activation of vascular PKC in diabetes [155].

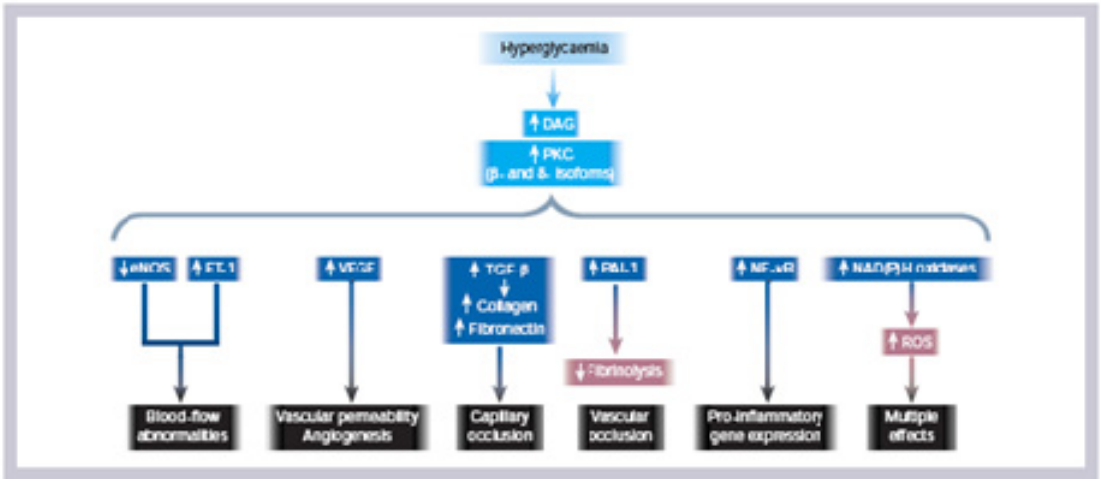



*Figure 8 Involvement of PKCs in the development of diabetic complications. High glucose levels characteristic of Type II Diabetes increases intracellular content of DAG, which in turn, activates PKCs (mainly β and δ isoforms), responsible for the onset of many complications of the disease [134] .*

Evidences, especially from knock-out studies, have indicated that several of PKC isoforms can modulate glucose or energy metabolism in an isoform-specific manner: inactivation of PKCλ increased whole-body insulin sensitivity, diminishing triglycerides content selectively in liver, without affecting their level in muscle [156]. Whereas inactivation of PKCα unaffected either basal glucose or energy metabolism [157], atypical PKC isoforms are required for insulin-stimulated glucose uptake in muscle and adipocytes, and defects in atypical PKC in human muscle are found in insulin resistance, Obesity and Type II Diabetes. In muscle, these defects are due to both impaired activation of PI3K and the direct activation of atypical PKCs by the lipid product of PI3K, PI-3,4,5-(PO4)3 [158]. Moreover, inactivation of PKCθ represents a risk factor for the onset of

Obesity and enhances dietary-induced insulin resistance [159]. Unlike many PKC isoforms, PKCβ is expressed as a major isoform in a variety of tissues, and knock out of PKCβ caused slight changes in glucose homeostasis [160]; moreover, PKCβ has been linked with many vascular abnormalities in retinal, renal, and cardiovascular tissues [161]. Of the various PKC isoforms in vascular cells, PKCβ and δ isoforms (aorta and heart), PKCα, βII and ε isoforms (retina) and PKCα, βI and δ (glomerulus) have been found to be increased in diabetes. Analysis of the retina, kidney and cardiovascular tissues of diabetic rats showed that PKCβ isoforms are preferentially activated.

All these evidences highlight the main role of PKC in the development of insulin resistance and Type II Diabetes as well as their involvement in the onset of diabetic complications, but the role of the isoform β has to be stressed. PKCs are activated in conditions of oxidative stress [162] and PKCβ is not an exception [163]; furthermore Metformin, a widely used antidiabetic agent, inhibits the glucose-induced activation of PKCβ through an antioxidant mechanism [164]. Recently, Liberman *et al*. demonstrated PKCβ implication in the rearrangements of insulin intracellular signal transduction. They found remarkably elevated expression level and activation state of PKCβII in the fat tissues of diabetic *ob*/*ob* mice and in high-fat diet-fed mice compared with that from lean animals, and these increases were associated with enhanced phosphorylation of IRS-1 at $Ser^{336/332}$, an important negative modulator of insulin signaling [165]. Furthermore, PKCβ inhibitor has shown beneficial effects in diabetic complications as diabetic retinopathy and neuropathy, suggesting PKCβ could represent a pharmacological target for Type II Diabetes therapy [166-169].

The isoform seems to be involved also in adipocyte differentiation and in the regulation of food intake: mice with targeted disruption of the PKCβ gene ($PKC\beta^{-/-}$), show decreased fat in adipose tissue, liver, and muscle. Although these mice consumed 20–30% more food than the wild type littermates, they lost body weight. Again, $PKC\beta^{-/-}$ mice exhibit increased fatty acid oxidation and upregulation of PGC-1α and UCP-2 genes [170].

This body of evidence suggests the primary role of PKCs, in particular the isoform β, in detecting stress conditions presents in metabolic disorders, and in transducing them to the detrimental cellular responses associated.

# Aim of the Work

Because of the important prevalence of metabolic disorders such as Obesity and Type II Diabetes, adipose tissue has been revaluated as an active and important organ in regulating many processes involved in the development of the disorders. Thus, the identity and regulation of the adipocyte precursor cell are actually a topic of great interest, in particular with regards to the signals occurring in patho-physiological conditions and in aging. Stem cells are present in the adipose tissue, and under appropriate differentiation signals can differentiate *in vitro* in adipocyte or other mesenchymal cells. Adipose-derived stem cells (ADSCs) represent the key cellular element for the control of body adiposity and they may substitute bone-marrow derived stem cells in their promising applications for tissue repair and regeneration. Indeed, subcutaneous adipose depots are accessible and abundant, thereby providing a potential adult stem cell reservoir for each individual.

Further complexity is added by the observation that intramuscular fat stores and substitution of muscle with adipose tissue have been reported to strongly correlate with insulin resistance and thus to the development of Type II Diabetes and other metabolic disorders. A key biological issue is how intra/intermuscular adipose tissue originates and proliferates and attention has been recently focused on the transdifferentiation of muscle progenitors to the adipogenic lineage, given that myopathic skeletal muscle is characterized by the replacement of myofibers by adipose tissue. Muscle-derived stem cells (MDSCs) include the so called satellite cells, i.e. the precursor cells responsible for the maintenance of the regenerative and proliferative potential of adult muscle tissue.

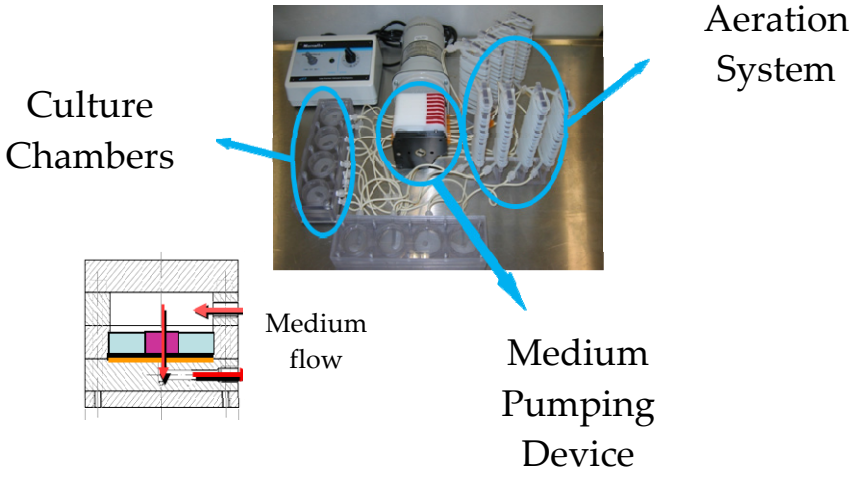
Aim of this work is investigate the major feature of Type II Diabetes, Hyperglycemia, as possible inductor of adipose differentiation of mesenchymal stem cells residing in both adipose and muscle tissue, and to better identify the precise molecular mechanism involved in adipogenic differentiation of Muscle-derived Stem Cells, in the contest of the

intense research concerning pathological signals involved in the development of epidemic metabolic diseases.

# Results

## Osteogenic, Chondrogenic and Adipogenic Differentiation Potential of Adipose-derived Stem Cells

Adipose-derived Stem Cells (ADSCs) were isolated from human lipoaspirates using standard protocols and their *in vitro* differentiation capacity into various mesenchymal tissues was first established. To maximize nutrients delivery and to mimic the dynamic stress conditions stimulating formation of extracellular matrix, ADSCs were seeded onto hyaluronan based sponge in a perfusion system, called Bioreactor, which allows the circulation of culture medium through the scaffold (figure below, for a more detailed description see Materials and Methods).



Three classical differentiation protocols into osteoblasts, chondrocytes and adipocytes (described in Materials and Methods) were tested to characterize the ADSCs differentiation capacity, and the results were verified by morphological analyses (histological and electron microscopy analyses) and molecular approaches.

Osteogenic differentiation was detected fixing and staining the cultured cells for alkaline phosphatase activity (fig.1 *Osteogenic Medium* A). The histochemical reactions turned out markedly positive (blue cells), whereas chondrocyte markers (immunocytochemistry for type II collagen, typical of mature collagen) and adipocyte markers (presence of lipid droplets, as revealed by Oil Red O) were completely negative. Ultrastructural analysis of the sample confirmed the bone differentiation (fig.1 *Osteogenic Medium* B), by showing a typical osteoblastic phenotype: cells with a large ovoid nucleus, a large amount of granular endoplasmic reticulum and completely surrounded by fully mineralized bone matrix. More, these results well matched the molecular analysis by real-time PCR (RT-PCR, fig.1 *Osteogenic Medium* C), carried out in parallel batches of cells, that revealed expression of bone differentiation markers, such as alkaline phosphatase, collagen type I, osteopontin, osteonectin and osteocalcin, and negativity for adipocyte markers (fatty acid binding protein 4 (FABP4), peroxisome proliferators activated receptor–gamma (PPARγ), lipoprotein lipase (LPL), glucose transporter 4 (GLUT4)) and chondrocyte markers (type II collagen).

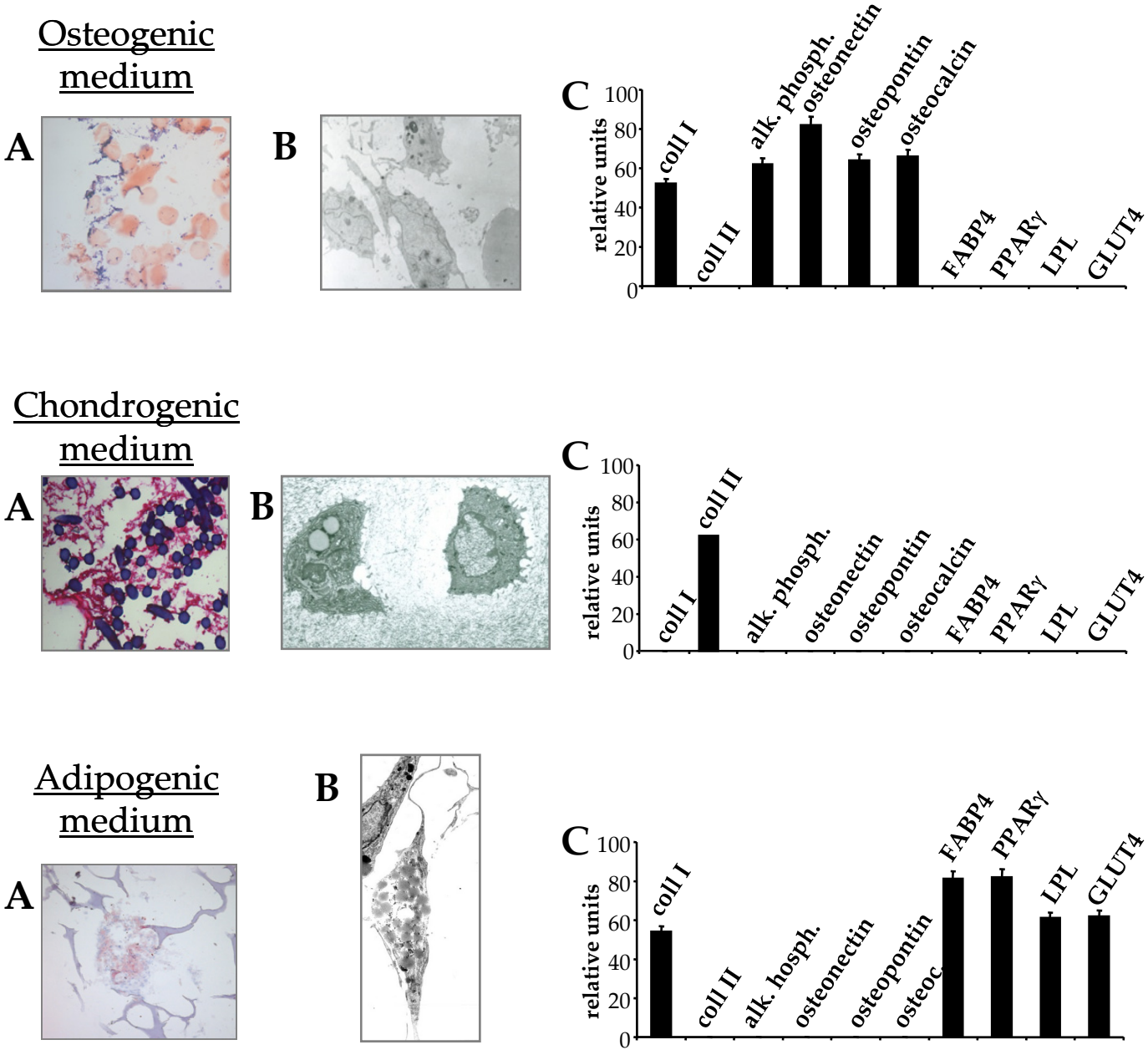
Chondrogenic differentiation was confirmed by positive cell-staining at the immunocytochemistry for type II collagen (fig.1 *Chondrogenic Medium* A), while completely negative for alkaline phosphatase and lipid staining. Transmission electron microscopy (TEM) showed (fig.1 *Chondrogenic Medium* B) that the cells contained a certain number of well preserved mitochondria, an expanded cytoplasm rich in free ribosomes and abundant rough endoplasmic reticulum, a well developed Golgi apparatus and a relevant number of glycogen granules. Cell membranes showed contact points and finger-like protrusions; microfibrils were present in the intercellular spaces, frequently connected to cell membranes from which they looked extruded. The RT-PCR results confirmed

chondrogenic differentiation, by revealing expression of type II collagen and total negativity for osteogenic and adipose marker (fig.1 *Chondrogenic Medium* C).

Finally, Adipogenic differentiation was detected by cell staining with the triglycerides-specific marker Oil Red O. Oil Red O staining of the cells revealed loading with lipid droplets (fig.1 *Adipogenic Medium* A), and negativity for alkaline phosphatase and type II collagen. The EM pictures (fig.1 *Adipogenic Medium* B) showed the typical features of adipocytes (presence of lipid droplets), and RT-PCR (fig.1 *Adipogenic Medium* C) revealed the expression of adipocyte markers, such as FABP4, PPARγ, LPL and GLUT4. Similar data have been obtained by us and others using ADSC from mouse or rats, or using more standard culture conditions [171].

These results indicate that, upon application of suitable differentiation protocols, mesenchymal stem cells derived from lipoaspirates (ADSCs) retained the capability of efficiently differentiating into bone, cartilage and adipose cells.

# Differentiation of Adipose-derived Stem Cells into Osteogenic, Chondrogenic and Adipogenic lineage




***Results fig. 1***

*Adipose-derived Stem Cells (ADSCs) cultured on Hyaff ®11 sponges in Osteogenic, Chondrogenic or Adipogenic Medium*

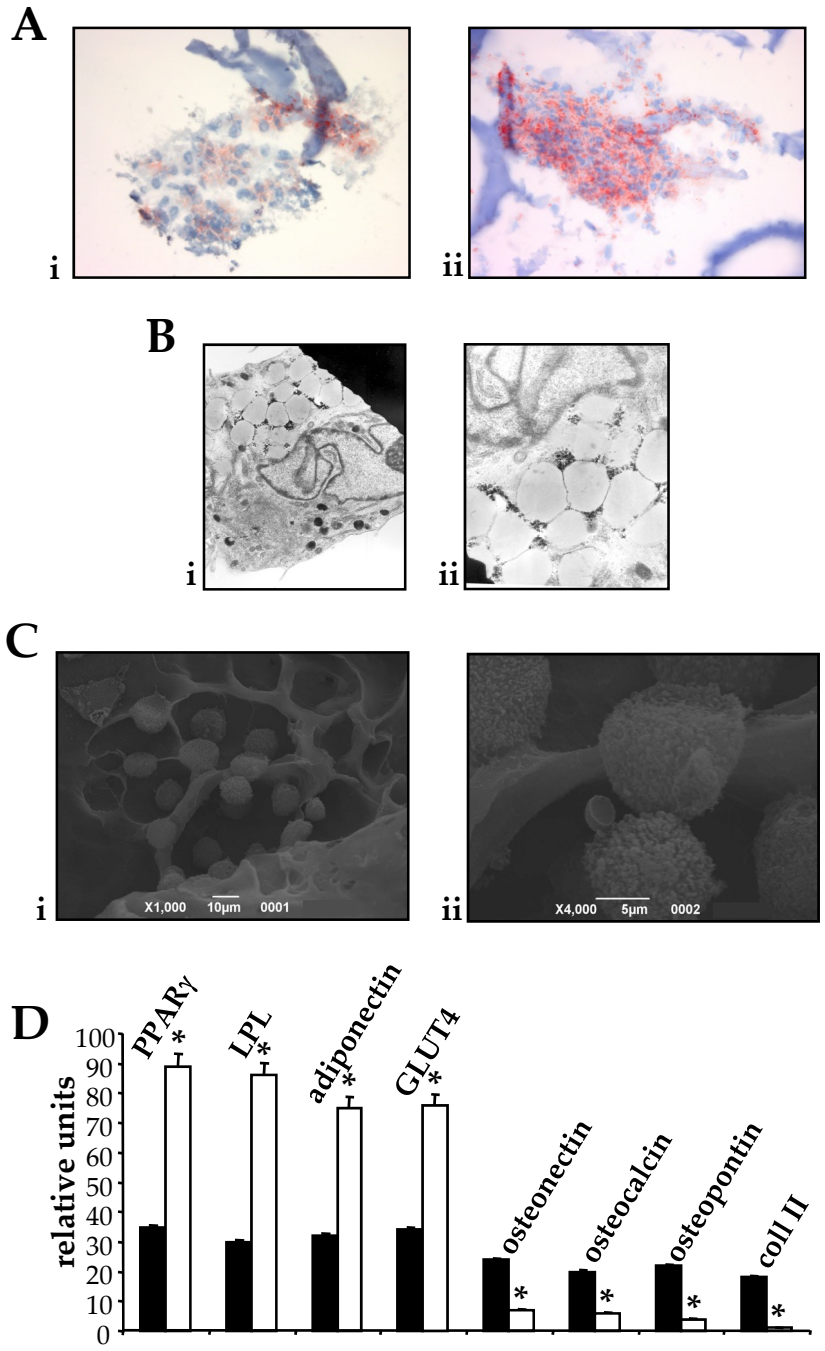
*A Histochemical reaction [Osteogenic Medium: alkaline phosphatase activity (blue cells)][Chondrogenic Medium: Type II Collagen positivity (blue cells)][Adipogenic Medium: Oil Red O Staining (red cells)] Magnification: 20X.* ***B:*** *Transmission electron microscopy (TEM) showing lineage-specific phenotypes. Magnification: 6500X.* ***C:*** *mRNA expression of adipogenic, chondrogenic and osteogenic markers analyzed after 14 days by semi-quantitative real time PCR. Results for each condition are from quadruplicate experiments and values are expressed in relative units as the mean ±SD. * $p<0.05$.*

# High glucose induces adipocyte differentiation of stem cells from adipose tissue

The physiological stimuli that control ADSCs differentiation into adipocytes *in vivo* remain unknown. In this work is investigated the potential of high glucose concentration, a condition leading to an increase in adipose tissue *in vivo [172]* to affect human ADSCs differentiation into mature adipocytes *in vitro* (fig. 2). ADSCs were maintained for 14 days in a low glucose medium (LG-DMEM, i.e. DMEM supplemented with 10% FBS and 5.5 mM glucose) or in a high glucose medium (HG-DMEM, i.e. DMEM supplemented with 10% FBS and 25 mM glucose), and then analyzed by morphological (histochemistry and electron microscopy) and molecular approaches. Staining with Oil Red O (fig. 2 A), selective for triglyceride depots, clearly revealed substantial adipose differentiation in cells grown in HG-DMEM (ii) compared to cells grown in lower glucose (ii), result confirmed also by Oil Red O staining quantification (HG-DMEM: 4.18±0.21 OD and LG-DMEM: 2.16±0.11 OD at 520 nm). Accordingly, EM analysis (fig. 2 B) showed numerous cells containing large lipid droplets surrounded by a thin ring of cytoplasm, indicating the typical adipose phenotype, and SEM microscopy (fig. 2 C) showed a population of rounded cells of various sizes, i.e. the typical appearance of adipose tissue. The lower magnification (fig. 2 Ci) reveals the presence of round adipocytes on both sides of the specimen.

In high glucose conditions, RT-PCR analysis of mRNA transcripts (fig. 2 D) revealed significant expression of adipocyte-specific proteins, such as PPARγ, LPL, adiponectin, GLUT4 and sterol regulatory element-binding protein 1c (SREBP1c). Conversely, chondrogenic (type II collagen) and osteogenic markers (osteopontin, osteonectin, osteocalcin) were downregulated in HG-DMEM compared to cells cultured in LG-DMEM.

# High glucose induces adipocyte differentiation of stem cells from adipose tissue




***Results fig. 2***

*Adipose-derived Stem Cells (ADSCs) cultured on Hyaff ®11 sponges in LG and HG-DMEM*

***A:*** *Oil Red O staining of ADSCs grown in LG (i) or HG-DMEM (ii). Lipid droplets are in red, biomaterial fibers in blue. Magnification: 20X.* ***B:*** *Transmission electron microscopy (TEM) of two representative fields. Magnification: 6500X.* ***C:*** *Scanning Electron microscopy (SEM) of ADSCs at two different magnifications.* ***D:*** *mRNA expression of adipogenic, chondrogenic and osteogenic markers analyzed after 14 days by semi-quantitative real time PCR in ADSCs cultured on 3D scaffolds in LG ■ and HG □ DMEM. Results for each condition are from quadruplicate experiments and values are expressed in relative units as the mean ±SD. * $p<0.05$.*

# Adipogenic conversion of muscle-derived stem cells upon high glucose

Incubation in high glucose *per se* can drive the differentiation of uncommitted stem cells into adipocytes, and this represents a novel mechanism for ADSCs (but not for pancreatic β cell lines: see [173]). However these stem cells were derived from adipose tissue and thus the effect observed with high glucose incubation could represent the enhancement of the default differentiation route of adipocyte precursors. To investigate whether it reflected conversely the differentiation of uncommitted mesenchymal precursors or even the transdifferentiation of stem cells of other mesenchymal tissues, the analysis was turned out to primary cultures of skeletal muscle. These cells can be easily obtained from animal models and thus the following experiments were carried out on cells derived from neonatal rats. Muscle-derived Stem Cells (MDSCs) from mouse or rats can be differentiated into large myotubes, carrying most phenotypic properties of skeletal muscle, in terms of contractile protein expression, morphological aspect (with diad and triad formation) and signalling properties [174].

In the experiments presented in fig. 3 is analyzed the effect of HG-DMEM on MDSCs, using the standard culture conditions, i.e. in 100 mm Petri dishes. Parallel batches of cells were maintained in LG-DMEM. In both cases, the medium was supplemented with 10 % FCS (a condition that prevents differentiation into myotubes) and cells were maintained in culture for 18-25 days before analysis. In LG-DMEM, as revealed by either counting the cells with large vacuoles in the cytoplasm (fig. 3 Ai) or those positive for the lipid specific dye Bodipy (fig. 3 Aii) the percentage of cells with a morphological adipocyte phenotype was only 3.3 ± 2.4%. In HG-DMEM, a highly significant increase in adipocyte differentiation was detected: 9.2 ± 2.9% of the cells showed a clear adipose phenotype, with either analysis methods (fig. 3Bi; ii), 2 to 4 fold higher than in LG-DMEM (HG: n = 6; LG: n = 4)**(*)**.

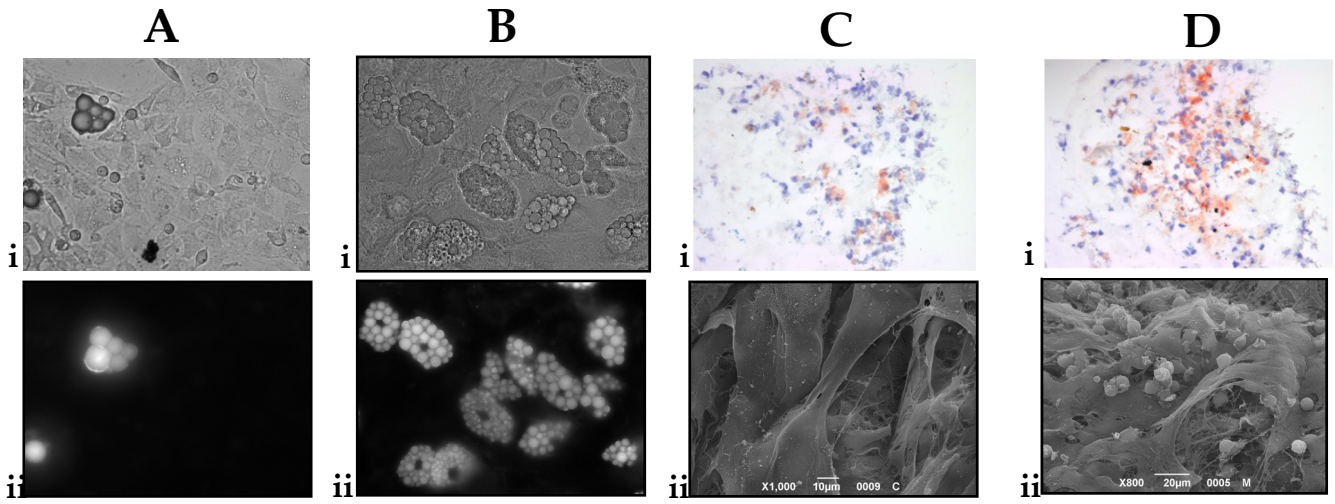
Then the adipose differentiation efficiency in the three-dimensional scaffold of the bioreactor was verified. MDSCs were thus seeded onto the hyaluronan based sponge and maintained in culture for 14 days under LG-DMEM or HG-DMEM perfusion. Then, histochemical and ultrastructural analyses of the cell culture were carried out. As to the former, 5 randomly chosen fields were analyzed for each condition (and the experiment was repeated with identical results in 3 different trials). The results of a typical experiment are presented in fig. 3. In LG-DMEM Oil Red O staining was hardly detectable (fig. 3 Ci) while the cells became loaded with lipid vesicles in HG-DMEM, as revealed by Oil Red O staining (fig. 3 Di); quantification of Oil Red O-stained cells showed a marked higher percentage of adipocytes in HG-DMEM compared to LG-DMEM (3.87±0.31 OD vs 1.89±0.15 OD at 520nm).

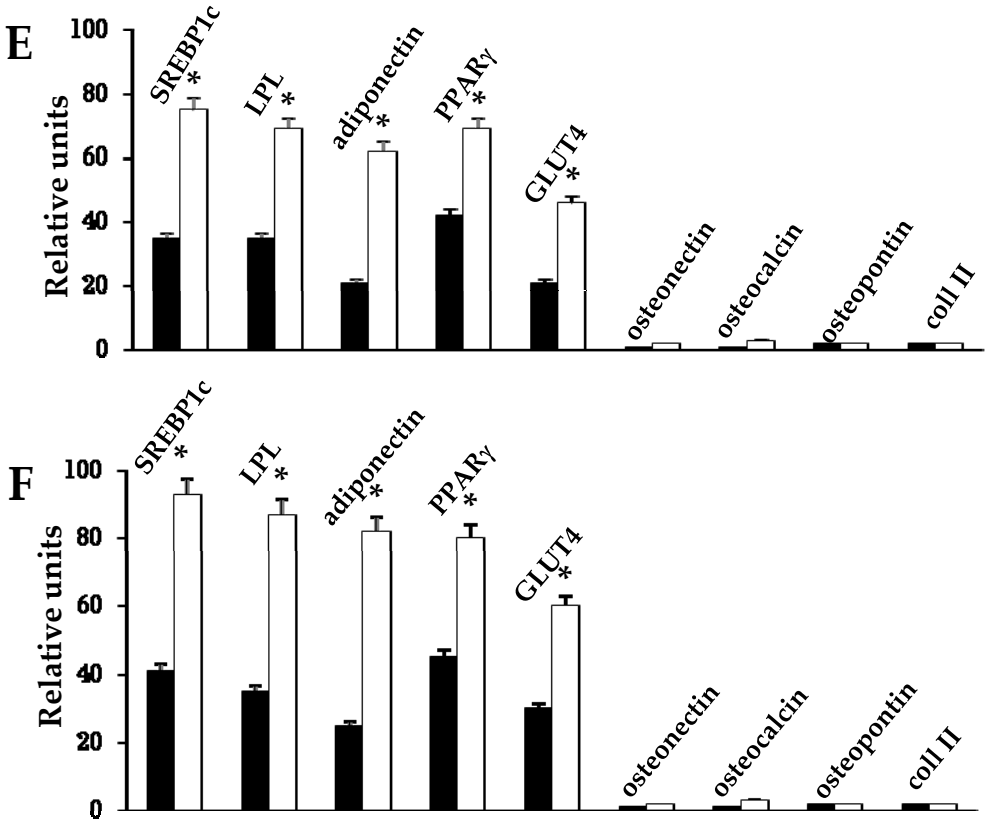
In agreement with these observations, electron micrographs showed mostly elongated fibroblast-like cells in LG-DMEM (fig. 3 Cii) and a high fraction of rounded cells, with the typical mature adipocytes appearance, in HG-DMEM (fig. 3 Dii). The percentage of cells with an adipocyte phenotype obtained with HG-DMEM from muscle cultures did not differ significantly from that observed under the same conditions using ADSCs (3.87±0.31 OD at 520nm in MDSCs and 4.18±0.21 OD at 520nm for ADSCs), indicating that the differentiated adipocytes in the former case cannot derive from a contamination from adipose tissue in the muscle cell cultures.

The adipogenic potential of high glucose on MDSCs was confirmed also by RT-PCR analyses of adipocyte-specific transcripts: cells cultured in the bioreactor and maintained in HG-DMEM for 7 (fig 3 E) and 14 (fig 3 F) days, shown an higher expression of PPARγ, LPL, adiponectin, GLUT4 and SREBP1c and a downregulation of chondrogenic and osteogenic markers, compared to cells grown in LG-DMEM.

* *To rule out that the process represents only the differentiation of a contaminating cellular population of adipocyte precursors, control experiments were done. Separate cultures were prepared from upper and lower limb muscles (the former may be partly contaminated by subcutaneous and visceral adipose tissue, while the latter is virtually devoid of it) and the efficiency of high glucose induced adipocyte differentiation was very similar (upper limbs: 8.9±2.7%; lower 10.3±1.8%).*

# High glucose induces adipocyte differentiation of Muscle-derived Stem Cells




***Results fig. 3***

*High glucose induces adipocyte differentiation of Muscle-Derived Stem Cells (MDSCs)*

**A** *and* **B**: *Phase contrast image (i) and Bodipy-staining (ii) of MDSCs cultured in LG-DMEM* **(A)** *or HG-DMEM* **(B)**. **C** *and* **D**: *Oil Red O staining (i) and SEM analysis (ii) of MDSCs cultured for 14 days on Hyaff ®11 sponges in LG-DMEM* **(C)** *and HG-DMEM* **(D)**. *Lipid droplets are in red, nuclei in blue (40X).* **E** *and* **F**: *Adipogenic, chondrogenic and osteogenic markers mRNA expression analyzed by semi-quantitative real time PCR in MDSCs cultured on 3D scaffolds days in LG■ and HG □ DMEM for 7* **(E)** *and 14* **(F)** *days. Results for each condition are from quadruplicate experiments and values are expressed in relative units as the mean ±SD. * p<0.05.*

## Oxidative stress, via effector PKCs, is a trigger of adipocyte differentiation

Adipocyte differentiation of Muscle-derived Stem Cells could be the cell model to get some insight into the molecular mechanisms that drive high glucose-induced adipocyte differentiation.

In the past years, major attention has been dedicated to hyperglycaemia-induced damage comprising both microvascular and macrovascular pathologies, and four main hypotheses about their development are given: increased polyol pathway flux; increased advanced glycation end-product (AGE) formation; activation of protein kinase C (PKC) isoforms; and increased hexosamine pathway flux. The unifying mechanism linking these hypotheses proposed by Brownlee [134] is the induction of oxidative stress by high glucose through three mechanisms, NADP(H) oxidase, xanthine oxidase and the mitochondrial respiratory chain.

PKC isoforms, PKCβ and δ in particular, have been shown to be activated by oxidizing conditions [163] and in this work was verified whether these mechanisms represent the signalling route in stem cells differentiation to adipocytes.

First, ROS production enhancement during high glucose incubation was verified. MDSCs were obtained as in fig. 3, but the cells were seeded onto 24 mm glass coverslip for microscopic analysis. 7 days after seeding, cells cultured in LG-DMEM or HG-DMEM were loaded with the ROS-sensitive fluorescent probe CM-$H_2$DCFDA. The coverslip with the cells was transferred to the stage of a confocal microscope and fluorescence emission at 520 nm was monitored, revealing continuous ROS production. The results, shown in fig. 4 A, demonstrated that ROS production is markedly higher if the cell culture is maintained in HG-DMEM.

This condition is known to activate PKCβ, as previously observed in endothelial cells [164] and verified also in this cell model. Endogenous PKCβ revealed by

immunofluorescence was mostly cytosolic in MDSCs maintained in LG-DMEM (fig. 4 Bi). Incubation for 1 hour in 25 mM glucose induced a partial translocation of the kinase to the plasma membrane in approximately 30% of the cells (fig. 4 Bii), with a similar pattern to that observed upon direct activation by phorbol 12-myristate13-acetate (PMA) (fig. 4 Biii).

It can be assumed that high glucose-induced ROS, by stimulating PKCβ (and possibly other effectors), can act as adipogenic differentiation signals for MDSCs. Thus, 3 different MDSCs differentiation conditions were tested, and the results, presented in fig. 5 A, strongly support the hypothesis:

i) the role of ROS in adipocyte differentiation was verified adding $H_2O_2$ (*in vitro* model of oxidative stress) to LG-DMEM; the enhancement of the differentiation rate raised to a level comparable to that observed in high glucose (9.34±2.9% vs 9.19±2.9%, respectively), suggesting that $H_2O_2$ could mimic the effect of high glucose and thus triggers adipocyte differentiation. No significant enhancement was detected if $H_2O_2$ was added to HG-DMEM (10.18±0.5%);

ii) involvement of PKCβ in the adipogenic route was first tested transducing MDSCs, maintained in HG-DMEM, with a vector carrying the PKCβ siRNA, and this condition drastically reduced adipocytic differentiation (1.88±0.5%, ~-80%) (fig. 5A) to levels below those found in LG-DMEM, whereas an unrelated siRNA (FHIT**(*)**) had no effect (5.5±0.5%);

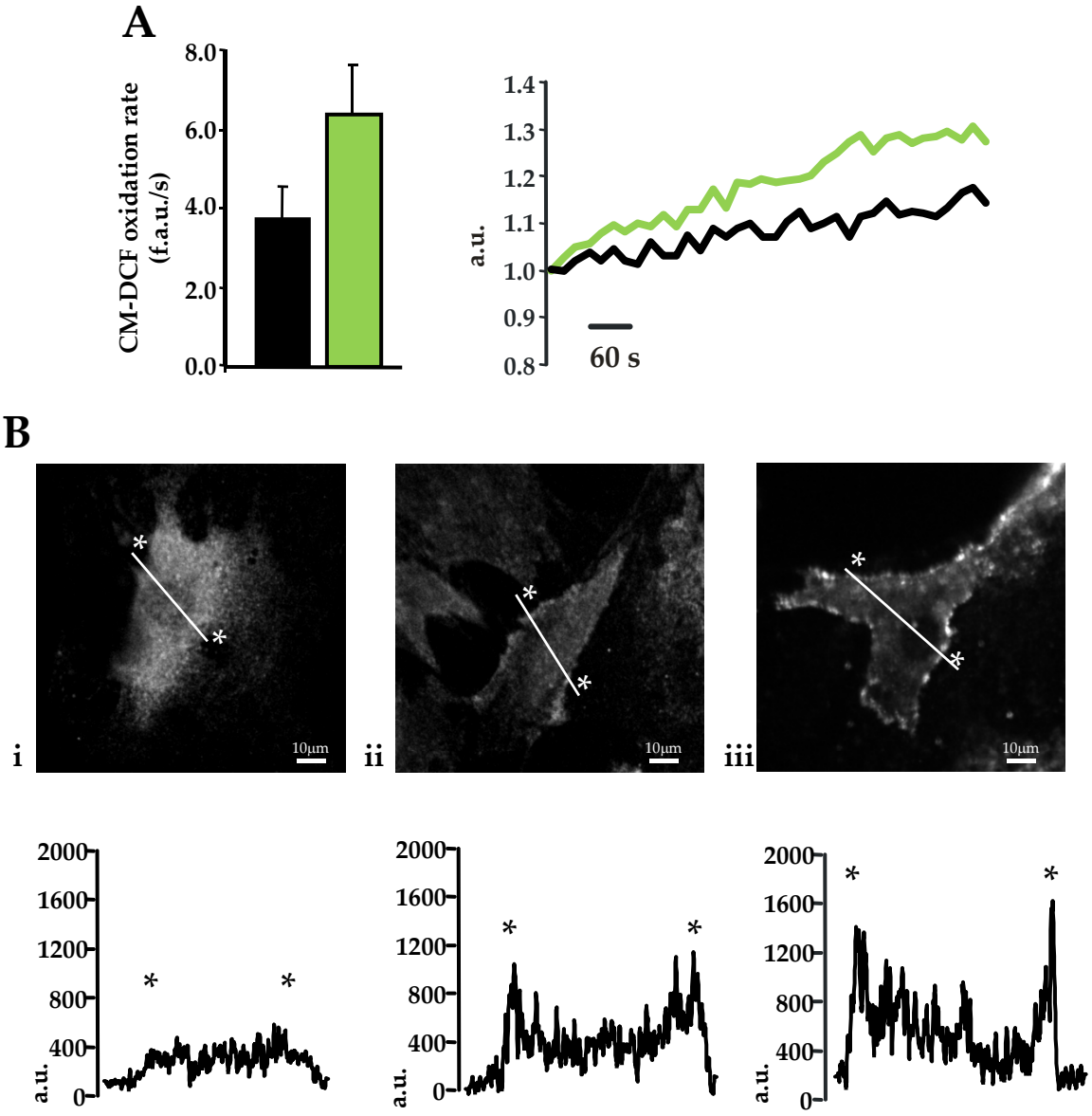
iii) then, transduction of the cells in HG-DMEM with a PKCβ-expression vector triggers a major enhancement of lipid-loaded cells (28.7±6.9%, ~+300%), while infection with a control viral vector (an adenoviral vector expressing mtGFP) and PKCβ if added to cells in LG-DMEM did not increase adipocyte differentiation (control experiment non presented).

The same results were obtained seeding MDSCs onto hyaluronan base sponge of the bioreactor (fig. 5 B): the enhancement of differentiation observed in cells treated with $H_2O_2$ or in PKCβ-overexpressing cells is clearly recognized by the large amount of Oil Red O

loaded cells (fig. 5 Bi and ii); vice versa, the differentiation fall obtained by PKCβ-silencing in HG-DMEM is demonstrated by the lack of loaded-cells (fig. 5 Biii).

**As a control, an unrelated gene was silenced (FHIT), since a siRNA scrambled mixture was previously shown [186] and confirmed in our studies to enhance adipocyte differentiation.*

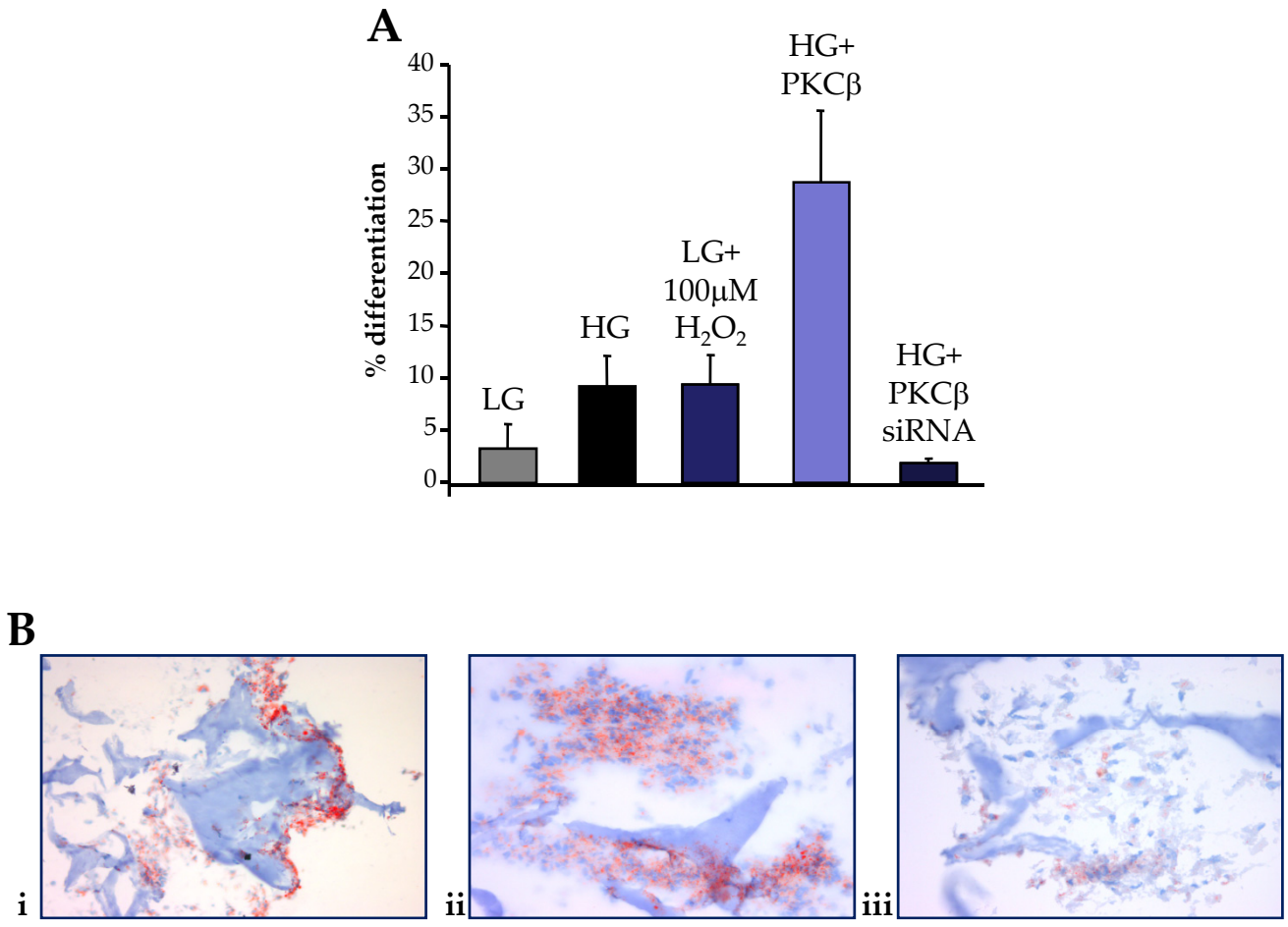
# Hyperglycemia induces Reactive Oxygen Species Production and PKCβ activation in MDSCs




***Results fig. 4***

*ROS production of MDSCs cultured in LG and HG-DMEM and glucose induced PKCβ-activation A: 7 days after seeding, cells were loaded with the CM-H2-DCFDA and analysed by confocal microscopy. Basal ROS production is expressed as mean of CM-DCF oxidation rate (F.a.u./s). Representative traces of CM-DCF oxidation kinetics are shown on the right (■ LG-DMEM and ■ HG-DMEM).* **B***: PKCβ membrane translocation, as revealed by immunofluorescence microscopy. Representative images show cells maintained in LG-DMEM (i), incubated for 1h in HG-DMEM (ii) and treated with PMA, 500nM (iii). The graphs show the quantitation of PKCβ fluorescence intensity along a line crossing the cell (thick white line in the micrograph).*

# ROS-induced activation of the PKCβ triggers adipocyte differentiation of MDSCs




***Results fig. 5***

*Quantitation of MDSCs adipocyte differentiation in different conditions*

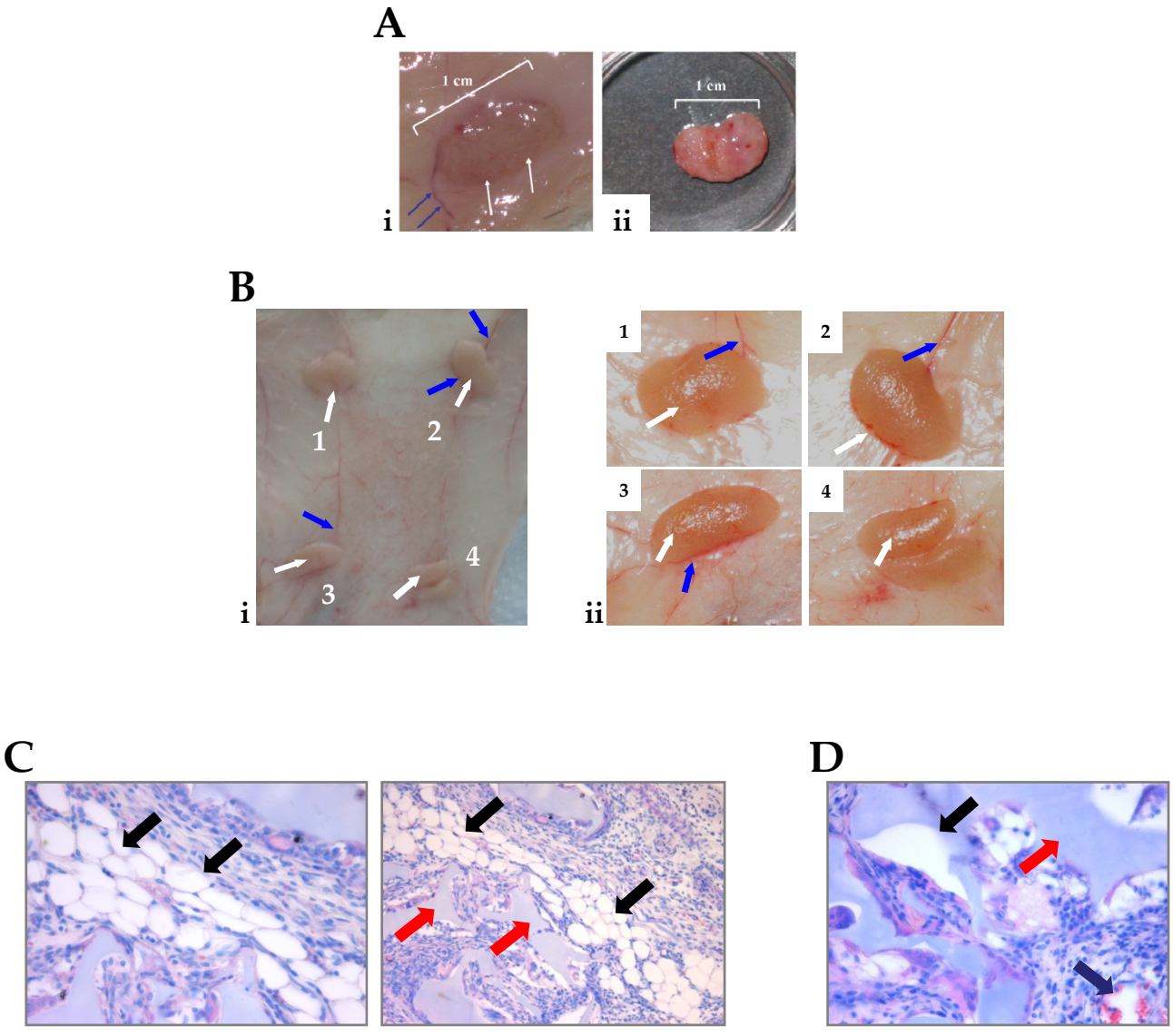
**A**: *MDSCs cultured in different conditions: HG-DMEM, LG-DMEM, LG-DMEM supplemented with 100μM $H_2O_2$, PKCβ-overexpression in HG-DMEM and PKCβ siRNA in HG-DMEM. The efficiency of MDSCs differentiation was quantitated by counting the percentage of Bodipy loaded cell. The histogram shows the percentage of differentiation ± SEM.* **B**: *MDSCs cultured on Hyaff®11 sponges in LG-DMEM supplemented with 100μM $H_2O_2$ (i), PKCβ-overexpression in HG-DMEM (ii) and PKCβ siRNA in HG-DMEM. Adipogenic differentiation was confirmed by Oil Red O staining: lipid droplets are in red, nuclei in blue. Magnification: 10x.*

## High glucose-differentiated adipocytes can be successfully implanted in vivo

The ability of *in vitro* differentiated adipocytes to generate mature and viable adipose tissue *in vivo* was tested. ADSCs seeded on HYAFF® sponges and cultured in the bioreactor for 7 days in high glucose, were implanted in the abdominal area of 8 female nude rats (Materials & Methods section). 1, 2, 3 and 4 weeks after insertion of the graft, the rats were sacrificed and the grafts analyzed macroscopically (colour, ingrowth and vessels formation, weight, thickness) and microscopically (pore size, specific and unspecific cellularity, vascularity). The implant appeared well integrated in the subcutaneous tissue and not surrounded by fibrotic tissue (fig. 6 Ai at 2 weeks and fig. 6 B at 3 weeks; white arrows). In addition, numerous capillaries (fig. 6 Ai and Bi; blue arrows) revealed significant vascularization of the engineered, re-implanted tissue. Harvesting of the sample confirmed that the sponge maintains its volume and soft consistence, and is easily dissected from the surrounding tissue (fig. 6 Aii).

Morphological characterisation of the explants confirmed a major distribution of preadipocytes over the whole cross-section (fig 6 Ci (10X), 6Cii (40X)). Significant adipose tissue formation was detected (black arrows) (fig. 6C i, ii) already at 3 weeks. In most cases, the cells exhibited the hallmarks of mature adipocytes, i.e. a large lipid droplet surrounded by a thin ring of cytoplasm, with a flattened nucleus located at the cell periphery. Adipocytes (fig. 6 C, black arrows) were closely attached to the scaffold (red arrows), and multiple capillaries were located near the preadipocytes and adipocytes (fig. 6 Ci and 6 D green arrows).

# High Glucose-Differentiated Adipocytes Implanted *in vivo*




***Results fig. 6***

*Macro and microscopic analyses of in vitro differentiated adipocytes implanted in vivo*

**A, B**: *Macroscopic aspect 2* **(A)** *and 3* **(B)** *weeks after implantation of the HYAFF®11 sponge seeded with adipocytes in HG-DMEM.* **A***i and* **B***i: images of the implanted tissue in situ;* **A***ii, image of the graft after dissection and* **B***ii, low magnification images of the 4 implanted grafts.*

**C, D**: *Histological analyses of the grafts 3 weeks after implantation in diverse magnifications(***C** *20X and* **D** *40X) by haematoxilin/eosin staining of newly formed tissues.*

*Black arrows indicate adipocytes, red arrows indicate the biomaterial and the green arrow indicates a capillary.*

# Discussion

Due to its primary role in the development of Obesity and type II Diabetes, the major interest on adipose tissue resides in the identity of the adipocyte precursor cell and regulation of the differentiation process. Since the molecular actors involved and the whole adipogenic transcriptional pathway is now fully elucidated, the signals occurring in pathological conditions and in aging are still incompletely understood. It is now well established that the increase of adipose tissue volume derives from differentiation of a pool of adipose precursors present in the tissue, called pre-adipocytes, committed to the adipogenic lineage and ready to receive the appropriate differentiation signal. Moreover, in the adipose tissue is present a reservoir of stem cells which, upon differentiation signals, allows tissue regeneration and increase in fat cells number. Thus, Adipose-derived stem cells (ADSCs) represent the key cellular element for the control of body adiposity and furthermore, depending on the stimulating signals they receive, can differentiate not only in adipocyte, but also in other mesenchymal cells, such as chondrocytes or osteocytes, and in endothelial and neuronal cells. Thus, they may substitute bone-marrow derived stem cells in their promising applications for tissue repair and regeneration. Indeed, subcutaneous adipose depots are accessible and abundant, thereby providing a potential adult stem cell reservoir for each individual.

A pool of mesenchymal stem cells retaining adipogenic potential can be found also in muscle fibres. Muscle-derived stem cells (MDSCs) include the so-called satellite cells, the precursor cells capable of differentiating *in vivo* to mature muscle fibres and *in vitro* up to multinucleated skeletal myotubes [174] and responsible for the high regenerative capacity maintained by adult skeletal muscle [175].

Human satellite cells retain broad differentiation capacity, including the generation of adipocytes in appropriate culture conditions (adipogenic medium) in a process that is enhanced by the insulin sensitizer rosiglitazone [176]. Moreover, activators of PPAR-γ cause in muscle the induction of genes involved in fatty acid uptake, storage, and metabolism [177] and that the myogenic cell line C2C12 can be converted to adipocytes by overexpression of PPAR-γ and C/EBP [178].

Intramuscular fat accumulation and substitution of muscle with adipose tissue have been reported to strongly correlate with insulin resistance and subsequently with type II Diabetes development [179]. For this reason, how intra/intermuscular adipose tissue originates and proliferates in Diabetes and in other metabolic or myodystrophic conditions is still a matter of debate. Attention has been recently focused also on the transdifferentiation of muscle progenitors to the adipogenic lineage, given that myopathic skeletal muscle is characterized by the replacement of myofibers by adipose tissue [180].
One of the major features of type II Diabetes, hyperglycemia, triggers many changes in both gene expression and protein function, contributing to the development of complications of Diabetes itself [134]. Furthermore, recently was discovered that hyperglycemia is able to induce de novo lipogenesis and intracellular lipid accumulation in adipocytes through the ERK1/2-PI3K/Akt-regulated PPARγ pathway) [172] and contracting myotubes (through upregulation of SREBP-1c) [181].

In this research, the experiments presented demonstrate that an increase in glucose concentration induced full adipogenic conversion of stem cells residing in both adipose and muscle tissue and a clear induction of adipogenic transformation was observed upon the application of high glucose culture conditions. This differentiation potential of high glucose had already been noticed in the pancreas; in fact, in response to high glucose levels, stem cells residing in the pancreas generate new β cells and also partly activate an adipogenic differentiation program [173, 182].

In our case, the process consists in a full conversion into adipocytes, as verified by morphological and molecular criteria and also by the capacity of these cultured cell to form viable and vascularized adipose depots when implanted *in vivo*. Stem cells from muscle tissue, when cultured in high glucose conditions, form viable and functional adipocytes in the same proportion as the same cells from the adipose tissue, since the percentage of high glucose-differentiated adipocytes from muscle tissue is very similar to that obtained from adipose-derived cells. Moreover, to rule out that the process represents only the differentiation of a contaminating cellular population of adipocyte precursors, control experiments were done. Separate cultures were prepared from upper and lower limb muscles (the former may be partly contaminated by subcutaneous and visceral adipose tissue, while the latter is virtually devoid of it) and the efficiency of high glucose induced adipocytic differentiation was very similar. Taken together, this findings indicate that the process observed is the same from both cell types, and represents the genuine differentiation of uncommitted mesenchimal precursors or a trans-differentiation of myogenic precursors. In this process, an important role is attributed to reactive oxygen species (ROS) and downstream effector kinases, such as PKCβ, recently shown to act as signalling link between ROS and mitochondrial targets implicated in age-dependent organ deterioration [163].

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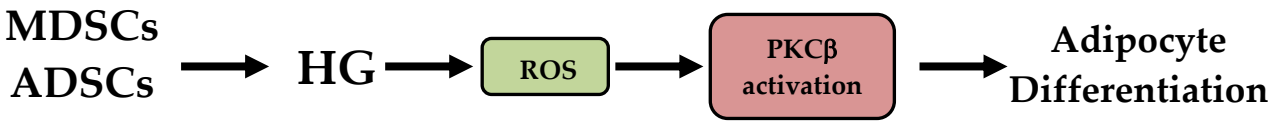


*Glucose-induced Adipocyte Differentiation of stem cells residing in both muscle and adipose tissue. The intracellular mechanism proposed involves the augmented ROS production stimulated by high glucose levels and the subsequent activation of PKCβ, driving adipose differentiation.*

The identification of a primary role of high glucose exposure in adipocyte differentiation has some major important conceptual implications. It shows that high glucose *per se* has an adipogenic potential, thus opening the search for novel signal transduction pathways operating in Obesity and Type II Diabetes. Secondly, it highlights the role of high glucose levels in committing stem cells specifically to the adipose lineage, since incubation in high glucose lowers the expression of markers of the other mesenchymal lineage. More, a direct link between hyperglycemia and the increase in adiposity highlights a feed-forward cycle that may play a key role in the progression of the metabolic dysfunction into an irreversible diabetic state. Indeed, the regenerative process and proliferative capacity of muscle fibres decrease as a function of age [183], partly for the reduced activity of stimulatory factors [184], with consequent loss of muscle mass. However these alterations do not account for other features of aged muscle, and in particular for the increased lipid content [185]. The concept that high glucose triggers *in vitro* adipogenic differentiation of MDSCs suggests that hyperglycemia could be responsible for intramuscolar fat accumulation and thus, to other muscle fibers alterations found in myodystrophies.

As to the signals driving the differentiation of MDSCs into adipocytes, the data suggest a crucial role for Reactive Oxygen Species (ROS). Interestingly, among the cellular consequences of high glucose, increased production of ROS is recognized as a major cause of the clinical complications associated with type II Diabetes and Obesity (*Brownlee)*. Various mechanisms appear to synergize in increasing ROS production, ranging from the activity of mitochondrial respiratory complexes to the activation of enzymatic systems, such as NOX isoforms, NO synthase and xanthine oxidase [117] , and despite the presence of scavenging systems (e.g., superoxide dismutase, catalase), the damaging effects of ROS have been proposed to be responsible for progressive organ degeneration [186]. However, the role of ROS is not only restricted to cell damage induction. Indeed, ROS may directly regulate the activity of transcription factors, such as NF-kB, in turn controlling pro-inflammatory gene expression [187].

The results presented in this work add an important conceptual mechanism, by revealing a morphogenetic signalling activity of ROS. In fact, ROS are able to lead to the neoformation of adipose cells, since they mime high glucose adipogenic potential. As in the diabetic complications development, ROS esercitare their adipogenic potential through activation of their downstream effectors PKCs, and in particular through the isoform β (as revealed by the strong inhibitory effect of siRNA and by the strong synergism of high glucose with PKCβ overexpression). A similar role is in keeping with previous reports showing a role of ROS in embryogenesis [188].

The results is in keeping with many previous observations, i. e. the translocation of PKCβ to the plasma membrane of endothelial cells upon high glucose culture conditions [164] and reinforces an important role for PKCs in the pathogenesis of Type II Diabetes and in adipocyte differentiation, expecially in regards to isoform β. The isoform seems to be involved in the rearrangements of insulin intracellular signal transduction [165] and furthermore in adipocyte differentiation and in the regulation of food intake [170].

In addition, this work provides additional support to the role of PKCs in detecting high glucose conditions and sustaining the ROS-mediated signalling route. This result well agrees with a number of previous observations, that besides the important role for PKC in Diabetes and in Obesity, highlights a link between PKCs, ROS production and cell degeneration [189]. In addition to the metabolic effects, PKCs appear to be involved in detecting and decoding a variety of stress conditions, including oxidative challenges. Recently our group showed that PKCβ recruits the 66kDa pro-apoptotic isoform of Shc (p66Shc) to act as oxidoreductase within mitochondria [190] and in triggering a feed-forward cycle of ROS production eventually leading to cell death [163]. The same actors (PKCs, ROS and eventually p66Shc) may come together in a radically different context, the insulin target tissues, where they regulate the production of cellular signals linking hyperglycemia and the trans-differentiation of stem cells. How the same signalling route can trigger uncommitted stem cell differentiation or induce apoptosis remains to be

elucidated, but it has to be stressed that the toxicity and the differentiation effects of $H_2O_2$ are observed at different concentrations, i.e. 1mM and 100μM, respectively, suggesting a dose-response model for the action of ROS.

In conclusion, this work demonstrted that high glucose has an adipogenic potential on stem cells derived from both the adipose tissue and skeletal muscle, and provides some insight into the signals and molecules that underlie this process. These results may deepen the understanding of the pathogenesis of Type II Diabetes and highlight novel potential targets for pharmacologically addressing this disease of dramatic social impact.

# Materials and Methods

## Cell cultures

### Muscle-derived stem cells

Primary cultures of muscle-derived stem cells (MDSCs) were prepared from newborn rats (2-3 days) as described previously [174]. Adipogenic conversion was observed both in the first preplating (15min or 2 hours), and in the replating of non-rapidly adherent cells. In the latter case, a small fraction of myotubes was still present, and their contraction in culture made the cell yield highly variable. For this purpose, the first pool of adherent cells was used for all experiments.

### Adipose-derived stem cells

Adipose-derived Stem Cells (ADSCs) were extracted from human adipose tissues of five healthy female patients undergoing cosmetic surgery procedures, following guidelines from Clinic of Plastic Surgery, University of Padova. Adipose tissues were digested with 0.075% collagenase (type 1A, Sigma Chemicals, St.Louis, MO) in Krebs-Ringer Buffer (modified KRB: 125mM NaCl, 5mM KCl, 1mM Na3PO4, 1mM MgSO4, 5.5mM glucose, 20mM HEPES, pH 7.4, 37°C) for 60 min at 37°C followed by 10 min with 0.25% trypsin. Floating adipocytes were discarded and cells from the stromal-vascular fraction were pelleted, rinsed with media, centrifuged and a red cell lysis step in $NH_4Cl$ was done for 10 min at RT. The viable cells obtained were counted using trypan blue exclusion assay and seeded at a density of $10\times10^5$ cells/$cm^2$ for in vitro expansion.

## Biomaterials

Sponges made of HYAFF®11, a linear derivative of hyaluronic acid, in which the carboxylic function of the monomer glucuronic acid in the hyaluronic acid chain is totally esterified with benzyl groups, were supplied by Fidia Advanced Biopolymers (Abano Terme, Italy). The structure of these sponges shows open interconnecting pores. Sponges were provided as cylinders. All carriers were sterilised by $\gamma$-irradiation before use.

## Characterization of the mesenchymal potential of ADSCs

The differentiation protocols used to test the differentiation capacity of ADSCs into osteoblasts, chondrocytes and adipocytes are described below.

The three different phenotypes were achieved maintaining ADSCs in culture for 14 days in presence of Dulbecco's modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, and with different lineage-specific inducing factors:

- for the osteogenic phenotype: 50 μg/ml L-ascorbic acid [Sigma], 10 ng/ml fibroblast growth factor (FGF) [Calbiochem, CA], dexamethasone 10nM, β glycerophosphate 10 mM ;
- for the chondrogenic phenotype: 50 μg/ml L-ascorbic acid [Sigma], 1 ng/ml transforming growth factor-β1 (TGF-β1) [Calbiochem, CA], 1 ng/ml of insulin [Sigma], 1 ng/ml epidermal growth factor (EGF), [Sigma] and 10 ng/ml basic fibroblast growth factor (EGF) [Sigma];
- finally, for adipogenic phenotype: 1 μm dexamethasone (Sigma), 0.1 mm indomethacin (Sigma), 0.5 mm 3-isobutyl-1-methylxanthine (IBMX) (Sigma), and 10 μm insulin (Sigma).

Then, every sample was analyzed by the three approaches, histochemical, ultrastructural and molecular analyses (discussed in the Results section).

## Adipocyte differentiation detection

To quantitate adipocyte differentiation, Petri dish-cultured cells at 18th-25th day were loaded for 10 minutes with 40μM Bodipy 493/503 (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene, Invitrogen) and 36μM bisbenzimide (Sigma-Aldrich), then washed with KRB. At the indicated times, Oil Red O (ORO, Sigma) staining of the cytoplasmic droplets of neutral lipids was performed according to a modification from (Ramirez-Zacarias J-L, 1992 Histochemistry). Cells cultured in the bioreactor were rinsed, fixed with 10% buffered formalin, stained with 0.3% ORO in isopropanol: water (3:2), photographed and extracted with 4% Nonidet (Sigma)/isopropanol. The optical density (OD) of the solution was measured at 520nm for quantification using a Victor 3 spectrometer (Perkin Elmer).

## Electron microscopy

SEM Samples of reconstructed adipose tissues were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h before being processed either with hexamethyldisilazan or at the critical point followed by gold-palladium coating. All micrographs were obtained at 30kV on a JEOL 6360LV SEM microscope (Tokyo, Japan).

TEM Cultures were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 for 3h, post-fixed with 1% osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in araldite. Semithin sections were stained with toluidine blue and used for light microscopy analysis. Ultrathin sections were stained with uranyl acetate and lead citrate, and analyzed with a Philips EM400 electron microscope.

## Real-time PCR (qRT-PCR)

Total RNA was extracted using a kit (RNeasy Mini; Qiagen) following the supplier's instructions. 1μg of RNA was treated with DNase (Ambion) and reverse-transcribed for 1h

at 37°C in a 50μl reaction containing 1×RT buffer, 150ng random hexamers, 0.5mmol/l deoxynucleotide triphosphates, 20 U of RNAsin ribonuclease inhibitor and 200 U of M-MLV RT (Promega Corporation, Madison, WI, USA). PCR was carried out using a DNA Engine Opticon 2 Continuous Fluorescence Detection System (MJ Research), all reactions were performed twice. Results were normalised by beta2microglobulin mRNA and reported as arbitrary units. The sequence of the primers are available upon request.

## Analysis of PKCβ translocation

To analyse PKCβ translocation, cells seeded onto 24 mm glass coverslips and grown in LG-DMEM for 3 days were fixed in 4% paraformaldehyde solution for 20min at RT, quenched with 0,1% glycine and permeabilized with 0.1% Triton X-100 for 20min. After three washes with PBS, nonspecific binding sites were blocked with 2% BSA for 1h. Immunostaining was performed with the monoclonal mouse anti-PKCβII primary antibody (diluted 1:20 in 2% BSA), followed by the visualization with the AlexaFluor 488 anti-mouse antibody (Invitrogen, Molecular Probes).

## Measurements of ROS production

Intracellular ROS generation was measured with 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA, Invitrogen) which is cell permeant. After ester hydrolysis the probe is trapped as a non fluorescent probe (CM-H2-DCF). After its oxidation by ROS, CM-DCF green emission was recorded at 520nM.
Cells were loaded with 10μM CM-H2DCFDA at 37°C in modified KRB at 5.5 or 25 mM glucose. 30 min later, laser scanning confocal microscopy (LSCM) images were obtained. Acquisitions were made every 1 s for 500 s.

## PKCβ overexpression and silencing

To overexpress PKCβ, the cells were transduced the day after seeding with a adenoviral vector expressing PKCβ-GFP (AdEasy/PKCβ-GFP). In the case of PKCβ overexpression in LG, we observed a decrease in cell viability 14 days after transfection, that affects the evaluation of the results. To evaluate the effect of PKCβ in LG, we compared in this experiment the efficiency of differentiation in PKCβ-expressing and control cells at an earlier time point, in which overall differentiation was lower but there was no detectable reduction in cell survival. PKCβ silencing was achieved by infecting cells the day after seeding with a commercially available lentiviral siRNA for PKCβ (Sigma-Aldrich), at a viral titol of 3.5 TU/ml (TU: transduction units). As a control, an unrelated gene was silenced (FHIT), since a siRNA scrambled mixture was previously shown [191] and confirmed in our studies to enhance adipocyte differentiation.

## Surgical implantation of hyaluronic acid sponges seeded with adipocytes

The protocol was approved by the Institutional Animal Care Committee of Padua University. 8 female Nude rats (Charles River Laboratories) weighing 150-200g were subjected to the surgical procedures under halothane anesthesia and aseptic conditions. 4 HYAFF®11 sponges seeded with adipocytes in HG-DMEM were implanted in the abdominal area: throughout 4 small (0.3 cm) skin incisions the sponges were inserted in a subcutaneous pocket above the abdominal fascia. Skin incisions were closed with 1 nylon 5.0 stitch. No anticoagulants were used before or after the operation, and no prophylactic antibiotic was administered. All surgical procedures were performed in the same way by a single surgeon. The animals were fed an unrestricted standard diet. After 1, 2, 3 and 4 weeks two rats were killed by an overdose of gaseous anesthetic and the grafts were explanted.

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# Commonly Used Abbreviations

| | |
|---|---|
| ADSCs | Adipose-Derived Stem Cells |
| Akt/PKB | Protein Kinase B |
| AMPK | AMP-Activated Protein Kinase |
| C/EBP | CAAT Enhancer Binding Protein |
| CM-H2DCFDA | 5-(and-6)-Chloromethyl-2',7'-Dichlorodihydrofluorescein Diacetate, Acetyl ester |
| Coll I | Type I Collagen |
| Coll II | Type II Collagen |
| DAG | Diacylglycerol |
| EM | Electron Microscopy |
| ETC | Electron Transport Chain |
| FHIT | Fragile Histidine Triad |
| FFA | Free Fatty Acids |
| GLUT4 | Glucose Transporter 4 |
| HG-DMEM | High Glucose-Dulbecco's Modified Eagle Medium |
| IRS1 | Insulin Receptor Substrate-1 |
| IR | Insulin Receptor |
| IRS2 | Insulin Receptor Substrate-2 |
| LG-DMEM | Low Glucose-Dulbecco's Modified Eagle Medium |
| LPL | Lipoprotein Lipase |
| MDSCs | Muscle-Derived Stem Cells |
| MHS | Melanocyte Stimulating Hormone |
| mtGFP | Mitochondria-Targeted Green Fluorescent Protein |
| PEPCK | Phosphoenolpyruvate Carboxykinase |
| PI3K | Phosphatidylinositol-3-OH Kinase |
| PKC | Protein Kinases C |
| PKCβ siRNA | Protein Kinase C β-Small Interfering RNA |
| PMA | Phorbol 12-Myristate 13-Acetate |
| PPARγ | Peroxisome Proliferator Activated Receptor γ |
| qRT-PCR or RT-PCR | (quantitative) Real-Time RT-PCR |
| ROS | Reactive Oxygen Species |
| SEM | Scansion Electron Microscopy |
| SREBP1 | Sterol Regulatory Element- Binding Protein 1 |
| TEM | Transmission Electron Microscopy |
| VAT | Visceral Adipose Tissue |

# High glucose induces adipogenic differentiation of muscle-derived stem cells

Paola Aguiari*, Sara Leo*, Barbara Zavan†, Vincenzo Vindigni‡, Alessandro Rimessi*, Katiuscia Bianchi*, Chiara Franzin§, Roberta Cortivo†, Marco Rossato§, Roberto Vettor§, Giovanni Abatangelo†, Tullio Pozzan¶‖, Paolo Pinton*, and Rosario Rizzuto*‖

*Department of Experimental and Diagnostic Medicine, Interdisciplinary Center for the Study of Inflammation, University of Ferrara, 44100 Ferrara, Italy; †Department of Histology, Microbiology and Medical Biotechnologies, University of Padua, 35122 Padua, Italy; ‡Clinic of Plastic Surgery, University of Padua, 35122 Padua, Italy; §Endocrine-Metabolic Laboratory, Internal Medicine, Department of Medical and Surgical Sciences, University of Padua, 35122 Padua, Italy; and ¶Department of Biomedical Sciences, CNR Institute of Neuroscience, and Venetian Institute of Molecular Medicine, University of Padua, 35122 Padua, Italy

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**Regeneration of mesenchymal tissues depends on a resident stem cell population, that in most cases remains elusive in terms of cellular identity and differentiation signals. We here show that primary cell cultures derived from adipose tissue or skeletal muscle differentiate into adipocytes when cultured in high glucose. High glucose induces ROS production and PKC$\beta$ activation. These two events appear crucial steps in this differentiation process that can be directly induced by oxidizing agents and inhibited by PKC$\beta$ siRNA silencing. The differentiated adipocytes, when implanted *in vivo*, form viable and vascularized adipose tissue. Overall, the data highlight a previously uncharacterized differentiation route triggered by high glucose that drives not only resident stem cells of the adipose tissue but also uncommitted precursors present in muscle cells to form adipose depots. This process may represent a feed-forward cycle between the regional increase in adiposity and insulin resistance that plays a key role in the pathogenesis of diabetes mellitus.**

adipocyte | hyperglycemia | PKC | ROS

The identity and regulation of the adipocyte precursor cell is a topic of great interest and intense study. Microarray analyses and functional genomics have revealed adipogenic transcriptional signatures (1), but the signals occurring in pathophysiological conditions and aging are still incompletely understood. Stem cells are present in the adipose tissue and, under appropriate differentiation, signals can differentiate *in vitro* in adipocytes or other mesenchimal cells. Adipose-derived stem cells (ADSCs) represent the key cellular element for the control of body adiposity and they may substitute bone marrow-derived stem cells in their promising applications for tissue repair and regeneration. Indeed, s.c. adipose depots are accessible and abundant, thereby providing a potential adult stem cell reservoir for each individual.

Further complexity is added by the observation that the adipose depots of the body differ in functional properties, reflecting the existence of different subpopulations of adipocytes. Visceral and s.c. adipocytes release different endocrine mediators, collectively called adipokines. Although both adipose depots are correlated with metabolic risk factors, visceral adipose tissue remains more strongly associated with an adverse metabolic risk profile, type 2 diabetes development, and an increased risk of coronary heart disease (2). Even more intriguing is the role of intramyocellular triglycerides and intramuscular fat, given that intramuscular fat stores and substitution of muscle with adipose tissue have been reported to strongly correlate with insulin resistance (3). Thus, in the context of diabetogenesis (but also for other metabolic or myodystrophic conditions), key biological issues are how intra- and intermuscular adipose tissues originate and proliferate. Attention has been recently focused on the transdifferentiation of muscle progenitors to the adipogenic lineage, given that myopathic skeletal muscle is characterized by the replacement of myofibers by adipose tissue (4).

Muscle-derived stem cells (MDSCs) include the so-called satellite cells, i.e., the precursor cells capable of differentiating *in vivo* to mature muscle fibers and *in vitro* up to multinucleated skeletal myotubes (5). Human satellite cells retain broad differentiation capacity, including the generation of adipocytes in appropriate culture conditions (adipogenic medium) in a process that is enhanced by the insulin sensitizer rosiglitazone (6). Activators of PPAR-$\gamma$ cause, in muscle, the induction of genes involved in fatty acid uptake, storage, and metabolism (7), and the myogenic cell line C2C12 can be converted to adipocytes by overexpression of PPAR-$\gamma$ and C/EBP (8). As in hyperglycemia, high-glucose concentrations up-regulating sterol regulatory element binding protein 1c (SREBP-1c) induce *de novo* lipogenesis and intracellular lipid accumulation in contracting myotubes (9).

We here demonstrate that both ADSCs and MDSCs differentiate into adipocytes upon high glucose exposure. In this process, an important role is attributed to reactive oxygen species (ROS) and downstream effector kinases, such as PKC$\beta$, recently shown to act as signaling link between ROS and mitochondrial targets implicated in age-dependent organ deterioration (10).

## Results

**High Glucose Induces Adipocyte Differentiation of Stem Cells from Adipose Tissue.** The isolation of ADSCs from human lipoaspirates and their *in vitro* differentiation capacity into various mesenchimal tissues was first established by using standard protocols. To maximize nutrients delivery and to mimic the dynamic stress conditions stimulating formation of extracellular matrix, ADSCs were seeded onto hyaluronan-based sponge in a perfusion system that allows the circulation of culture medium through the scaffold (bioreactor). Further details on the isolation procedure and bioreactor are given in *Materials and Methods*. Under these conditions, the classical adipogenic, osteogenic, and chondrogenic media were highly efficient in causing specific differentiation into the expected cell lineage, as confirmed by morphological criteria (histological and electron microscopy analyses) and molecular hallmarks. Similar data have been obtained by us and others, using ADSC from mouse or rats, or using more standard culture conditions (11). The procedures used and the full characterization of the differentiated mesenchimal cells are described in supporting information (SI) Fig. 6.

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The authors declare no conflict of interest.

‖To whom correspondence may be addressed. E-mail: tullio.pozzan@unipd.it or rzr@unife.it.

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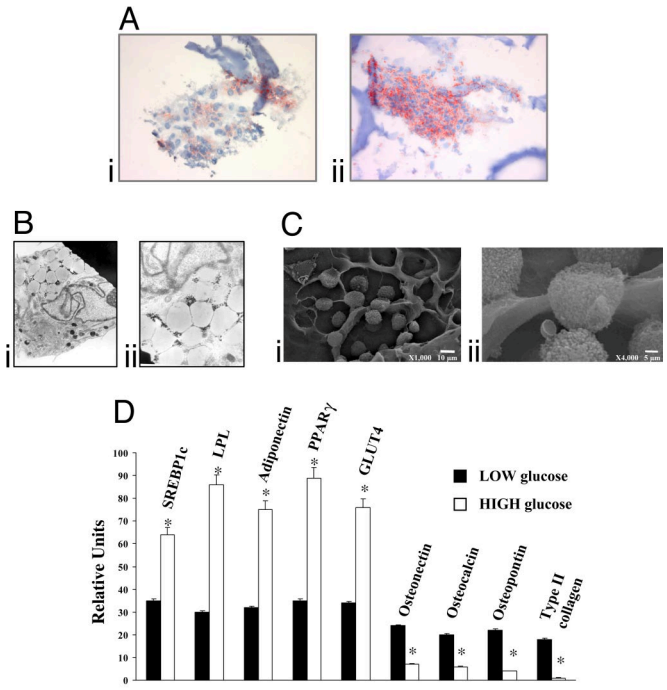


**Fig. 1.** Adipose-derived Stem Cells (ADSCs) cultured on Hyaff 11 sponges in LG- and HG-DMEM. (*A*) Oil red O staining of ADSCs grown in LG-DMEM (*i*) or HG-DMEM (*ii*). Lipid droplets are in red, and biomaterial fibers in blue. (Magnification: ×20.) (*B*) Transmission electron microscopy (TEM) of two representative fields. (Magnification: ×6,500.) (*C*) Scanning Electron microscopy (SEM) of ADSCs at two different magnifications. (*D*) mRNA expression of adipogenic, chondrogenic, and osteogenic markers analyzed after 14 days by semiquantitative real time PCR in ADSCs cultured on 3D scaffolds in LG- and HG-DMEM. Results for each condition are from quadruplicate experiments, and values are expressed in relative units as the mean ± SD. *, $P < 0.05$.

The physiological stimuli that control ADSCs differentiation into adipocytes *in vivo* remain unknown. We investigated whether one of the most common conditions leading to an increase in adipose tissue (12) *in vivo*, i.e., high-glucose concentration, can affect human ADSCs differentiation into mature adipocytes *in vitro* (Fig. 1). ADSCs were maintained for 14 days in a low-glucose (LG)-DMEM (i.e., DMEM, 10% FBS, and 5.5 mM glucose) or in a high-glucose (HG)-DMEM (i.e., DMEM, 10% FBS, and 25 mM glucose), and then analyzed by morphological (histochemistry, Fig. 1*A*, and electron microscopy, Fig. 1 *B* and *C*) and molecular (Fig. 1*D*) approaches. Staining with oil red O (ORO), selective for triglyceride depots, revealed substantial adipose differentiation in cells grown in HG-DMEM (Fig. 1*Aii*, 4.18 ± 0.21 OD at 520 nm), compared with cells grown in lower glucose DMEM (LG-DMEM) (Fig. 1*Ai*, 2.16 ± 0.11 OD at 520 nm). Accordingly, EM analysis showed numerous cells with a typical adipocytic phenotype, i.e., containing large lipid droplets surrounded by a thin ring of cytoplasm (Fig. 1*B*). SEM microscopy showed a population of rounded cells of various sizes, i.e., the typical appearance of adipose tissue (Fig. 1*C*). The lower magnification (Fig. 1*Ci*) reveals the presence of round adipocytes on both sides of the specimen.

In high-glucose conditions, RT-PCR analysis of mRNA transcripts (Fig. 1*D*) revealed significant expression of adipocyte-specific proteins, such as peroxisome proliferators activated receptor gamma (PPARγ), lipoprotein lipase (LPL), adiponectin, glucose transporter 4 (GLUT4), and SREBP1c. Conversely, chondrogenic (type II collagen) and osteogenic markers (osteopontin, osteonectin, and osteocalcin) were down-regulated in HG-DMEM compared with cells cultured in LG-DMEM.

**Adipogenic Conversion of Muscle-Derived Stem Cells on High Glucose.** We concluded that incubation in high glucose *per se* can drive the differentiation of uncommitted stem cells into adipocytes, a previously uncharacterized mechanism for ADSCs (but see refs. 13 and 14 for pancreatic β cell lines). However, these stem cells were derived from adipose tissue, and thus the observed effect could represent the enhancement of the default differentiation route of adipocyte precursors. To investigate whether it reflected conversely the differentiation of uncommitted mesenchimal precursors or even the transdifferentiation of stem cells of other mesenchimal tissues, we turned our analysis to primary cultures of skeletal muscle. These cells can be easily obtained from animal models, and thus the following experiments were carried out on cells derived from neonatal rats. Muscle-derived stem cells (MDSCs) from mouse or rats can be differentiated into large myotubes, carrying most phenotypic properties of skeletal muscle in terms of contractile protein expression, morphological aspect (with diad and triad formation), and signaling properties (5).

In the experiments shown in Fig. 2, we analyzed the effect of HG-DMEM on MDSCs, using the standard culture conditions, i.e., in 100-mm Petri dishes. Parallel batches of cells were maintained in LG-DMEM. In both cases, the medium was supplemented with 10% FCS (a conditions that prevents differentiation into myotubes), and cells were maintained in culture for 18–25 days before analysis. In LG-DMEM, as revealed by either counting the cells with large vacuoles in the cytoplasm (Fig. 2*Ai*) or those positive for the lipid specific dye 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-*s*-indacene (BODIPY) (Fig. 2*Aii*) the percentage of cells with a morphological adipocyte phenotype was only 3.3 ± 2.4%. In HG-DMEM, a highly significant increase in adipocyte differentiation was detected: 9.2 ± 2.9% of the cells showed a clear adipocytic phenotype, with either analysis methods (Fig. 2*B*), 2- to 4-fold higher than in LG-DMEM (HG: $n = 6$; LG: $n = 4$).

We then verified adipose differentiation efficiency in the 3D scaffold of the bioreactor. MDSCs were thus seeded onto the hyaluronan-based sponge and maintained in culture for 14 days under LG-DMEM or HG-DMEM perfusion. Then, histochemical and ultrastructural analyses of the cell culture were carried out. For the former, five randomly chosen fields were analyzed for each conditions, and the experiment was repeated with identical results in three different trials. The results of a typical experiment are presented in Fig. 2. In LG-DMEM, oil red O staining was hardly detectable (Fig. 2*Ci*) although the cells became loaded with lipid vesicles in HG-DMEM, as revealed by oil red O staining (Fig. 2*Di*); quantification of oil red O-stained cells showed a marked higher percentage of adipocytes in HG-DMEM (3.87 ± 0.31 OD at 520 nm) compared with LG-DMEM (1.89 ± 0.15 OD at 520 nm). In agreement with these observations, electron micrographs showed mostly elongated fibroblast-like cells in LG-DMEM (Fig. 2*Cii*) and a high fraction of rounded cells with the typical mature adipocytes appearance, in HG-DMEM (Fig. 2*Dii*). The percentage of cells with an adipocyte phenotype obtained with HG-DMEM from muscle cultures did not differ significantly from that observed under the same conditions, using ADSCs (3.87 ± 0.31 OD at 520 nm in MDSCs and 4.18 ± 0.21 OD at 520 nm for ADSCs), indicating that the differentiated adipocytes in the former case cannot derive from a contamination from adipose tissue in the muscle cell cultures.

The adipogenic potential of high glucose on MDSCs was confirmed also by RT-PCR analyses of adipocyte-specific transcripts: cells cultured in the bioreactor and maintained in HG-DMEM (Fig. 2*E*) for 7 and 14 days, shown an higher expression of PPARγ, LPL, adiponectin, GLUT4, and SREBP1c and a down-regulation of chondrogenic and osteogenic markers, compared with cells grown in LG-DMEM. Expression of the adipocyte markers is shown in Fig. 2*E* for 14 days of analysis. The comparison of the RT-PCR data at 7 and 14 days both for the ADSCs (Fig. 1) and the MDSCs (Fig. 2) is presented in SI Fig. 7.

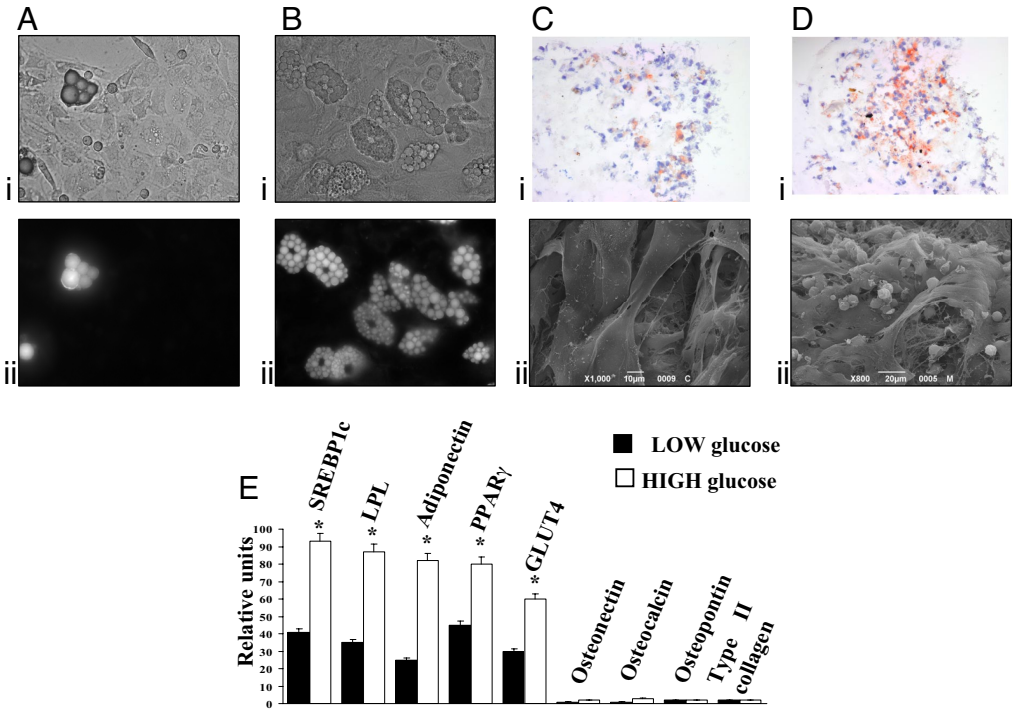


**Fig. 2.** High glucose induces adipocyte differentiation of muscle-derived stem cells (MDSCs). (*A* and *B*) Phase-contrast image (*i*) and BODIPY staining (*ii*) of MDSCs cultured in LG-DMEM (*A*) or HG-DMEM (*B*). (*C* and *D*) Oil red O staining (*i*) and SEM analysis (*ii*) of MDSCs cultured for 14 days on Hyaff 11 sponges in LG-DMEM (*C*) and HG-DMEM (*D*). Lipid droplets are in red, and nuclei are in blue. (Magnification: ×40). (*E*) Adipogenic, chondrogenic, and osteogenic markers mRNA expression analyzed by semiquantitative real time PCR in MDSCs cultured on 3D scaffolds for 14 days in LG- and HG-DMEM. Results for each condition are from quadruplicate experiments, and values are expressed in relative units as the mean ± SD. *, $P < 0.05$.

**Oxidative Stress by Effector PKCs Is a Trigger of Adipocytic Differentiation.** We decided to use this cell model to get some insight into the mechanisms that drive high-glucose-induced adipocytic differentiation. In the past years, major attention has been dedicated to the induction of oxidative stress by high glucose (through three mechanisms, NADP(H)oxidase, xanthine oxidase, and the mitochondrial respiratory chain) and its relevance for the development of diabetes complications. Regarding the effector mechanisms, PKC isoforms (PKC$\beta$ and PKC$\delta$ in particular) have been shown to be activated by oxidizing conditions (10). We thus verified whether these mechanisms represent the signaling route in stem cells differentiation to adipocytes. We first measured ROS production during high-glucose incubation. MDSC were obtained as in Fig. 2, but the cells were seeded onto 24-mm glass coverslip for microscopic analysis. Seven days after seeding, cells cultured in LG-DMEM or HG-DMEM were loaded with the ROS-sensitive fluorescent probe CM-$H_2$DCFDA. The coverslip with the cells was transferred to the stage of a confocal microscope, and fluorescence emission at 520 nm was monitored, revealing continuous ROS production. The results, shown in Fig. 3*A*, demonstrated that ROS production is markedly higher if the cell culture is maintained in HG-DMEM.

We then verified whether this condition activates PKC$\beta$, as observed in endothelial cells (15). Endogenous PKC$\beta$ revealed by immunofluorescence was mostly cytosolic in MDSCs maintained in LG-DMEM (Fig. 3*Bi*). Incubation for 1 h in 25 mM glucose induced a partial translocation of the kinase to the plasma membrane in ≈30% of the cells (Fig. 3*Bii*), with a pattern similar to that observed upon direct activation by phorbol 12-myristate 13-acetate (PMA) (Fig. 3*Biii*).

The simplest interpretation of the data are that ROS produced in response to high glucose, by stimulating PKC$\beta$ (and possibly other effectors), act as a differentiation signal for adipogenic conversion of MDSCs. We tested this hypothesis with three approaches: (*i*) LG-DMEM was supplemented with $H_2O_2$ to test whether it could mimic the effect of high glucose and thus trigger adipocytic differentiation; (*ii*) the cells, maintained in HG-DMEM, were transduced with a vector carrying the PKC$\beta$ siRNA to test whether this could inhibit adipocytic differentiation; and (*iii*) the cells in HG-DMEM were transduced with PKC$\beta$ expression vector to verify whether its overexpression could synergize with high glucose. The results presented in Fig. 4*A* (quantitated by counting the percentage of BODIPY-loaded cells) strongly support the hypothesis. In particular, (*i*) in LG-DMEM, addition of 100 $\mu$M $H_2O_2$ enhanced the differentiation rate to a level comparable to that observed in high glucose (9.34 ± 2.9% vs. 9.19 ± 2.9%, respectively) and no significant enhancement was detected if $H_2O_2$ was added to HG-DMEM (10.18 ± 0.5%); (*ii*) PKC$\beta$ siRNA, added to cells incubated in HG-DMEM, drastically reduced adipocytic differentiation (−80%) (Fig. 4*A*) to levels below those found in LG-DMEM, whereas an unrelated siRNA (*FHIT*) had no effect (5.5 ± 0.5%); (*iii*) a major (300%) enhancement of lipid-loaded cells was observed in cells overexpressing PKC$\beta$ and incubated in HG-DMEM. Infection with a control viral vector (an adenoviral vector expressing mtGFP) and PKC$\beta$, *per se*, did not increase adipocytic differentiation if added to cells in LG-DMEM (data not shown). The same results were obtained by seeding MDSCs onto hyaluronan base sponge of the bioreactor (Fig. 4*B*).

**High-Glucose-Differentiated Adipocytes Can Be Successfully Implanted *in Vivo*.** Finally, we verified whether *in vitro*-differentiated adipocytes can generate mature and viable adipose tissue *in vivo*. For this purpose, sponges enriched with ADSCs cultured in the bioreactor for 7 days in high glucose, were implanted in the abdominal area of eight female nude rats, as described in *Materials and Methods*. At 1, 2, 3, and 4 weeks after insertion of the graft, the rats were killed and the grafts analyzed macroscopically (for color, ingrowth and vessel formation, weight, and thickness) and micro-

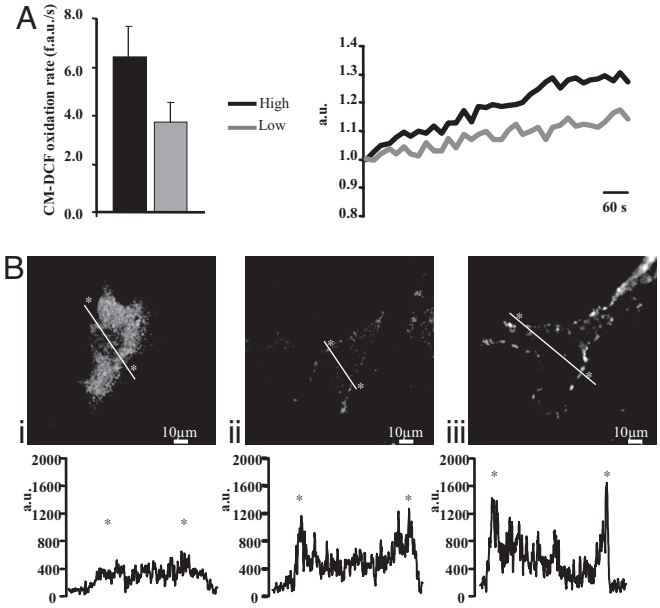


**Fig. 3.** ROS production of MDSCs cultured in LG- and HG-DMEM and glucose-induced PKC$\beta$-activation. (*A*) Seven days after seeding, cells were loaded with the CM-$H_2$DCFDA and analyzed by confocal microscopy. Basal ROS production is expressed as mean of CM-DCF oxidation rate (fluorescent arbitrary units per second). Representative traces of CM-DCF oxidation kinetics are shown at *Right* (black, high-glucose condition; gray, low-glucose condition). (*B*) PKC$\beta$ membrane translocation, as revealed by immunofluorescence microscopy. Representative images show cells maintained in LG-DMEM (*i*), incubated for 1 h in HG-DMEM (*ii*), and treated with 500 nM PMA (*iii*). The graphs show the quantitation of PKC$\beta$ fluorescence intensity along a line crossing the cell (thick white line in the micrograph).

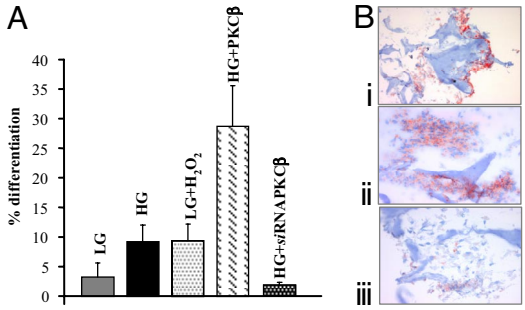


**Fig. 4.** Quantitation of MDSCs adipocyte differentiation in different conditions. (*A*) MDSCs cultured in different conditions: HG-DMEM, LG-DMEM, LG-DMEM supplemented with 100 $\mu$M $H_2O_2$, PKC$\beta$-overexpression in HG-DMEM, and PKC$\beta$ siRNA in HG-DMEM. The efficiency of MDSCs differentiation was quantitated by counting the percentage of BODIPY-loaded cell. The histogram shows the percentage of differentiation $\pm$ SEM. (*B*) MDSCs cultured on Hyaff 11 sponges in LG-DMEM supplemented with 100 $\mu$M $H_2O_2$ (*i*), PKC$\beta$-overexpression in HG-DMEM (*ii*), and PKC$\beta$ siRNA in HG-DMEM. Adipogenic differentiation was confirmed by oil red O staining. Lipid droplets are in red, and nuclei are in blue. (Magnification: $\times$10.)

scopically (for pore size, specific and unspecific cellularity, and vascularity). The implant appeared well integrated in the s.c. tissue and not surrounded by fibrotic tissue (Fig. 5*Ai* at 2 weeks and Fig. 5*B* at 3 weeks, white arrows). In addition, numerous capillaries (Fig. 5 *Ai* and *Bi*, blue arrows) revealed significant vascularization of the engineered, reimplanted tissue. Harvesting of the sample confirmed that the sponge maintains its volume and soft consistence and is easily dissected from the surrounding tissue (Fig. 5*Aii*).

Morphological characterization of the explants confirmed a major distribution of preadipocytes over the whole cross-section (Fig. 5*C*). Significant adipose tissue formation was detected (black arrows) (Fig. 5*C*) already at 3 weeks. In most cases, the cells exhibited the hallmarks of mature adipocytes, i.e., a large lipid droplet surrounded by a thin ring of cytoplasm, with a flattened nucleus located at the cell periphery. Adipocytes (Fig. 5*C*, black arrows) were closely attached to the scaffold (red arrows), and multiple capillaries were located near the preadipocytes and adipocytes [Fig. 5 *Ci* and *D* (green arrows)].

## Discussion

In this article, we investigated the effect of an increase in glucose concentration on the differentiation route of stem cells residing in both adipose tissue and skeletal muscle. In both cases, a clear induction of adipogenic transformation was observed upon the application of high-glucose culture conditions. The capacity of high glucose to partly activate an adipogenic differentiation program had already been noticed in pancreatic $\beta$ cells (13, 14). In our case, the process consists in a full conversion into adipocytes, as verified by morphological and molecular criteria and the capacity of these cultured cell to form viable and vascularized adipose depots when implanted *in vivo*. In muscle-derived cells, the process appears to be the genuine differentiation of uncommitted mesenchimal precursors or a transdifferentiation of myogenic precursors. This conclusion is supported by the following evidences: (*i*) when separate cultures were prepared from upper- and lower-limb muscles (the former may be partly contaminated by adipose tissue, whereas the latter is virtually devoid of it), the efficiency of high-glucose-induced adipocytic differentiation was very similar (upper limbs: 8.9 $\pm$ 2.7%; lower limbs 10.3 $\pm$ 1.8%) and (*ii*) the percentage of differentiated adipocytes induced by HG-DMEM is approximately the same whether the culture was derived from muscle or from adipose tissue.

Adult skeletal muscle has a remarkable regenerative capacity, largely mediated by satellite cells residing between the sarcolemma and basal lamina of myofibers (16). The regenerative process and proliferative capacity decrease as a function of age (17), partly for the reduced activity of stimulatory factors (18), with consequent loss of muscle mass. However, these alterations do not account for other features of aged muscle, and, in particular, for the increased lipid content (19). It has been reported that aged mice myoblasts, compared with those from adult mice, show increased expression of genes normally restricted to the adipocyte lineage (20), although a fully differentiated adipocyte phenotype is not achieved. The identification of a primary role of high-glucose exposure in adipocytic differentiation has at least two major important conceptual implications. It shows that high glucose *per se* has an adipogenic potential, thus opening the search for signal transduction pathways operating in obesity and diabetes. Second, a direct link between hyperglycemia and the increase in adiposity highlights a feed-forward cycle, which may play a key role in the progression of the metabolic dysfunction into an irreversible diabetic state.

Regarding the signals driving the differentiation of muscle stem cells into adipocytes, our data suggest a crucial role for reactive oxygen species (ROS). Interestingly, among the cellular consequences of high glucose, increased production of ROS is recognized as a major cause of the clinical complications associated with diabetes and obesity (21). Various mechanisms appear to synergize in increasing ROS production, ranging from the activity of mitochondrial respiratory complexes to the activation of enzymatic systems, such as NOX isoforms, NO synthase, and xanthine oxidase (22). Despite the presence of scavenging systems (e.g., superoxide dismutase and catalase), the damaging effects of ROS have been proposed to be responsible for progressive organ degeneration (23). However, the role of ROS is not only restricted to cell damage induction. Indeed, ROS may directly regulate the activity of transcription factors, such as NF-$\kappa$B, in turn controlling proinflamma-

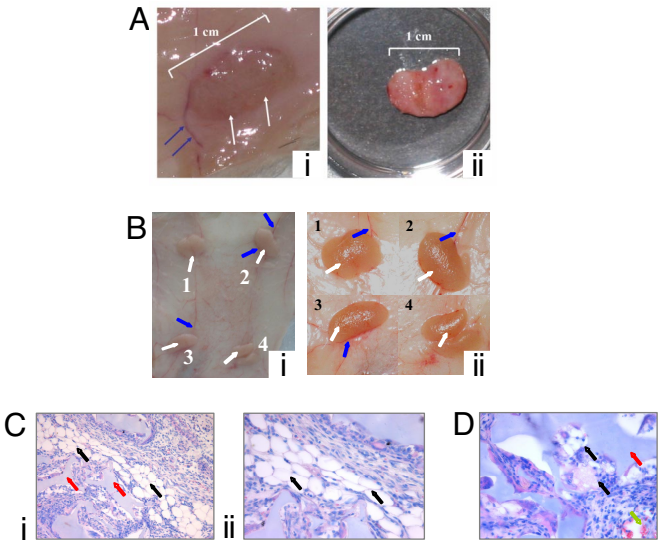


**Fig. 5.** Macro and microscopic analyses of *in vitro*-differentiated adipocytes implanted *in vivo*. (*A* and *B*) Macroscopic aspect 2 (*A*) and 3 (*B*) weeks after implantation of the Hyaff 11 sponge seeded with adipocytes in HG-DMEM. (*Ai* and *Bi*) Images of the implanted tissue *in situ*. (*Aii*) Image of the graft after dissection. (*Bii*) Low-magnification image of the four implanted grafts. (*C* and *D*) Histological analyses of the grafts 3 weeks after implantation. Haematoxilin/eosin staining of newly formed tissues is shown. (Magnification: *Ci*, ×20; *Cii*, ×40.)

tory gene expression (24). Our results add an important conceptual mechanism by revealing a morphogenetic signaling activity of ROS. In fact, ROS, through downstream effectors and in particular through PKC$\beta$ (as revealed by the strong inhibitory effect of siRNA and by the strong synergism of high glucose with PKC$\beta$ overexpression), lead to the neoformation of adipose cells. In turn, adipocytes are known to release adipokines that regulate, among other targets, the insulin sensitivity of peripheral tissues (25). A similar role is in keeping with previous reports showing a role of ROS in embryogenesis (see, for example, the role of NOX4 in promoting the spontaneous beating of cardiac cells within embryoid bodies of mice) (26).

Finally, the present data provide additional support to the role of PKCs in detecting high-glucose conditions and sustaining the ROS-mediated signaling route. This result well agrees with a number of previous observations that on the one hand highlighted an important role for PKC in diabetes and in adipogenesis and on the other established a link between PKCs, ROS production, and cell degeneration (27). Indeed, PKC isoforms have been shown to translocate to the plasma membrane of endothelial cells upon high-glucose culture conditions (15) and to play an important role in the pathogenesis of diabetes complications. It has been recently reported that PKC-λ muscle-specific knockout mice recapitulate most of the features of the metabolic syndrome including impaired glucose tolerance or diabetes, islet $\beta$ cell hyperplasia, and abdominal adiposity (28). In this case, the key dysfunction appears to be the impairment of GLUT4-mediated glucose transport across the plasma membrane of skeletal muscle, with ensuing release of anti insulinic factors and, thus, insulin resistance. In turn, insulin resistance and hyperinsulinemia can trigger abdominal obesity and adipose tissue growth in extra adipose sites such the intramuscular site. In addition to these metabolic effects, PKCs appear to be involved in detecting and decoding a variety of stress conditions, including oxidative challenges. We recently showed that PKC$\beta$ recruits the 66-kD proapoptotic isoform of Shc (p66Shc) to act as oxidoreductase within mitochondria (29) and in triggering a feed-forward cycle of ROS production eventually leading to cell death (10). The same actors may come together in a radically different context, i.e., the production of cellular signals linking hyperglicemia to the regulation of a transdifferentiation scheme of stem cells residing in insulin target tissues. How the same signaling route can trigger uncommitted stem cell differentiation or induce apoptosis remains to be elucidated. Here, we can only stress that the toxicity and the differentiation effects of $H_2O_2$ are optimally observed at different concentrations, i.e., 1 mM and 100 $\mu$M, respectively.

In conclusion, we demonstrate that high glucose has an adipogenic potential on stem cells derived from both the adipose tissue and skeletal muscle, and we provide some insight into the signals and molecules that underlie this process. These results may deepen our understanding of the pathogenesis of type 2 diabetes and highlight potential targets for pharmacologically addressing this disease of dramatic social impact.

## Materials and Methods

**Cell Cultures.** ***Muscle-derived stem cells.*** Primary cultures of muscle-derived stem cells (MDSCs) were prepared from newborn rats (2–3 days) as described in ref. 5. Adipogenic conversion was observed both in the first preplating (15 min or 2 h) and in the replating of non-rapidly adherent cells. In the latter case, a small fraction of myotubes was still present, and their contraction in culture made the cell yield highly variable. For this purpose, the first pool of adherent cells was used for all experiments.

***Adipose-derived stem cells.*** Adipose-derived stem cells (ADSCs) were extracted from human adipose tissues of five healthy female patients undergoing cosmetic surgery procedures, following guidelines from the Clinic of Plastic Surgery, University of Padova. Adipose tissues were digested with 0.075% collagenase (type 1A; Sigma–Aldrich) in Krebs–Ringer buffer (modified KRB) [125 mM NaCl, 5 mM KCl, 1 mM $Na_3PO_4$, 1 mM $MgSO_4$, 5.5 mM glucose, and 20 mM Hepes (pH 7.4)] for 60 min at 37°C followed by 10 min with 0.25% trypsin. Floating adipocytes were discarded, and cells from the stromal-vascular fraction were pelleted, rinsed with media, and centrifuged, and a red cell lysis step in $NH_4Cl$ was done for 10 min at room temperature. The viable cells obtained were counted by using trypan blue exclusion assay and seeded at a density of $10 \times 10^5$ cells per square centimeter for *in vitro* expansion.

**Biomaterials.** Sponges made of Hyaff 11, a linear derivative of hyaluronic acid, in which the carboxylic function of the monomer glucuronic acid in the hyaluronic acid chain is totally esterified with benzyl groups, were supplied by Fidia Advanced Biopolymers. The structure of these sponges shows open interconnecting pores. Sponges were provided as cylinders. All carriers were sterilized by $\gamma$-irradiation before use.

**Adipocyte Differentiation Detection.** To quantitate adipocyte differentiation, Petri dish-cultured cells at days 18–25 were loaded for 10 min with 40 $\mu$M BODIPY 493/503 (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-*s*-indacene; Invitrogen) and 36 $\mu$M bisbenzimide (Sigma–Aldrich), then washed with KRB. At the indicated times, oil red O (ORO) (Sigma–Aldrich) staining of the cytoplasmic droplets of neutral lipids was performed according to a modification from ref. 30. Cells cultured in the bioreactor were rinsed, fixed with 10% buffered formalin, stained with 0.3% ORO in isopropanol:water (3:2), photographed, and extracted with 4% Nonidet (Sigma–Aldrich) and isopropanol. The optical density (OD) of the solution was measured at 520 nm for quantification, using a Victor 3 spectrometer (Perkin–Elmer).

**Electron Microscopy.** ***SEM.*** Samples of reconstructed adipose tissues were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h before being processed either with hexamethyldisilazan or at the critical point followed by gold-palladium coating. All micrographs were obtained at 30 kV on a JEOL 6360 LV SEM microscope.

***TEM.*** Cultures were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 for 3 h, postfixed with 1% osmium tetroxide, dehydrated in a graded series of ethanol, and embedded in araldite. Semithin sections were stained with toluidine blue and used for light microscopy analysis. Ultrathin sections were stained with uranyl acetate and lead citrate and analyzed with a Philips EM400 electron microscope.

**Real-Time RT-PCR and Quantitative RT-PCR.** Total RNA was extracted by using a kit (RNeasy Mini; Qiagen) according to the supplier's instructions. RNA (1 $\mu$g) was treated with DNase (Ambion) and reverse-transcribed for 1 h at 37°C in a 50-$\mu$l reaction containing 1× room temperature buffer, 150 ng of random hexamers, 0.5 mmol/liter deoxynucleotide triphosphates, 20 units of RNAsin ribonuclease inhibitor, and 200 units of M-MLV RT (Promega). PCR was carried out by using a

DNA Engine Opticon 2 Continuous Fluorescence Detection System (MJ Research). All reactions were performed twice. Results were normalized by beta2microglobulin mRNA and reported as arbitrary units. The sequences of the primers are available upon request.

**Analysis of PKC$\beta$ Translocation.** To analyze PKC$\beta$ translocation, cells seeded onto 24-mm glass coverslips and grown in LG-DMEM for 3 days were fixed in 4% paraformaldehyde solution for 20 min at room temperature, quenched with 0.1% glycine, and permeabilized with 0.1% Triton X-100 for 20 min. After three washes with PBS, nonspecific binding sites were blocked with 2% BSA for 1 h. Immunostaining was performed with the monoclonal mouse anti-PKC$\beta$II primary antibody (diluted 1:20 in 2% BSA), followed by the visualization with the Alexa Fluor 488 anti-mouse antibody (Molecular Probes).

**Measurements of ROS Production.** Intracellular ROS generation was measured with 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-$H_2$DCFDA) (Invitrogen), which is cell-permeant. After ester hydrolysis, the probe is trapped as a nonfluorescent probe, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein. After its oxidation by ROS, 5-(and-6)-chloromethyl-2′,7′-dichlorofluorescein (CM-DCF) green emission was recorded at 520 nM.

Cells were loaded with 10 $\mu$M CM-$H_2$DCFDA at 37°C in modified KRB at 5.5 or 25 mM glucose. After 30 min, laser scanning confocal microscopy images were obtained. Acquisitions were made every 1 s for 500 s.

**PKC$\beta$ Overexpression and Silencing.** To overexpress PKC$\beta$, the cells were transduced the day after seeding with a adenoviral vector expressing PKC$\beta$-GFP (AdEasy/PKC$\beta$-GFP). In the case of PKC$\beta$ overexpression in LG, we observed a decrease in cell viability 14 days after transfection, which affects the evaluation of the results. To evaluate the effect of PKC$\beta$ in LG, we compared in this experiment the efficiency of differentiation in PKC$\beta$-expressing and control cells at an earlier time point, in which overall differentiation was lower but there was no detectable reduction in cell survival. PKC$\beta$ silencing was achieved by infecting cells the day after seeding with a commercially available lentiviral siRNA for PKC$\beta$ (Sigma–Aldrich), at a viral titol of 3.5 transduction units per milliliter. As a control, an unrelated gene (*FHIT*) was silenced, because a siRNA scrambled mixture was shown (31) and confirmed in our studies to enhance adipocyte differentiation.

**Surgical Implantation of Hyaluronic Acid Sponges Seeded with Adipocytes.** The protocol was approved by the Institutional Animal Care Committee of Padua University. Eight female nude rats (Charles River Laboratories), weighing 150–200 g, were subjected to the surgical procedures under halothane anesthesia and aseptic conditions. Four Hyaff 11 sponges seeded with adipocytes in HG-DMEM were implanted in the abdominal area. Throughout four small (0.3-cm) skin incisions, the sponges were inserted in a s.c. pocket above the abdominal fascia. Skin incisions were closed with one nylon 5-mm stitch. No anticoagulants were used before or after the operation, and no prophylactic antibiotic was administered. All surgical procedures were performed in the same way by a single surgeon. The animals were fed an unrestricted standard diet. After 1, 2, 3, and 4 weeks, two rats were killed by an overdose of gaseous anesthetic, and the grafts were explanted.

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