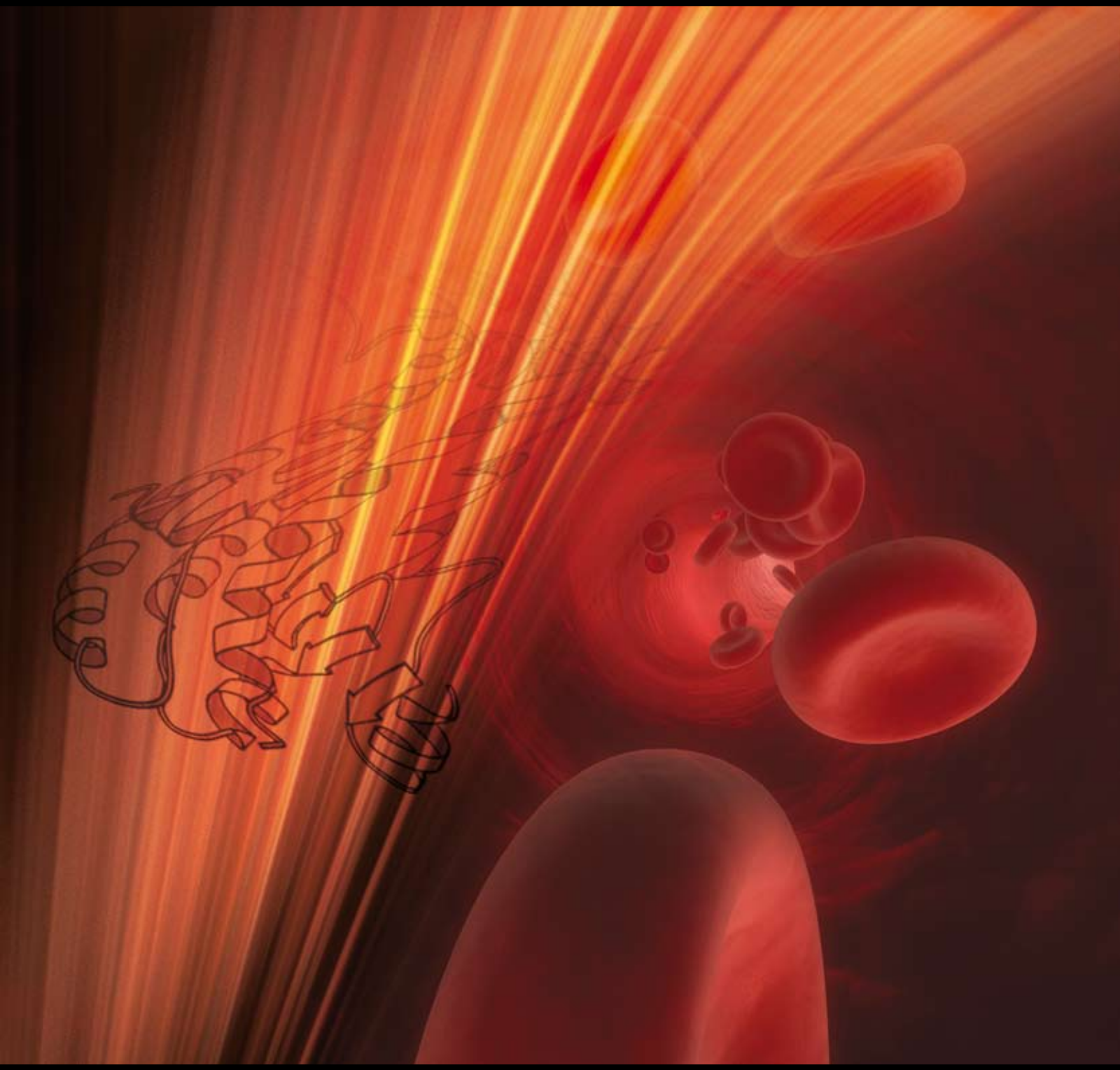
PPAR Research

PPARs, RXRs, and Stem Cells

Guest Editors: Z. Elizabeth Floyd and Jeffrey M. Gimble



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Editorial
PPARs, RXRs, and Stem Cells

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Welcome to this special issue of *PPAR Research: PPARs, RXRs, and Stem Cells*. Within the past decade, there has been a burgeoning interest regarding the mechanisms regulating stem cell regeneration and differentiation in embryonic and adult tissues. Recent studies have identified stem or progenitor cells within most, if not all, somatic tissues. Cell biologists have explored a number of transcriptional regulatory pathways in the context of stem cell self-renewal and lineage commitment. While there has been a wealth of attention given to the Wnt pathway, Oct4, nanog, and STAT transcription factors in this context, the role of PPARs and related nuclear hormone receptors in regulating stem cells remains relatively unexplored. The current issue of *PPAR Research* has called for manuscripts that will spotlight the PPAR-Stem Cell relationship. We are fortunate to have received a mixture of excellent primary research manuscripts and comprehensive review articles from experts in the field. Mullen, Gu, and Cooney (Houston, Tex) have explored the role of nuclear hormone receptors in murine embryonic stem cell differentiation and function. Purton (Boston, Mass) has comprehensively reviewed the literature concerning the role of retinoid receptors in hematopoietic stem cells. Casteilla, Cousin, and Carmona (Toulouse, Fla) evaluate the classical role of PPARγ as an adipogenic regulator in adipose tissue-derived stromal/stem cells (ASCs). Three investigators use bone marrow-derived mesenchymal stem cell (MSC) models. Isales et al. (Augusta, Ga) provide novel findings relating to the role of mystatin and GILZ on adipogenesis in response to PPAR ligands. Shockley et al. (Bar Harbor, Me & Little Rock, Ark) report the transcriptomic response of MSCs to PPARγ agonists. Duque, Rivas, and Akter (Montreal, Canada) describe a role for farnesylation in modulating MSC adipogenesis. Finally, Cimini et al. (L'Aquila, IT) provide novel insights into the effect of PPARγ during neural stem cell (NSC) astroglial differentiation. We hope that this issue will stimulate other investigators to pursue novel avenues related to the converging themes of PPARs, nuclear hormone receptors, and stem cell biology. The outcomes of such investigations will have far reaching implications regarding fundamental questions relating to normal development, tumor biology, and tissue engineering and regenerative medicine.

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Review Article

Nuclear Receptors in Regulation of Mouse ES Cell Pluripotency and Differentiation

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Embryonic stem (ES) cells have great therapeutic potential because they are capable of indefinite self-renewal and have the potential to differentiate into over 200 different cell types that compose the human body. The switch from the pluripotent phenotype to a differentiated cell involves many complex signaling pathways including those involving LIF/Stat3 and the transcription factors Sox2, Nanog and Oct-4. Many nuclear receptors play an important role in the maintenance of pluripotence (ERRβ, SF-1, LRH-1, DAX-1) repression of the ES cell phenotype (RAR, RXR, GCNF) and also the differentiation of ES cells (PPARγ). Here we review the roles of the nuclear receptors involved in regulating these important processes in ES cells.

1. INTRODUCTION

The nuclear receptor peroxisome proliferator activated receptor gamma (PPARγ) plays an important role in the differentiation of adipose cells and osteoblasts, and thus has the potential to direct embryonic stem (ES) cells to differentiate into these cell types for future therapeutic uses in disease treatment. This potential is real as nuclear receptor family members regulate many of the key functions of ES cells, and they are capable of unlimited self-renewal and can potentially differentiate into any of over 200 cell types in the body. They are derived from the inner cell mass of the mammalian blastocyst [1–4]. The pluripotency of ES cells is maintained by several key regulatory transcription factors and signaling molecules, which establish precise patterns of gene expression that are characteristics of the undifferentiated phenotype of ES cell [5]. Some of these key regulators are leukemia inhibitory factor (LIF) and the transcription factors Oct-4, Sox2 and, Nanog [5]. LIF belongs to the interleukin-6 cytokine family and binds to a heterodimeric receptor, which then leads to activation of the Jak/Stat pathway. Activation of Stat3 is essential and sufficient to maintain the mouse ES cell pluripotence, however, the LIF STAT3 pathway is mouse specific (related to diapause) and does not play a role in human embryonic stem cells [6–8]. Wnt3A is also important in the maintenance of ES cell pluripotence [9]. It was found that its presence in the media can maintain the pluripotent nature of ES cells, but it appears that this action occurs synergistically with LIF [10].

Oct-4 is a member of the POU homeodomain family of transcription factors, which acts as a gatekeeper to prevent ES cell differentiation by maintaining pluripotent gene expression and inhibiting expression of lineage determination factors. When repressed or inactivated in ES cells, differentiation occurs along the trophoectodermal lineage. Over expression of Oct-4 causes ES cells to differentiate mainly into primitive endoderm-like derivatives [11]. These divergent effects of Oct-4 suggest that it regulates the transcription of genes involved in coordination of multiple cellular functions and early cell fate decisions. Oct-4 usually binds to the octamer DNA sequence ATGCAAAT in ES cell-specific genes, and this binding often occurs in conjunction with Sox2 (a member of The SRY HMG box family), which binds to a neighboring Sox element [12, 13]. Nanog is an NK2 class homeobox transcription factor that was identified as a factor, which when over expressed, can maintain pluripotency even in the absence of LIF. Nanog-null embryos fail shortly after implantation, and at first give rise to pluripotent cells but these quickly differentiate along the extraembryonic endoderm lineage [14, 15].

It has been proposed that there are two mechanisms by which transcription factors play a role in the maintenance of pluripotency. First, Boyer et al. showed that the Oct-4, SOX2 and, Nanog co-occupy a substantial proportion of their target genes, which are mainly transcription factors. In addition, Oct-4, SOX2, and Nanog collaborate to form regulatory circuitry consisting of regulatory and feed forward loops that lead to coordinated auto regulation of their own expression [16]. Second, Ivanova et al. showed that the transcription factor ERRβ, along with TBX3 and TCL1 can also regulate pluripotency in ES cells, independently of the regulation by Oct-4, Sox2, and Nanog, thus forming a second regulatory axis [17].

ERRβ is a member of the nuclear receptor gene superfamily of ligand activated transcription factors [18–20]. The nuclear receptor gene superfamily includes a related, but diverse, array of transcription factors; which include nuclear hormone receptors such as the steroid receptors (NHRs) and orphan nuclear receptors [21]. NHRs are receptors for which hormonal ligands have been identified, whereas orphan receptors are so named because their ligands are unknown, at least at the time the receptor is identified. Nuclear receptors share structural motifs and domains that determine their function: a central DNA binding domain (DBD), an intervening hinge region, and a carboxy-terminal ligand binding domain (LBD), which mediates ligand-induced transactivation and participates in receptor dimerization. Nuclear receptors can exist as monomers, or homo- or heterodimers with each partner binding to specific sequences that exist as half sites separated by variable length nucleotide spacers between direct or inverted half-site repeats [22–24]. ERRβ is not the only nuclear receptor that has been implicated in regulation of ES cells, here we review the contributions of other nuclear receptors to the maintenance of pluripotency, repression of the ES cell phenotype during differentiation, and differentiation of ES cells.

1.1. Nuclear receptor contribution to the maintenance of pluripotence

1.1.1. ERRβ (NR3B2)

The ERR subfamily of nuclear receptors consists of 3 members, ERRα, ERRβ, and ERRγ. They display a high degree of homology within their DBDs and LBDs, which indicates that they probably bind to similar ligands and target the same promoters and/or enhancers [25–29]. ERRα is broadly expressed in both the developing embryo and in the adult [30–32]. ERRβ is expressed in the developing placenta in a subset of cells in extraembryonic endoderm destined to become the chorion. Knockout mice of ERRβ have impaired trophoblast stem cell differentiation and the placenta fails to develop normally [33, 34]. ERRβ is highly restricted in the adult, being detected at low levels in the liver, stomach, skeletal muscle, heart, and kidney [25, 27]. Interestingly, Ivanova et al. identified ERRβ as having a role in the maintenance of pluripotency. Although an ES cell-based phenotype is not observed in the ERRβ KO, this might be due to maternal contribution of protein, as it is expressed in the ovulated egg or due to redundancy of expression with either ERRα or ERRγ, which would be lost in cultured ES cells. They assessed the loss of various proteins on ES cell capacity for self-renewal. Upon loss of ERRβ by shRNA knockdown, ES cells differentiated suggesting that ERRβ appeared necessary to repress differentiation. Similar studies with TBX3 and TCL1 showed similar results and microarray analysis of gene alterations in the absence these factors identified a significant overlapping set of genes. Expression of 272 genes was up regulated by the loss of ERRβ, TBX3, or TCL1. This set of genes was distinct from those regulated by Oct-4, Sox2, and Nanog. In the same set of experiments microarray analysis showed that expression of 474 genes was either up or down regulated by knockdown of Nanog, Oct4, or Sox2 but unaffected by knockdown of ERRβ, TBX3, or TCL1. This data provides evidence that two independent transcriptional pathways are operating in ES cells. The first is controlled by Oct-4, Sox2, and Nanog and could be mainly responsible for maintenance of pluripotency and repression of differentiation. The second pathway involving ERRβ, TBX3, and TCL1 seems to be responsible for repression of differentiation along specific cell lineages. However, there appears to be cross-talk between the two pathways since slight over expression of Nanog compensated for loss of ERRβ, TBX3, and TCL1 [17]. Wang et al. also identified ERRβ as interacting with Nanog [35]. However, Sauter et al. showed that there was no change in ERRβ levels when cells are induced to differentiate upon removal of LIF [36]. Since ERRα and ERRγ are involved in regulating metabolism and mitochondrial activities, it is possible that ERRβ might not be involved in maintenance of pluripotency directly but alternatively may play a role in regulating ES cell metabolism [25, 28–31, 33, 34].

1.1.2. SF-1 (NR5A1)

Steroidogenic Factor 1 (SF-1; NR5A1), an orphan nuclear receptor, is, as its name suggests, expressed in steroidogenic tissues. SF-1 constitutively expressed in all three layers of the adrenal cortex, testis Leydig, and Sertoli cells, placenta, pituitary, and the hypothalamus [37, 38]. It has been shown to regulate the expression of each of the steroidogenic cytochrome P450 enzyme genes involved in steroid production [39–47], Mullerian inhibitory substance [48], and the alpha and beta subunits of the gonadotropins [49–53]. It is expressed in the urogenital ridge as early as day 9 of embryogenesis and displays dynamic expression profile in the developing gonads [37]. Disruption of SF-1 in mice leads to complete lack of adrenal glands and gonads due to adrenal and gonadal agenesis [38, 54]. A combination of the data shows that SF-1 has a central role in the regulation of steroidogenesis, development, and reproduction. Crawford et al. showed that stable expression of SF-1, which is not expressed in ES cells, directs the cells toward a more steroidogenic phenotype, which was demonstrated by the generation of progesterone. The directed differentiation of ES cells by SF-1 did not specifically require the AF2 domain but did require the proximal ligand binding domain [55].

Although SF-1 is expressed in the inner cell mass of mouse blastocysts, it is not expressed in mES cell lines.

However, it was noted that the proximal promoter of murine Oct-4 contains a consensus SF-1 responsive motif (PyCAAGGpyCPu). SF-1 was found to bind to this sequence and activate transcription in embryonic carcinoma (EC) cell lines P19 and NCCIT cells, where it is expressed [56, 57]. SF-1 and Oct-4 are coexpressed in these cell lines and when SF-1 is over expressed there is approximately a 3-fold increase in Oct-4 promoter activity in NCCIT cells. It was found that there are 3 putative SF-1 sites in the human Oct-4 promoter and that one SF-1 binding site in the evolutionarily conserved region 1 (CR1) was primarily responsible for SF-1-mediated transcription of the human Oct-4 promoter. Differentiation of these EC cells with retinoic acid (RA) causes a loss in expression of both SF-1 and Oct-4, thus indicating the role of SF-1 in the maintenance of pluripotency in EC cells [56, 57].

1.1.3. LRH-1 (NR5A2)

Comparison of SF-1 and Oct-4 knockout mouse models suggests that although SF-1 can regulate Oct-4 expression in EC cells, it is essential only in late organogenesis, therefore there must be another factor that compensates for SF-1 to maintain Oct-4 expression during early embryogenesis [56]. The orphan nuclear receptor liver receptor homolog-1 (LRH-1; NR5A2) is closely related to SF-1 particularly in its DNA binding domain and has the same DNA response element as SF-1 [58]. LRH-1 is expressed in endoderm derived tissues such as the liver, pancreas, and the intestines in the adult and in developing embryos [58–60]. It is involved in bile acid metabolism [61–63] and plays a role in liver development by activating genes such as HNF4α, HNF1α, and HNF3β, which coordinate hepatic gene expression [64, 65]. Like SF-1, LRH-1 also regulates the expression of genes involved in steroidogenesis. Importantly, LRH-1 is expressed at the inner cell mass of the blastocyst, in the embryonic ectoderm at the epiblast stage of embryonic development. Inactivation results in death at day 6.5 before the initiation of liver development [66]. In contrast to SF-1, which is expressed in EC cells, LRH-1 is expressed in ES cells. Upon differentiation with RA, Oct-4, and LRH-1 expression is down regulated. LRH-1 was found to bind to response elements in both the Oct-4 proximal promoter and proximal enhancer, which are evolutionarily conserved and activate its transcription. LRH-1 KO mice die at embryonic days 6.5–9.5 depending on the model analyzed. Gu et al. observed a penetrant phenotype with no embryos detected at day 7.5. However, Labelle-Dumais et al. observed a less-penetrant phenotype. Oct-4 is expressed in LRH-$1^{-/-}$ ES cells. However, upon RA differentiation, Oct-4 expression is more rapidly lost than in WT ES cells. Sox2, FGF4, UTF1, and REX1, which are regulated by Oct 4 and function in conjunction with it in ES cells, are also more rapidly lost in LRH-1 KO ES cells than in WT cells. The decreased expression of these genes is unlikely to be a direct result of LRH-1 as they contain no putative LRH-1 binding sites in their promoters and is most likely indirect due to the precocious loss of Oct-4 expression [56]. Maintenance of Oct-4 expression is probably not the only function of LRH-1 in ES cells, there are likely too numerous other target genes. For example, in intestinal stem cells, LRH-1 and β-catenin synergistically play an important role in regulating proliferation through direct interaction and regulation of cyclin G1 expression [67]. Inactivation of the β-catenin gene is embryonic lethal at the same stage as LRH-1 and presents a similar phenotype. Thus, LRH-1 and β-catenin may cooperate to regulate ES cell proliferation and expansion from an ICM in the blastocyst to a pregastrulation epiblast [68]. Recently, it has also been found that a novel promoter directs expression of LRH-1 in ES cells and hence a novel transcript with the first ATG start codon being in exon 3 of the regular LRH-1 transcript. The novel and regular transcripts have partially overlapping tissue distribution but have important temporal and spatial differences [69]. Thus, the ES cell LRH-1 isoform may have different transcriptional properties from other isoforms of LRH-1.

1.1.4. DAX1 (NR0B1)

DAX1, which stands for dosage sensitive sex reversal (DSS), adrenal hypoplasia congenital (AHC), locus on the X chromosome, gene 1, is another orphan nuclear receptor that appears to be critical in early embryonic development [70]. In contrast to canonical nuclear receptors, which have both a DBD and an LBD, DAX1 contains only an LBD. In the N-terminus there are 4 repeats purported to act as a DBD by binding to stem loop structures [70–72]. DAX1 has a known role in the establishment and maintenance of steroid producing tissues such as the testis and the adrenal cortex [73, 74]. DAX1 and SF-1 were shown to have a colocalized tissue expression in developing tissues [75, 76] and it has been shown that DAX1 acts as a repressor of SF-1 in these tissues. This transcriptional repression seems to involve direct protein-protein interactions between DAX1 and DNA-bound SF-1 via the DAX1 N-terminal domain and with subsequent recruitment of corepressors to the promoters of target genes via a DAX1 c-terminal transcriptional silencing domain [77, 78]. DAX1 has also been shown to repress LRH-1, ER, AR, and PR expression [79]. However, in contrast to molecular studies a genetic analysis of SF-1 and DAX1 in gonad development showed that rather than DAX1 antagonizing the function of SF-1 it worked in concert with it to maintain Cyp17 expression [80]. Generation of a DAX1 KO mouse model presented some problems as the gene is X-linked. The failure to generate a DAX1 knockout mouse suggests that DAX1 plays an earlier role in embryogenesis than just steroidogenesis. DAX1 was found to be expressed in early preimplantation embryos as well as in ES cells [81]. Differentiation of ES cells with RA caused a decrease in the expression of DAX1 similar to that observed for Oct-4. Disruption of the expression of DAX1 by RNA interference as well as a conditional knockout in ES cells caused their differentiation [82]. DAX-1 has been further implicated in the maintenance of pluripotence since it was discovered that it interacted with Nanog. Knockdown of DAX1 using shRNAs led to a loss of pluripotence in ES cells [35].

1.2. Nuclear receptor mediated repression of the ES cell phenotype

During ES cell differentiation two events must occur; one is a loss of the original phenotype and two is the induction of a new phenotype. Nuclear receptors play a role in both downregulation of the ES cell phenotype and the induction of a new cell fate.

1.2.1. RARs and RXRs (NR1B1-3 and NR2B1-3)

The retinoid receptors play a prominent role in RA-mediated differentiation of ES cells. There are three genes encoding Retinoic Acid Receptors (RARα, β and γ), which bind both all-*trans* RA and 9-*cis* RA and in response activate target gene expression [83, 84]. There are also three genes encoding Retinoid X receptors (RXRα, β, and γ), which bind 9-*cis* retinoic acid (9-*cis* RA) and activate target gene expression. RARs form functional heterodimers with RXRs [21]. Gene targeting experiments in mice provided evidence that the RXR/RAR heterodimer transduces the retinoid signal during mouse development [85]. RXR enhances RAR's efficiency of binding to RA response elements (RAREs), the specificity of RARE recognition, and modulate RAR signaling [86, 87]. Work in the EC cell line PCC7 suggested that RXRα and RARγ are required for endodermal differentiation. Zechel found that selective agonists of RARα, β, and γ cause the downregulation of Oct-4, up regulation of GCNF, and the induction of neuronal markers although these agonists had distinct efficacy indicating a differential requirement of RAR isotypes during the initial stages of neuronal differentiation [88]. Since absence of RXR is embryonic lethal in mice due to myocardial malformation, it is possible that RXR plays a role in the differentiation of ES cells into cardiomyocytes. Honda et al. found that the number of beating cardiomyocytes was increased significantly following treatment with the agonist PA024 in the absence of serum and that the number was significantly decreased in the presence of the antagonist PA452, suggesting that RXR signaling regulates cardiomyocyte numbers during ES cell differentiation and maybe in normal development [89].

Early development is RA sensitive, yet thyroid hormone Receptor alpha (TRα) is expressed along with the RARs. Loss of TRα in mouse ES cells led to an increase in basal and RA-induced expression [90]. This combined with transient transfection experiments of RA responsive elements showed that TR inhibits RA-responsive gene expression and modulates RA-stimulated neural differentiation in ES cells [90].

Treatment of ES cells with RA induces not only differentiation but also repression of pluripotency genes such as Oct-4. Although there is evidence for direct regulation of Oct-4 expression by RARs in P19 cells, the inhibition of Oct-4 by RA is likely indirect. Treatment of P19 cells with RA induces expression of the orphan receptor COUP-TF, which can bind to a hormone response element in the Oct-4 proximal promoter that overlaps with the LRH-1 element. However, the expression and binding of COUP-TF occurs late in the differentiation process, after Oct-4 has been repressed. Thus, COUP-TFs are not likely to physiological mediator of Oct-4 repression in response to RA treatment [91, 92].

2. GCNF (NR6A1)

In contrast to COUP-TFs the orphan nuclear receptor germ cell nuclear factor (GCNF) is induced early during P19 cell differentiation and thus was a likely candidate for Oct-4 repression. GCNF is involved in regulating early embryonic development and reproduction [93–96]. It is essential for embryonic survival, normal development of the anterior-posterior axis as well as organogenesis [95, 97]. In the adult female, GCNF mRNA was detected in the growing oocytes but not in oocytes in primordial follicles, suggesting a role in oogenesis [94, 98, 99]. It also appears to play a role in spermatogenesis and its expression is restricted to certain stages of spermatogenesis [99]. GCNF-deficient mouse embryos die at 10.5 dpc due to cardiovascular defects and failure to establish the correct chorioallantoic connection [95]. One of the molecular defects in the GCNF KO embryos is an inability to repress and silence the Oct-4 gene [30]. In GCNF knockout embryos Oct-4 expression was present in both the primordial germ cells after gastrulation (normal) and in somatic cells (abnormal). There was also no repression of Nanog in these embryos [100].

GCNF expression is induced in response to RA treatment in P19 and ES cells and it binds to an evolutionarily conserved DR0 element in the Oct-4 proximal promoter [100]. Recombinant GCNF can bind to DNA as a monomer, homodimer, or heterodimer [101]. Dimerization of GCNF is DNA-dependent and is initiated upon binding to a DR0 element. However, endogenous GCNF induced by RA in ES cells and EC cells forms a slower migrating form of GCNF; that was shown not to be a homodimer but instead is composed of a GCNF hexamer [100, 101]. This hexamer is termed the transiently retinoid-induced factor (TRIF), which binds to and represses transcription from the DR0 on the Oct-4 promoter [96, 100, 102]. The expression pattern of GCNF inversely correlates with that of Oct-4 and Nanog in mouse embryos, P19 cells, and ES cells. Generation of GCNF$^{-/-}$ ES cells showed that GCNF is required to repress the expression of Oct-4, Nanog, and Sox2 upon differentiation with RA [100]. This was a direct effect mediated through binding to DR0 elements in the Oct-4 and Nanog promoters; and likely an indirect effect on Sox2, which itself is an Oct-4 target gene [100]. Analysis of the repression mechanism of GCNF showed that it plays an essential role in the repression and silencing of Oct-4 through epigenetic modifications, especially DNA methylation. GCNF binding to the Oct-4 promoter triggers initiation of promoter DNA methylation. GCNF-dependent methylation of the Oct-4 promoter is mediated by recruitment of MBD (methylated CpG binding domain) factors, which previous studies have shown to be components of NURD repression complexes MBD3 and MBD2 and de novo DNA methyltransferases [103, 104]. In addition, GCNF interacts with DNA methyl transferase 3 (DNMT3) and likely recruits them to the Oct-4 promoter [103, 104]. The Oct-4 promoter is hypomethylated and recruitment of MBD3 and MBD2 is lost in GCNF$^{-/-}$ embryos.

Table 1: Summary of involvement of nuclear receptors in mouse ES cell pluripotency and differentiation.

Nuclear receptor	Function
ERRβ	Maintenance of pluripotency and repression of differentiation. Repression of differentiation along specific cell lineages
SF-1	Maintenance of Oct-4 expression in embryonic carcinoma cells
LRH-1	Maintenance of Oct-4 expression in ES cells. Interaction with β-catenin may play role in cell proliferation
DAX-1	May act as a repressor of SF-1, LRH-1, ER, AR, and PR. Conditional KO causes loss of pluripotency and differentiation
RAR	Downregulation of Oct-4. Upregulation of GCNF. Neuronal differentiation
RXR	May play role in differentiation of cardiomyocytes
GCNF	GCNF required for repression of Oct-4, Nanog, and Sox2 upon differentiation with RA. Repression of ES cell phenotype
PPARγ	Required in the early stages of adipose differentiation. Differentiation down osteogenic lineage in siRNA experiments. PPARγ agonist downregulated LIF-mediated self-renewal

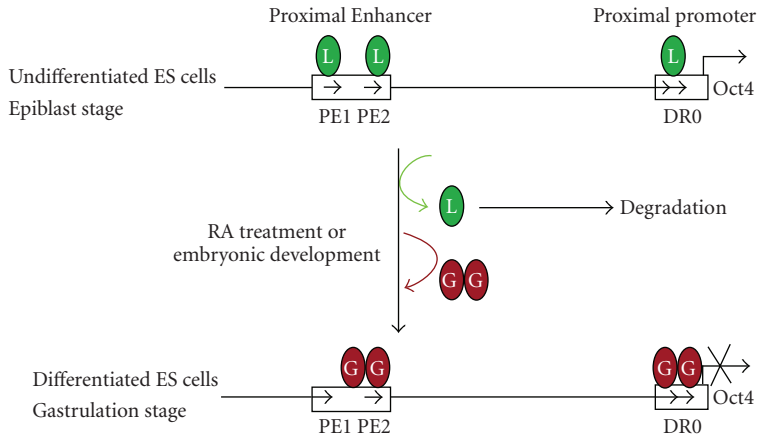

Figure 1: Yin-yang regulation of Oct-4 expression during ES cell differentiation by LRH-1 and GCNF, which compete for the same element. In undifferentiated ES cells LRH-1 binds to elements in the Oct-4 proximal enhancer and proximal promoter to maintain its expression during the very earliest stages of differentiation. As differentiation progresses LRH-1 expression decreases and GCNF expression is induced. At an intermediate point GCNF displaces LRH-1 and represses Oct-4 by recruiting the DNA methylation machinery that ultimately leads to the silencing of Oct-4 expression in somatic cells.

RNAi-mediated knockdown of MBD3 and MBD2 leads to reduced Oct-4 repression. Thus, GCNF appears to initiate repression and leads to the methylation [103, 104]. In MBD3 knockout ES cells, there is still repression of Oct-4 which is likely due to the reduction in the expression of activators such as LRH-1 after RA treatment [105, 106]. However, maintained low-level expression of Oct-4 and hypomethylation of the promoter were observed in the MBD3 KO ES cells treated with RA after six days (unpublished data AJC and PG), which means that precise repression and silencing of Oct-4 requires both GCNF and MBD3.

Thus, GCNF is essential for the repression of pluripotency genes such as Oct-4 and Nanog, and also in the initiation of differentiation where both transcriptional and epigenetic mechanisms play a role in its function (see Figure 1).

2.1. Nuclear receptor involvement in ES cell differentiation

Because of the pluripotent nature of ES cells, many nuclear receptors will, at some stage, play a role in their differentiation to anyone of the 200 cell types found in our bodies. The exact role of each nuclear receptor will depend on the cell type that the ES cells are being differentiated into. An example of the roles of nuclear receptors in ES cell differentiation is the role of the nuclear receptor PPARγ in differentiation of ES cells into adipocytes.

The peroxisome proliferator activated receptor gamma (PPARγ) is expressed in adipose, heart, kidney, spleen, intestine, colon, epithelial cells, and skeletal muscle and has been implicated in the differentiation of numerous cells and

tissues including macrophages, breast, colon, and adipose [107, 108]. Targeted disruption of PPARγ is embryonic lethal and mice die and 10 dpc due to defects in the placental and cardiac development and also displays adipose tissue defects [109]. Rosen et al. showed that PPARγ is required for adipose differentiation. Analysis of PPAR$\gamma^{+/+}$ $\leftrightarrow$ PPAR$\gamma^{-/-}$ chimeric mice revealed that the adipose tissue in these mice derived preferentially from WT cells and not the inserted PPAR$\gamma^{-/-}$ ES cells. Most other tissues had an almost even distribution of cells derived from both WT and PPAR knockout cells. They also found that when PPAR$\gamma^{-/-}$ ES cells were differentiated using a protocol to differentiate them into fat cells, no fat cells developed [110]. Vernochet et al. showed that PPARγ is expressed early in embryoid bodies and in mouse embryos at day E8.5. Addition of RA caused an increase in adipogenesis, and addition of RA and PPARγ ligand caused a further increase. However, upon addition of a PPARγ ligand alone to developing embryoid bodies overexpressing PPARγ, there was no commitment to the adipose lineage. When PPAR$\gamma^{-/-}$ embryoid bodies were differentiated, only the preadipose markers C/EBPγ and C/EBPδ were expressed. Although PPARδ was present it did not compensate for PPARγ in terminal differentiation. They proposed that PPAR is critical only in stages of adipose differentiation but is not required for early differentiation of pluripotent ES cells. The early steps of adipose differentiation are RA dependent and the latter stages are PPARγ dependent [111]. In a recent study, PPARγ expression was knocked down in ES cells using RNA interference. When the cells were induced to differentiate down an adipogenic lineage, they instead differentiated down an osteogenic lineage shown by the expression of the osteoblast markers collagen type 1, osteopontin, Cbfa1, and osteocalcin [112]. An investigation of PPARγ expression during ES cell proliferation and self-renewal showed that the PPARγ agonist 15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2(15d-PGJ2) down-regulated LIF-mediated self-renewal and proliferation and that this PPARγ-mediated regulation occurred via the JAK-STAT pathway [113].

2.2. Perspective

The maintenance of pluripotency and subsequent differentiations of ES cells involves a great deal of complexity. There are undoubtedly multiple mechanisms involved including signal transduction pathways and transcription factors, all of which interact to yield the phenotype of pluripotency, or of a differentiated cell. Nuclear receptors interact with these pathways and can either maintain the pluripotent phenotype, repress the acquisition of a differentiated phenotype, or aid in the acquisition of a differentiated cell type. As nuclear receptors are ligand-activated transcription factors they are part of what is now known as the druggable genome. They are obvious targets to manipulate ES cells in culture with small molecules. Based on genetic models, ligands for LRH-1 or GCNF would be predicted to affect the maintenance or repression of pluripotent gene expression mediated by these factors [56, 100] (see Figure 1). Thus, agonists for LRH-1 or antagonists for GCNF would be expected to maintain ES cell pluripotence and self-renewal, which would be optimum for large-scale culture of ES cells in the absence of LIF for therapeutic purposes. Likewise, LRH-1 antagonists or GCNF agonists would promote the silencing of pluripotency genes like Oct-4 and Nanog, which would be beneficial for differentiating ES cells into target cells. Similarly nuclear receptors can be targeted by small molecules to influence ES cell differentiation along specific pathways, for example, PPARγ agonists could promote osteoblast differentiation of ES cells. The realization of the therapeutic potential of ES cells will be greatly enhanced by the application of strategies that target nuclear receptors, or other components of the druggable genome, to push these cells into the desired cell type. Much of the pioneering works in ES cells has been performed in the mouse and each significant finding and potential target needs to be validated in human ES cells.

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Review Article

Roles of Retinoids and Retinoic Acid Receptors in the Regulation of Hematopoietic Stem Cell Self-Renewal and Differentiation

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Multipotent hematopoietic stem cells (HSCs) sustain blood cell production throughout an individual's lifespan through complex processes ultimately leading to fates of self-renewal, differentiation or cell death decisions. A fine balance between these decisions in vivo allows for the size of the HSC pool to be maintained. While many key factors involved in regulating HSC/progenitor cell differentiation and cell death are known, the critical regulators of HSC self-renewal are largely unknown. In recent years, however, a number of studies describing methods of increasing or decreasing the numbers of HSCs in a given population have emerged. Of major interest here are the emerging roles of retinoids in the regulation of HSCs.

1. INTRODUCTION

1.1. Cell fate decisions of HSCs

HSCs maintain hematopoiesis through fine processes involving cell self-renewal, differentiation, or death decisions (Figure 1). A balance between these choices is required for homeostasis of the blood cell system. Imbalances can result in severe consequences to the health of the individual: bone marrow failure can result from reduced HSC self-renewal or enhanced cell differentiation or death, whereas bone marrow diseases such as leukemia largely result from inhibition of cell differentiation or death of a progenitor cell in addition to enhanced self-renewal of the leukemia-initiating cell.

1.2. Functional self-renewal of HSCs

HSC self-renewal is defined in this review as the retention of the functional capacity of the HSC after cell division. Studies investigating the regulation of HSC self-renewal have predominantly focused on *ex vivo* culture systems, which, while allowing more direct examination of the roles of certain factors in the absence of others, may not provide information as to physiological regulators of HSC self-renewal, especially with regards to microenvironmental influences. The importance of the bone marrow microenvironment in regulating hematopoiesis has been demonstrated by the capacity of in vitro bone marrow stromal cell cultures to support hematopoietic stem cells [1] . To date, however, both in vitro and in vivo studies of the microenvironmental regulators of HSC self-renewal have been relatively few. This is due in part to the complexity of the multicellular stromal cell system in addition to the lack of identification of the HSC-regulatory cells within the stromal cell compartment, also known as the HSC niche. The recent identification that a key component of the in vivo HSC niche is the osteoblast, or bone-forming cells [2, 3], in addition to the observation that a change in the osteoblast niche size has a marked impact on the ability of the HSCs to self-renew, has now opened the field for further investigation in the context of the microenvironmental regulation of HSC self-renewal. The studies reported here have predominantly been performed independent of the HSC niche, thus represent intrinsic, or cell-autonomous, roles in HSC function.

1.3. Retinoids

Vitamin A (retinol) and its derivatives, collectively referred to as retinoids, are essential for normal development and

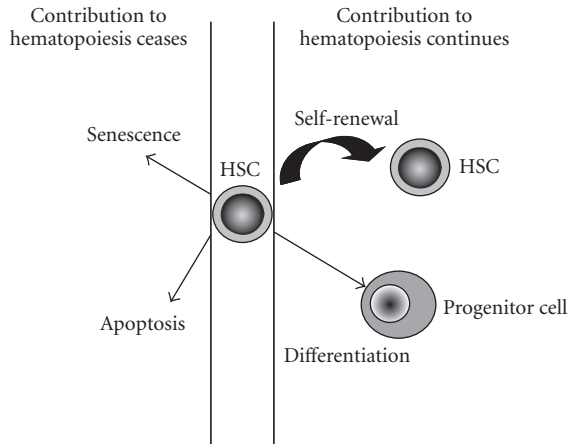

Figure 1: Hematopoietic stem cells (HSCs) undergo fate decisions including self-renewal (resulting in the production of more HSCs), differentiation (producing more mature progenitor cells), senescence, or apoptosis, the latter two resulting in cell death.

homeostasis of vertebrates as shown by their profound effects as morphogens during embryonic development [4] and by their crucial role in the physiology of many organs. The mechanisms responsible for the diverse effects of retinoids have yet to be fully elucidated but are ultimately dependent on the specific binding of retinoid ligands to nuclear receptors, which as ligand-dependent transcription factors regulate complex programs of gene expression in various target cells and tissues.

Retinol is not synthesized by animals, but is obtained from a variety of food sources in the form of carotenoids (from fruits and vegetables) or retinyl esters (from animal sources, especially liver). Retinol is a teratogenic agent: vitamin A deficiency results in multiple and severe developmental defects in many different organs. Paradoxically, excess retinol (hypervitaminosis A) causes many serious developmental defects. These findings not only highlight the importance of retinoids in regulating many developmental processes, but they also emphasize how critical it is to regulate retinoid levels within the body [5, 6].

Retinol is not biologically active, but is metabolized within the body by a series of enzymes into a range of biologically active forms of aldehyde or carboxylic acids [7]. The major aldehyde form, 11-*cis* retinal, is crucial for normal processes involved in vision. The major carboxylic acid form, all-*trans* retinoic acid (ATRA), is required for the regulation of gene transcription by vitamin A [7], and is the form of vitamin A that has roles in hematopoiesis.

1.4. Retinoic acid receptors

The biologic effects of ATRA and other retinoids are mediated by two families of receptors belonging to the nuclear hormone receptor superfamily: the retinoic acid receptor (RAR) and retinoid X receptor (RXR) families [8]. These receptors are encoded by a number of related genes, each of which generates distinct subtypes (designated α, β, and γ), and each subtype has at least 2 different isoforms generated by alternative splicing [9, 10]. The retinoid receptors are highly conserved between species and show complex stage- and tissue-specific patterns of expression, suggesting a molecular basis for the diverse biological effects of retinoids. RAR/RXR heterodimers are the functional units responsible for the transduction of retinoid signals [11], binding to specific retinoic acid response elements (RAREs) present in the promoters of their target genes to regulate transcription [8]. The retinoid receptors have two contrasting roles in the regulation of transcription. When not bound to ligand, the RAR/RXR heterodimers repress transcription. In contrast, in the liganded state, these receptors activate transcription. ATRA preferentially binds to RARs but not RXRs. RARs are specific to the retinoid signalling pathway, whereas RXRs also heterodimerize with other members of the nuclear hormone receptor superfamily. There are very few reports on the roles of the RXRs in hematopoiesis, however, a recent report showed that mice lacking RXRα, which is widely expressed by hematopoietic cells, have normal hematopoiesis in vivo [12].

1.5. The effects of retinoids on hematopoiesis

In hematopoiesis, the best documented action of retinoids is the induction of differentiation of primary leukemic blasts from patients with acute promyelocytic leukemia (APML), and therapies that include ATRA treatment achieve sustained remission in approximately 75% of patients [13].

Numerous studies investigating the effects of ATRA on normal human and murine hematopoiesis reached variable and often contrasting conclusions. Some reports suggested that ATRA enhanced the proliferation of human progenitor cells [14–16], whereas others demonstrated an inhibitory effect on both proliferation and differentiation of both human and murine progenitor cells [17–21]. It must be noted that inhibition of proliferation could be interpreted in different ways depending on the cell type: inhibition of proliferation in maturing cells is associated with cell cycle arrest accompanied by differentiation of the cell, as observed when immature granulocytes differentiate in response to ATRA [22]. In contrast, in the context of immature hematopoietic cells, especially HSCs, which are relatively quiescent cells [23], inhibition of proliferation (or more appropriately, slowing of proliferation) may be associated with maintenance of a primitive state of the cell. This has been observed when immature hematopoietic cells enriched for HSCs were cultured with ATRA [18, 24, 25].

Indeed, these contradictory effects of ATRA in hematopoiesis may be resolved by the recent finding that ATRA has pleiotropic effects on murine hematopoietic cells. In accord with its effects in APML, ATRA was found to be a potent inducer of terminal maturation of normal promyelocytes into granulocytes [24]. However, on more immature populations of hematopoietic cells enriched in hematopoietic stem and progenitor cells (lineage-negative, c-*kit* -positive, Sca-1-positive cells [LKS+]) [26], ATRA exhibited the opposite effect. The addition of ATRA to *ex vivo* liquid suspension media containing cytokines markedly prolonged and enhanced the production of colony-forming

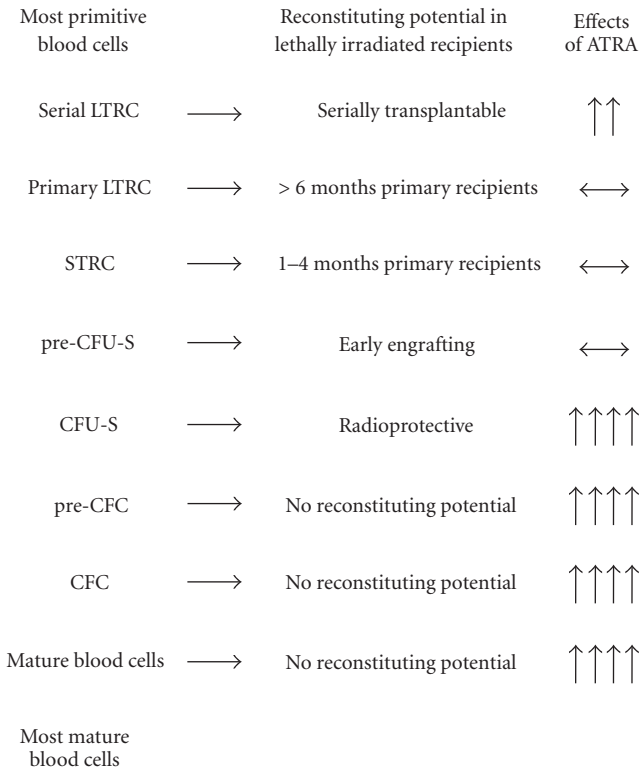

Figure 2: A summary of the effects of ATRA on HSC-containing LKS+ cells. LTRC= long-term repopulating HSCs, STRC = short-term repopulating HSCs, CFU-S = colony-forming unit-spleen, CFC = colony-forming cell. Upward pointing arrows indicate increase in potential, the number of arrows indicates the magnitude of increase, sideways arrows indicate maintenance of potential. Modified from [25].

cells (CFCs) and colony-forming unit-spleen (CFU-S) and maintained pre-CFU-S production from cultured LKS+ [24, 25]. In addition, ATRA enhanced the maintenance of in vivo repopulating HSCs from this cultured cell population [25]. Additional studies demonstrated that ATRA enhanced the self-renewal of serially transplantable HSCs [27]. These effects of ATRA were restricted to a relatively primitive cell population: in contrast to that observed for LKS+ cells, lineage-negative, c-*kit*-positive, Sca-1-negative cells (LKS−), which exhibit CFU-S and CFC potential, but do not contain HSCs [26], differentiated in response to ATRA [24]. A summary of the effects of ATRA on the production of hematopoietic cell types from LKS+ are given in Figure 2.

The different effects of ATRA in hematopoiesis may be due to the cell target, the RAR(s) activated in such cells, or both. We and others have recently examined the expression of the different RARs in purified populations of murine hematopoietic cells and have found that the RARs are differentially expressed in different cell types [27, 28]. LKS+ cells (which contain HSCs and which have increased repopulating potential in response to ATRA) express RARα1, RARα2, RARβ2, RARγ1, and RARγ2 [27]. In contrast, LKS− cells (which do not contain HSCs and which differentiate in response to ATRA treatment) have similar RAR expression to LKS+ but do not express RARβ2 or RARγ1 [27]. Additional data using RAR knockout mice have revealed distinct roles for the RARs in hematopoiesis.

1.6. Roles of retinoic acid receptors in hematopoiesis

Previous studies have investigated the role of pharmacological levels of ATRA in cultured hematopoietic cells. Such studies do not, however, provide insight of the physiological roles of the RARs in hematopoiesis. The importance of RARα in granulopoiesis is demonstrated in APML patients, whose leukemic cells have aberrant chromosomal translocations that result in fusion of the RARα gene with other genes, such as PML and PLZF [29]. These fusion gene products ultimately result in a block in promyelocytic differentiation, resulting in leukemia. Additional support for physiological roles of RARs in hematopoiesis comes from studies of mice either given a vitamin-A-deficient diet [30] or fed with a pan-RAR antagonist [31], who exhibit a dramatic increase in myeloid cells in bone marrow, spleen, and peripheral blood. However, while this underscores the importance of RARs in hematopoiesis, it does not discriminate between roles of each of the different RAR subtypes in hematopoiesis. ATRA, the most widely used retinoid in therapeutic applications at present, activates all three RAR subtypes. Each of the three RARs were previously considered to have similar effects in different organs, however recent data using mouse models or RAR-specific ligands are now emerging to challenge and even disprove this concept. Some studies on RAR knockouts have also begun to delineate the different roles of the RARs in hematopoiesis, and are discussed below.

1.7. Studies of HSCs in RAR-knockout mice

Mice null for RARα, RARβ, or RARγ all survive birth, but both RARα- and RARγ knockout mice exhibit early lethality [32–34]. Subsequent double null mice generated from these RAR subtype null mice have more profound defects, and die at the latest by 12 hours after caesarean delivery at E18.5 [35]. The triple null mouse has not been reported to date.

Previous reports on hematopoiesis in RAR null mice have been two separate studies on granulocyte development in RARα1 and full RARα knockouts. Both demonstrated that RARα is not an important physiological regulator of granulocytes [28, 36]. Mice lacking both RARα1 and RARγ did exhibit a block in in vitro terminal differentiation into granulocytes, but this was not observed in vivo, suggesting that in vivo compensatory mechanisms in these double null mice restore normal granulopoiesis [28].

Both RARα and RARγ are the most widely expressed in hematopoiesis, including HSCs, hence we have investigated the HSC content in 8-week-old RARα and RARγ null mice. The RARα null mice had normal HSC content, as assessed by limiting dilution analysis [27]. In contrast, whole bone marrow obtained from RARγ null mice had a 3.3-fold reduction in the number of long-term repopulating HSCs in primary transplant recipients compared to that of their wild-type littermates [27]. Interestingly, bone marrow from RARγ heterozygous mice had 2-fold fewer HSCs than the wild-type

littermates, further highlighting the importance of RARγ signalling in the regulation of HSCs [27]. The reduced numbers of HSCs observed in RARγ null bone marrow was accompanied by increased numbers of more mature progenitor cells (CFU-S and CFCs), suggesting that RARγ is critical for maintaining a balance between HSC self-renewal and differentiation [27].

The response of enriched populations of HSCs (LKS+) obtained from RAR mutants to ATRA treatment was also monitored in *ex vivo* cultures. HSCs obtained from RARα null mice retained a normal response to ATRA treatment, as measured by prolonged and enhanced cell proliferation and their ability to reconstitute mice after 14 days of *ex vivo* culture [27]. In contrast, ATRA-treated LKS+ isolated from RARγ null mice had markedly impaired proliferation and did not reconstitute mice after 14 days of culture [27]. Collectively, these studies demonstrate that ATRA-induced HSC self-renewal requires RARγ signalling.

RARγ has therefore been identified as being a key regulator of HSC self-renewal: activation of RARγ enhances self-renewal, whereas inactivation of RARγ enhances HSC differentiation, resulting in increased numbers of more mature progenitor cells.

The recent generation of RAR-specific ligands [37] has made future studies of the effects of gain of function of different RARs on hematopoietic cells possible, and will likely lead to further therapeutic applications for retinoids in hematopoiesis.

1.8. Regulators of retinoid signaling: aldehyde dehydrogenase family

Little is known about the regulators of RARs in organogenesis. One major way of regulating activity of the RARs is by altering the availability of the biologically active retinoic acid ligands. A series of sequential enzymes with different specificities regulate the production of retinoic acid from retinol [7]. The important enzymes involved in the NAD-dependent oxidation of the aldehyde forms of vitamin A into ATRA and 9-*cis* retinoic acid are those of the aldehyde dehydrogenase (ALDH) family.

Like RARs, the ALDHs are highly conserved amongst vertebrates. There are numerous members of this family, not all of which can use retinoids as substrates. The cytosolic class 1 enzymes, retinaldehyde dehydrogenase 1 (RALDH1), RALDH2, and RALDH3, are the ALDH forms important for the conversion of retinal into retinoic acid forms [7]. All three enzymes are expressed differentially in embryogenesis and throughout later mouse organogenesis [38].

RALDH1 (also known as ALDH1, ALDH1A1, RalDH1, and Ahd2) is expressed in both embryonic and adult tissues and is capable of converting both all-*trans* retinal and 9-*cis* retinal into their respective carboxylic acid forms, hence providing ligands for both the RARs and RXRs [7]. *Raldh1* knockout mice are viable, with no apparent defects in growth or survival [39]. RALDH2 (also known as ALDH1A2) is more important embryonically, and *Raldh2* knockout mice die by E10.5, exhibiting multiple defects and a block in embryonic retinoic acid synthesis [40]. Interestingly, this lethal phenotype can almost be completely overcome by maternal retinoic acid administration, demonstrating that the defects in these mice are predominantly due to lack of retinoic acid. RALDH3 (also known as ALDH1A3 and ALDH6) is expressed in the ventral retina in the developing eye, olfactory regions, and other organs [38, 41]. *Raldh3* knockout mice are born, but die from respiratory distress within 10 hours of birth [42]. To date there have been no reports on hematopoiesis in any of the *Raldh* mutants.

Table 1: Expression of RARs and ALDH1 in murine HSCs and progenitor cells. Positive expression is indicated by (+) and negative expression by (−). Summary of data is obtained from references [27] and [44].

	Hematopoietic cell population	
	HSCs	Progenitors
RARα1	+	+
RARα2	+	+
RARβ2	+	−
RARγ1	+	−
RARγ2	+	+
ALDH1	+	−

A series of recent reports have shown that both murine and human primitive HSCs and progenitors are contained within the lineage-negative, ALDH high fraction, and can be isolated based on ALDH activity [43–47]. In contrast, the population of murine hematopoietic cells lacking ALDH1 expression did not contain HSCs [44]. These data therefore not only reinforce the importance of RAR signalling in HSCs, as shown in our recent studies, but also provide evidence that HSCs themselves are capable of generating ATRA and 9-*cis* retinoic acid from retinal. A summary of the expression of RARs and ALDH1 in murine HSCs and progenitor cells is given in Table 1.

One study to date has reported that inhibiting ALDH1 in human hematopoietic stem/progenitor cells in vitro induces their expansion and prevents their differentiation [48]. Further studies of the roles of the aldehyde dehydrogenases in the regulation of HSCs are therefore of interest.

1.9. Therapeutic applications of retinoids for HSCs

Given that the retinoid pathway is highly conserved between human and mouse, it is now of interest to determine whether ATRA has the same effects on human HSCs. Some obstacles to these translational studies are that (1) the population enriched for human HSCs is much more heterogeneous than the one that can be obtained for murine HSCs, which presents potential problems given the pleiotropic effects of ATRA and (2) the NOD/SCID mouse repopulating assay, which to date is the best small animal model for in vivo transplantation studies of human HSCs, may not be reflecting true HSC activity of the cell population [49]. Nevertheless, a recent report demonstrated that ATRA could support the expansion of SCID-repopulating cells (SRC), human hematopoietic cells that are capable of repopulating NOD/SCID mice [50]. These effects of ATRA on human

hematopoietic stem/progenitor cells relied on the presence of a stromal feeder layer, but did not require contact between the stromal cells and HSPCs [50]. It is therefore likely that ATRA induced the secretion of substances from the stromal cells that were capable of expanding HSPC. The potential use of retinoids to expand human HSCs for therapeutic purposes therefore warrants further investigation: in particular, given its profound roles in murine HSCs, it is of interest to determine the effects of specifically activating RARγ in these cells.

2. CONCLUSION

It is becoming apparent that the roles of retinoids and their receptors in hematopoiesis are complex, having pleiotropic effects depending on the hematopoietic target cell. In contrast to its potent differentiation-inducing effects on granulocyte progenitor cells, ATRA enhanced the self-renewal of HSCs. These different effects are likely due to the effects of the distinct RARs in hematopoiesis. RARα has a clear role in enhancing granulocyte maturation, as demonstrated by both its involvement in APML [29] and also the potent effects of an RARα-specific ligand on granulocyte differentiation [22]. We also recently reported that an RARα-specific ligand enhanced the mobilization of murine hematopoietic stem and progenitor cells into the peripheral blood for transplantation purposes via increasing the numbers of immature granulocyte progenitors in vivo [51]. Interestingly, these effects were not seen when ATRA was used in place of the RARα-specific ligand, perhaps due to contrasting effects obtained by activating all three different RARs concurrently, a possibility that further adds to the complexity of the effects of retinoids in hematopoiesis. In contrast, RARγ is a major regulator of HSC self-renewal: gain of function of RARγ enhances HSC self-renewal, whereas loss of function of RARγ promotes differentiation of HSCs [27]. These distinct effects of the RARs in hematopoiesis suggests that, in the future, therapeutically targeting the RARs via RAR-specific ligands may have a more profound effect on the target cell than by using the pan-RAR agonist ATRA. Such studies will also permit further delineation of the roles of the RARs in HSC biology.

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Review Article

PPARs and Adipose Cell Plasticity

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Due to the importance of fat tissues in both energy balance and in the associated disorders arising when such balance is not maintained, adipocyte differentiation has been extensively investigated in order to control and inhibit the enlargement of white adipose tissue. The ability of a cell to undergo adipocyte differentiation is one particular feature of all mesenchymal cells. Up until now, the peroxysome proliferator-activated receptor (PPAR) subtypes appear to be the keys and essential players capable of inducing and controlling adipocyte differentiation. In addition, it is now accepted that adipose cells present a broad plasticity that allows them to differentiate towards various mesodermal phenotypes. The role of PPARs in such plasticity is reviewed here, although no definite conclusion can yet be drawn. Many questions thus remain open concerning the definition of preadipocytes and the relative importance of PPARs in comparison to other master factors involved in the other mesodermal phenotypes.

1. INTRODUCTION

Adipose tissues have long been associated with the invasive prevalence of obesity and metabolic disorders. Recent advances have focused the attention on the presence of intriguing cells able to differentiate towards various phenotypes and recall the old use of fat in plastic and reconstructive surgery [1–3]. Two rather distinct medical and scientific domains share these perspectives but irrespective of field, a good understanding of adipogenesis is absolutely requisite to manipulating and controlling adipocyte differentiation. This point is emphasized by the fact that adipocyte differentiation belongs to the mesenchymal stroma or stem cell hallmark. We thus should consider that any of these cells can acquire the adipocyte phenotype depending on its environment. In this view, peroxysome proliferator-activated receptor (PPAR) family of transcription factors appears to be a key and unavoidable actor.

After a brief overview on adipose tissues, this review will focus on the importance of PPARs and PPARγ in particular in adipose-derived cell phenotype and plasticity.

2. ADIPOSE TISSUES: WHAT ROLE DO PPARS PLAY?

2.1. Adipose tissue or adipose tissues?

Three functionally different types of adipose tissues are described in mammals: brown adipose tissue (BAT), white adipose tissue (WAT), and bone-marrow adipose tissue (BMAT) [4–6].

BAT and WAT participate differently in energy balance and homeostasis. BAT is heavily involved in nonshivering thermogenesis (cold and diet-induced thermogenesis). In contrast, WAT is mainly involved in energy storage and is now considered an endocrine organ [4–7]. Brown and white adipocytes similarly display lipogenesis, triglyceride accumulation, and lipolysis, but brown adipocytes are also specialized in energy dissipation in the form of heat. These cells are smaller than white adipocytes, contain abundant mitochondria and highly express the uncoupling protein-1 (UCP-1) [8]. This protein, by uncoupling ATP synthesis from the respiratory-chain function, subsequently promotes the dissipation of energy as heat [9]. It is noteworthy that only 40%–60% of the whole cell population in BAT as well as in WAT is composed of mature adipocytes. Endothelial, haematopoietic cells, and adipocyte precursors, the so-called preadipocytes, or adipose-derived stromal cells (ASCs) are also found in adipose tissues [10]. It has long been known that adipocyte precursors are present throughout adulthood and can proliferate and/or be recruited depending on physiological or physiopathological situations [7]. BAT and WAT develop at specific and different locations and WATs various locations also determine its different metabolic and molecular features [11, 12].

In contrast to brown and white adipocytes, the role of medullar adipocytes has been poorly investigated and is not well-understood [5]. The number and size of adipocytes seem to be inversely correlated to haematopoietic activity in bone marrow based on the physiological or physiopathological situation. Nevertheless, it is noteworthy that many scattered adipocytes are observed in active haematopoietic bone marrow. Highly similar gene expression has been described between human subcutaneous and medullary mature adipocytes, suggesting that medullary adipocytes may share some of the functions exhibited by subcutaneous and visceral adipocytes [13].

Beside the physiological anatomy of adipose tissues, ectopic location of adipocytes is also observed in degenerative tissues. The origin of these adipocytes infiltrating the degenerative tissues is still unclear.

2.2. PPARs and white adipocyte differentiation

As usual in any differentiation program, a subtle balance and interconnection exist between cell cycle and commitment to adipocyte differentiation. In this context, overexpression of both C/EBPα and PPARγ, mediates the cell cycle arrest after clonal expansion. Indeed, PPARγ induces cell cycle withdrawal by the inhibition of the transcription factor E2F/DP DNA-binding activity via downregulation of the protein phosphatase 2A (PP2A) [14]. In the opposite, E2Fs trigger clonal expansion, and terminal adipocyte differentiation through regulation of PPARγ expression [15]. In the same manner, p107 protein belonging to retinoblastoma family is also involved in regulating PPARγ and its recovery into deficient cells reduces adipocyte differentiation through the downregulation of PPARγ activity [16]. The potency of PPARγ and its interplay with cell cycle is illustrated by a report studying liposarcoma cells. Treating cells from these aggressive tumours with ligands for both PPARγ and the retinoid X receptor (RXR) forces their terminal differentiation [17]. Indeed, the antiproliferative activity of PPARγ is now investigated to limit and treat tumour cell proliferation although several PPARγ ligand-mediated antiproliferative effects act through a complexity of PPARγ-independent mechanisms [18]. Nevertheless, this antigrowth effect seems to be specific to cell type because PPARγ activation promotes the proliferation of neural stem cells [19], instead of their differentiation.

The crucial role for PPARγ as a dominant regulator of white-adipocyte differentiation is now well documented and largely reviewed [20, 21]. It interplays with several other nuclear factors such as CAAT Enhancer Binding Protein (C/EBP) and Sterol regulatory element-binding proteins (SREBP) families and numerous coactivators or corepressors. The use of genetic ablated cells demonstrates that while the lack of C/EBPα can be overcome by the overexpression of PPARγ, the opposite does not hold true that PPARγ is absolutely required for the adipogenic program [22]. During this program, the expression of both factors is interdependent not only in promoting adipocyte differentiation but also in sustaining and maintaining the fully differentiated adipocyte phenotype. Any signal able to modulate one or the other of both transcription factors subsequently induces change in adipocyte differentiation. Thus, insulin/IGF-I signalling modulates adipogenesis by the upregulation of PPARγ expression via the insulin/IGF-I receptor-AktSH2-B-Foxo1-pathway [23].

Whereas PPARγ was described as a factor involved in white adipocyte differentiation, PPARα and δ were described for their role in fatty acid oxidation in several tissues including adipose tissue [24–27]. However, gain-of-function experiments suggest that both PPARδ and PPARγ isoforms are required to facilitate maximal lipid accumulation and differentiation during white adipogenesis [28].

As PPARγ stimulates white adipogenesis, its negative control inhibits this process. In this way, hypoxia-mediated inhibition of adipogenesis can be partly explained via the repression of PPARγ expression by the hypoxia-inducible factor-1 (HIF-1)-regulated gene DEC1/Stra13, a member of the Drosophila hairy/enhancer of split transcription repressor family. Similarly, the inhibition of adipogenesis by cytokines is mediated via the repression of PPARγ function through kinase cascade [29, 30]. It has also been demonstrated that the constitutive expression of GATA-2 and GATA-3 resulted in a decrease in PPARγ expression and a consequent inhibition of adipocyte differentiation [31].

2.3. Preadipocytes, circulating cells, and PPARs

The first report in this field was published in 2005. Its authors described a circulating human cell population able to differentiate towards adipocytes when cultured under adipogenic conditions. Moreover, these human progenitors engrafted and formed adipose tissue following injection into SCID mice [32]. This report is now supported by a recent investigation suggesting that new fat cells arise not only from resident precursor cells within the tissue, but also from other sources, such as BM-derived circulating progenitor cells. One difference between both reports is the nature of the newly-formed adipocytes. In the first study, unilocular white adipocytes were observed whereas in the second, mutlilocular cells, expressing low levels of UCP1, were detected. In this work, the appearance of bone-marrow-derived adipocytes in the fat pad is triggered by PPARγ ligand [33]. No explanation is proposed for this effect of PPARγ ligand, although it appears to be at least partly a specific mechanism because this phenomenon is not totally reproduced by a high fat diet. Obviously, the underlined mechanisms and the relevance of such observations need further investigation, but could participate in the ectopic emergence of fat cells through the local recruitment of circulating adipocyte progenitor cells.

2.4. Role of PPARs in white and brown adipocytes

The relationship between brown and white fat is complex because besides the typical interscapular brown adipose tissue, an extensive analysis of the different fat deposits revealed that

scattered brown adipocytes are present in any white adipose depot also in primates [34, 35]. Furthermore, brown fat can extensively change into white-like fat and conversely, according to physiological or physiopathological situations and the species [36]. These properties led us to use the term "plasticity of adipose tissue." The main pitfall is that a lack of uncoupling protein-1 (UCP-1) expression is taken as the gold standard for white fat and no positive marker is yet characterized to clearly identify white adipose phenotype. Thus, with the present knowledge, it is not possible to determine whether brown adipocytes can be transformed into white-like adipocytes with different metabolic properties (termed masked brown adipocyte) or into true white fat cells. The establishment of different preadipocyte cell lines and the use of primary culture favor the existence of distinct precursor cells [4]. First depending upon the origin of cultured cells (i.e., from brown or white fat) a corresponding brown or white mature phenotype is obtained [37]. Second, genetic manipulations allowing irreversible labelling of brown adipocytes show that they do not convert into white adipocytes during normal mouse development [38]. Third, no differentiation of pluripotent mouse embryonic stem cells into brown adipocytes even after PPARγ activation take place whereas white phenotype can be easily obtained [39]. In this regard, it is noteworthy that brown adipocyte is the only cell type that coexpresses high levels of the three PPAR subtypes. Furthermore, PPARα and PPARγ are strictly subjected to opposite regulation by retinoids in brown fat, supporting the notion of specific physiological roles of each transcription factor in controlling brown fat differentiation and thermogenic activity [40]. A further step was undertaken when PGC-1, for PPARγ coactivator 1, was identified in brown fat. Initially claimed as a specific factor to brown fat, it was proposed to be a coactivator involved in brown versus white adipocyte differentiation. This coactivator has been shown to control a subset of genes involved in mitochondrial activity and biogenesis [21, 41]. This is consistent with the adenovirus-mediated expression of human PGC-1α that increases the expression of UCP1, respiratory chain proteins, and fatty acid oxidation enzymes in human adipocytes differentiated in primary culture [42]. Nevertheless it appears that this protein displays numerous functions in many tissues, all of them related to mitochondrial function [43]. Other factors have been proposed to be central switches for white to brown adipocyte differentiation, including the retinoblastoma protein [44] and the corepressor RIP140 [45].

3. PPARS AND MESODERMAL FATES

Although, it is well admitted that adipose cells arise from mesodermal origin, the exact origin of adipocyte precursors is not well defined as illustrated by the recent work on circulating adipose precursors [32]. Recently, it appears that cells named previously preadipocytes can differentiate towards various mesodermal origins [46–48]. These cells were thus named adipose derived stromal cells (ADSC). Conversely, it seems thatadipocyte phenotype could be obtained from different cells including myoblasts [49]. PPARs may thus be involved in the preferential cell commitment towards one or the other lineage and/or in maintaining pluripotency. These aspects are discussed in the second part of this review.

3.1. Adipocyte versus osteogenic potential

Many reports have demonstrated that adipocytes and osteoblasts share a common precursor. Osteoblastic genes are expressed in cell lines able to differentiate towards adipocyte. Bipotent cells with features specific to both osteoblasts and preadipose cells have been cloned from bone marrow [50] and more recently from human adipose tissue [47, 48, 51, 52]. Recently, Birk et al. have shown that the 3T3-F442A preadipocyte clonal cell line differentiate into osteoblasts [53]. It has also been reported that stromal colonies, either undifferentiated or differentiated into mature adipocytes, are able to give rise to osteogenic cells when transplanted in intraperitoneal diffusion chambers. These observations suggest that stromal cells, first differentiated along the adipogenic lineage, are able to dedifferentiate and then to redifferentiate in an osteoblastic phenotype.

Several transcription factors, such as PPARγ and Runx2, have been proposed as playing a critical role in the commitment of bipotent stem cells towards the adipogenic or the osteogenic lineages [54]. However, it has to be noted that the effect of genes of interest are often investigated in different cell lines already committed in either one or the other lineage, making dubious their role in the regulation of the commitment of a multipotent stem cell. For example, ectopic overexpression of adipogenic transcription factors such as PPARγ induces transdifferentiation of mouse osteoblastic MC3T3-E1 cells into mature adipocytes [55]. A unique investigation really addressed the issue of the commitment of stem cells on embryonic stem (ES) cells. In this report, the authors transiently suppressed PPARγ expression. This genetic manipulation directs ES cells into an osteoblastic lineage suggesting that PPARγ can be considered a proadipogenic as well as an anti-osteoblastic factor [56]. The role of the transcription factor deltaFosB has been investigated in further detail. Overexpression of deltaFosB under the control of a promoter-driving transgene both in osteoblasts and adipocytes led to increased bone mass and decreased adipocyte formation. Given the assumption of a reciprocal development of both lineages, it has been proposed that differentiation into one cell type could be a consequence of the action of deltaFosB in the other [57]. More recently, elegant experiments from the same group generating transgenic mice that express deltaFosB in a bone-specific manner, led the authors to conclude that the change in osteoblast and adipocyte differentiation results from independent cell-autonomous mechanisms [58]. Therefore, factors playing a role in the switch of stem-cell differentiation towards the adipogenic or osteogenic lineages remain to be definitively identified. The 14-3-3-binding protein, TAZ (transcriptional coactivator with PDZ-binding motif), which coactivates Runx2-dependent gene transcription while repressing PPARγ-dependent gene transcription could be also involved [59].

3.2. Myoblast and Preadipocyte

The deciphering and understanding of myogenesis is far beyond our current understanding of adipogenesis, but both programs are closely linked by their mesodermal origin and the well-described emergence of adipocytes in denervated muscle. From such observations, the question of a putative role of PPARs in directing cell fate towards one of the differentiation programs seems reasonable. As described for fibroblasts, overexpression of PPARγ or activation through its ligands in satellite cells transdifferentiates myoblasts towards the adipogenic phenotype. This characteristic is shared by the other key adipogenic factor, C/EBPα [60]. This transdifferentiation was observed for myoblasts in all mammals and during the whole development [49]. In addition, the activation of PPARδ abolishes the development of multinucleated myotubes while inducing the expression of PPARγ gene. Loss and gain function experiments are consistent with a role for PPARδ as an inducer of transdifferentiation into adipocyte-like cells, which precedes and triggers PPARγ expression [61].

On the other hand, PPARγ can be controlled by Wnt proteins and especially Wnt 1 and Wnt-10b that govern adipogenesis. Indeed, PPARγ as well as C/EBP proteins is inhibited by Wnt signalling, which in turn maintains preadipocytes in an undifferentiated state. When Wnt signalling in preadipocytes is prevented by the overexpression of Axin or dominant-negative T-Cell Factor-4 (TCF4), these cells differentiate into adipocytes. These results are strengthened by the fact that the lack of Wnt signalling also induces transdifferentiation of myoblasts into adipocytes [62]. However, the relationship between Wnt signalling and PPARγ seems to be complex because the impairment or activation of Glycogen Synthase Kinase-3β (GSK3β)/β-catenin only affects a subset of PPARγ-dependent genes [63].

Finally, it has been suggested that myostatin, a potent negative regulator of skeletal muscle growth member of the transforming growth factor beta (TGF-β) family, blocks adipocyte differentiation via down regulation of PPARγ in 3T3-L1 [64]. However, recent data reports that in pluripotent C3H10T1/2 cell line, myostatin treatment may promote the differentiation of multipotent mesenchymal cells into the adipogenic lineage and inhibit myogenesis [65, 66].

3.3. Preadipocytes and macrophages

Analysis of the literature lined out that adipocyte and monocyte/macrophage lineages have many features in common including aP2 and PPAR expression. More surprisingly, we demonstrated that preadipocytes efficiently phagocytoze yeasts and apoptotic bodies in a similar manner, albeit to a lesser extent, than specialized phagocytic cells such as macrophages [67, 68]. This suggests the involvement of adipocyte progenitors in tissue remodelling and plasticity through the discarding of apoptotic bodies. A profiling analysis between adipocyte and macrophage lineages expanded the known similarities between these cell phenotypes [69]. Finally, preadipocytes can be very rapidly converted into macrophage-like cells when injected into the peritoneal cavity, considered a likely environment for supporting macrophage phenotype [69]. The rapidkinetics of change suggests a transdifferentiation process and/or a stronger lineage relationship between adipocyte progenitors and macrophages than expected. Since only 80% of the macrophages come from bone marrow in obesity [70], one could thus postulate that the remaining 20% of adipose-tissue-resident macrophages derive from preadipocytes. According to the importance of PPARγ to direct cells towards adipocyte differentiation, it is reasonable to wonder if PPARs could be involved in such plasticity.

PPARγ is expressed at low levels in circulating monocytes and its expression increases significantly upon differentiation to macrophages, including in the foam cells of atherosclerotic lesions. Numerous studies have thus focused on its role in macrophage metabolism and activation and its possible involvement in the process of atherosclerosis. These studies have provided evidence supporting a role of PPARγ in both lipid uptake and lipid efflux pathways in macrophages [71]. The fact that PPARγ could influence both lipid uptake and efflux raised the question of whether the net effect of PPARγ ligands on macrophages within an atherosclerotic lesion would be to promote or impede foam cell formation. PPARγ is not essential for myeloid development or for mature macrophage functions, such as phagocytosis and inflammatory cytokine production [72]. It is also a negative regulator of macrophage activation and inhibits macrophage production of inflammatory cytokines, although some of this activity may not be mediated by PPARγ [73]. In addition, PPARγ agonists inhibit foam-cell formation in vivo [74]. However, such an effect could be cell-type dependent [75]. Although PPARγ seems fundamental in preadipocyte and macrophage biology, adipocytes and foam cells are probably distinct cell types and thus far no study has described a potential role of PPARγ in the differentiation of preadipocytes into macrophage-like cells.

4. CONCLUSION

The importance and the role of PPARs in adipogenic process is definitively demonstrated and brought numerous new insights in our understanding of adipogenesis but many questions remain open concerning the definition of preadipocytes and the relative importance of PPARs compared to other master genes involved in other mesodermal phenotypes. Altogether, the negative regulation of several master proteins involved in other mesodermal differentiation programs suggests that at least PPARγ can play its role only when all negative regulators are missing. This could suggest that preadipocyte status results from a default pathway.

Similarly, the tissue origin of preadipocytes is now challenged and needs to be clarified because it can help to build a more dynamic view of adipose tissue development and the role of PPAR proteins.

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Review Article

Energy Balance, Myostatin, and GILZ: Factors Regulating Adipocyte Differentiation in Belly and Bone

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Peroxisome proliferator-activated receptor gamma (PPAR-γ) belongs to the nuclear hormone receptor subfamily of transcription factors. PPARs are expressed in key target tissues such as liver, fat, and muscle and thus they play a major role in the regulation of energy balance. Because of PPAR-γ's role in energy balance, signals originating from the gut (e.g., GIP), fat (e.g., leptin), muscle (e.g., myostatin), or bone (e.g., GILZ) can in turn modulate PPAR expression and/or function. Of the two PPAR-γ isoforms, PPAR-γ2 is the key regulator of adipogenesis and also plays a role in bone development. Activation of this receptor favors adipocyte differentiation of mesenchymal stem cells, while inhibition of PPAR-γ2 expression shifts the commitment towards the osteoblastogenic pathway. Clinically, activation of this receptor by antidiabetic agents of the thiazolidinedione class results in lower bone mass and increased fracture rates. We propose that inhibition of PPAR-γ2 expression in mesenchymal stem cells by use of some of the hormones/factors mentioned above may be a useful therapeutic strategy to favor bone formation.

1. INTRODUCTION

The peroxisome proliferator-activated receptor gamma (PPAR-γ) family of transcription factors belongs to the nuclear hormone receptor subfamily of transcription factors that can bind to specific DNA response elements in the regulatory regions of target genes. Other isotypes of this family include PPAR alpha (PPAR-α) and PPAR beta/delta (PPAR-β/-δ). Each of these isotypes is encoded by a different gene and has different functions and different tissue distribution [1]. Many excellent reviews have been published recently on the regulation of nuclear receptors belonging to the PPAR family [2–12] and the reader is referred to one of these reviews for an overview on these nuclear receptors.

The current review more narrowly focuses on PPAR-γ2, its role in bone formation, and its regulation by the energy state. Bone, like other tissues in the body, requires a positive energy balance to grow. However, even with a positive energy balance, bone progenitor cells can differentiate into osteoblast, adipocyte, or muscle cells. PPAR-γ2 is a key regulator of this differentiation step in bone marrow progenitor cells. Tissues in the body important in regulating this energy balance include skeletal muscle, adipocytes, and liver (see Figure 1). The crosstalk between these target tissues occurs via both central nervous system (CNS) output and peripheral hormones from enteric, pancreatic, or adipocytic sources. Studies from our laboratories have focused on enteric hormones like glucose-dependent insulinotropic peptide (GIP), adipocytic hormones like leptin, and skeletal muscle-derived factors such as myostatin in addition to transcriptional regulators such as the glucocorticoid-induced leucine zipper (GILZ) in the regulation of osteoblast/adipocyte differentiation from bone marrow progenitor cells. The impact of changes in the organism's energy balance on mesenchymal stem cell differentiation will be discussed in more detail below.

2. PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR

PPAR-γ is highly expressed in adipose tissue although it is also expressed in other tissues including skeletal muscle,

intestine, endothelium, prostate, and white blood cells [13]. The gene for PPAR-γ is localized on chromosome 3 [13] and there are two protein products, PPAR-γ1 and PPAR-γ2, which are isoforms transcribed from the same gene with different promoter usage [14, 15]. PPAR-γ2 is predominantly expressed in adipose tissue, while PPAR-γ1 is more widely expressed [10]. Of these two isoforms, PPAR-γ2 is a key regulator of adipogenesis [16–18] and is expressed at an early stage of the adipogenesis program [19]. PPAR-γ2 can activate a battery of genes necessary for lipid metabolism, including lipoprotein lipase (LPL) [20], phosphoenolpyruvate carboxykinase (PEPCK) [21], fatty acid-binding and transport proteins [22], and stearoyl-CoA desaturase-1 (SCD-1) [23]. Functional PPAR response elements (PPREs) have been identified in the promoter regions of these genes. In addition, activation of PPAR-γ2 by its ligand [24, 25] induces cell cycle withdrawal and terminal adipocyte differentiation in a variety of mesenchymal cell lines [26–29]. Thus, the pivotal regulatory role that PPAR-γ2 plays in adipocyte differentiation is recognized by its early and tissue-specific expression [19] and its ability to direct fibroblasts and myoblasts to differentiate into adipocytes when it is ectopically expressed in these cells [28, 30].

Most importantly, a recent in vivo study has demonstrated that PPAR-γ insufficiency in mice (PPAR-$\gamma^{+/-}$) results in a dramatic decrease (by 50%) in adipogenesis with a concomitant increase in osteogenesis through osteoblast formation from marrow progenitors [31]. This study suggests the possibility of interrupting the PPAR-γ pathway as a novel treatment of osteoporosis.

3. MESENCHYMAL STEM CELLS

Mesenchymal stem cells (MSCs) are multipotent cells that, under appropriate culture conditions, can differentiate into multiple cell lineages, including osteoblasts, myoblasts, and adipocytes [32–35]. Considerable evidence has shown that the commitment between osteoblast and adipocyte lineages from MSCs is reciprocal, that is, when the adipogenic pathway is blocked, MSCs enter the osteogenic pathway, and vice versa [36–40]. Increased marrow adipogenesis negatively impacts bone formation because mesenchymal precursor cells are directed towards the adipocyte lineage rather than to the osteoblast lineage [41, 42]. Marrow adipocytes can also inhibit osteoblast proliferation in vitro, and adipocytes secrete factors such as IL-6 and TNFα [43] that stimulate the differentiation of the bone-resorbing cells, osteoclasts [44]. The negative impact of marrow adipogenesis on bone health is further indicated by the fact that bone formation rate is inversely correlated with adipocyte number in bone biopsies of adult men and women [45], and women with osteoporosis have higher number of marrow adipocytes than those with healthy bone [46].

Clinically, much recent attention has focused on drugs belonging to the thiazolidinedione class. These medications (e.g., rosiglitazone and pioglitazone) are PPAR agonists used to treat patients with diabetes mellitus, and have recently been associated with impaired bone quality, increased marrow fat, and increased fracture rates [47, 48].

Understanding the regulators of MSC differentiation between fat and bone has gained increasing importance with increasing human longevity since, as humans age, the number of adipocytes increases and the number of osteoblasts decreases resulting in weakened bone, age-related osteoporosis, and fragility fractures. Because of the importance of PPAR-γ2 in MSC differentiation into adipocytes or osteoblasts, we will briefly discuss some of the regulators of the PPAR-γ2 receptor.

4. REGULATION OF THE PPAR-γ RECEPTOR

Regulation of the PPAR-γ receptor activity can occur via (1) changes in receptor expression levels or (2) changes in transcriptional activity (see Figure 2).

A number of transcription factors can either positively or negatively modulate PPAR-γ receptor expression in adipocytes [49]. Major transcription factors activating PPAR-γ receptor expression include CCAAT/enhancer-binding protein (C/EBP) family of transcription factors and these have been reviewed extensively elsewhere [2, 50]. Although GATA-1, -2, Wnt (Wnt 10b), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and transforming growth factor beta (TGF-β) play important roles in PPAR-γ regulation, they will not be discussed in this review.

PPAR-γ receptor transcriptional activity is regulated by two distinct processes: repression of receptor activity by phosphorylation (by kinases such as mitogen-activated protein, MAP kinases, which activate Jun N-terminal kinase, or JNK, and extracellular signal-regulated kinase 2, or ERK-2) and increased receptor activity by ubiquitination. Agonists for the nuclear PPAR-γ receptor include protein kinase A, natural fatty acids, eicosanoids, and oxidized lipoproteins. Less well studied are negative regulators of the nuclear PPAR-γ receptor. Activators of MAPK and thus inhibitors of PPAR-γ receptor transcriptional activity include growth factors like epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor β's (TGF-β's) 1 and 2, and GILZ. Both insulin and glucocorticoid induce the expression of C/EBP-β and -δ, which in turn induce the expression of PPAR-γ and C/EBP-α and initiate the adipogenesis program.

5. NUTRITION-RELATED HORMONES

Enteric hormones represent the mechanism by which ingested nutrients are distributed to the various tissues in the body so as to maximize their utilization. These hormones play a key role in regulating the energy balance, in part through modulation of PPAR expression. In fact, elevation of incretin hormones, through use of inhibitors of the enzyme that breaks them down (DPP-IV inhibitors), has been shown to increase PPAR expression in the kidney [51].

Nutritional hormones are also known to be important in bone turnover as evidenced by the fact that as soon as a meal is ingested, bone breakdown is suppressed. Many nutrition-related hormones have been shown to have effects on bone turnover through in vitro or in vivo studies including (a) *Intestinal Hormones* such as (1) GIP, (2) Ghrelin, and (3)

Glucagon-like peptide- (GLP-2); (b) *Pancreatic Hormones* such as (1) Insulin, (2) Amylin, (3) Adrenomedullin, and (4) Preptin; (c) *Adipocyte-secreted Hormones* such as (1) Leptin, (2) Adiponectin, and (3) Resistin, as recently reviewed by Clowes et al. [52] and Reid et al. [53]. For purposes of this review, we will focus more extensively on GIP and leptin but discuss these other hormones briefly below.

5.1. Intestinal hormones

Ghrelin is a 28-amino acid peptide expressed predominantly in the gastric epithelium and small intestine, though it is also expressed to a lower extent in the brain, pancreatic islets, adrenal cortex, kidney, and bone [54]. Ghrelin's physiologic function is to stimulate growth hormone secretion, and systemic elevations of ghrelin stimulate food intake and weight gain. Ghrelin's systemic effects on energy metabolism appear to oppose those of leptin. Ghrelin receptors are expressed on osteoblasts and ghrelin stimulates osteoblastic proliferation and differentiation [55, 56]. In addition, intraperitoneal infusion of ghrelin for four weeks resulted in significant increases in bone mineral density in Sprague-Dawley rats [55]. In humans, the data supporting a role for ghrelin in bone turnover are less clear. Ghrelin levels have a significant negative correlation with markers of bone breakdown at baseline, although not with bone mineral density, and ghrelin infusion has no acute effect on these markers [57, 58].

GLP-2 is a 33-amino acid peptide expressed mainly in the L cells of the small intestine. GLP-2 is secreted in response to nutrient ingestion and its physiologic function appears to be to regulate intestinal motility and stimulate intestinal cell growth; it is also antiapoptotic [59]. GLP-2 receptors are expressed in osteoclasts and the administration of GLP-2 to human subjects inhibits bone resorption and increases bone mass [60–62].

5.2. Pancreatic hormones

Insulin has long been considered the main anabolic hormone, stimulating bone formation in vitro. However, in vivo, although insulin infusion is known to decrease markers of bone breakdown, this effect is only about 30% of the decline in resorption markers that occurs postprandially. In fact, it has been suggested that this effect is due to hypoglycemia and the attendant impairment in skeletal cellular activity rather than to a direct antiresorptive effect [63].

Amylin is a 37-amino acid hormone cosecreted from the pancreatic β cells with insulin in response to a meal. Amylin lowers serum calcium, inhibits bone resorption, and increases bone mass in mice [64–66].

Adrenomedullin is a 52-amino acid peptide related to amylin; it is expressed in the adrenal medulla, vasculature brain, kidney, and bone [67]. Adrenomedullin stimulates osteoblastic proliferation and injection of adrenomedullin to mice increases bone formation and strength without a major effect on bone breakdown [68, 69].

Preptin is a 37-amino acid peptide cosecreted from the pancreatic islet with amylin and insulin. Preptin stimulated osteoblastic proliferation, and the daily injection of this peptide for five days over the calvaria resulted in increased bone area and mineralized surface through increased bone formation rather than through inhibition of bone breakdown [70].

5.3. Adipocytic hormones

Adiponectin is a 247-amino acid protein strongly expressed in mature adipocytes (particularly in subcutaneous versus visceral adipocytes) and the levels correlate with the degree of differentiation [71]. Thus, PPAR-γ agonists (e.g., thiazolidinediones) are potent stimulators of adiponectin expression. Adiponectin suppresses both cell proliferation and release of other inflammatory cytokines [71]. Both the adiponectin protein and its receptor are expressed in osteoblasts and osteoclasts, and its effects on bone turnover are complex [72, 73]. In humans, adiponectin levels have been shown to be negatively correlated with bone mineral density [74], particularly in postmenopausal female patients [75].

Resistin is a 137-amino acid protein secreted from adipocytes [76]. In addition to adipocytes, resistin is also expressed in pancreas, brain, and bone marrow. In the adipocyte, resistin expression is regulated by PPAR-γ with PPAR-γ agonists such as rosiglitazone resulting in an inhibition of resistin expression [77]. Resistin secretion results in insulin resistance. In the bone, resistin is expressed in osteoblast, osteoclast, and mesenchymal stem cells [76] and resistin levels are negatively correlated with bone mineral density [78].

5.4. Glucose-dependent insulinotropic peptide

There are two major intestinal hormones that potentiate glucose-induced insulin secretion (incretin effect), that is, GIP and glucagon-like peptide-1 (GLP-1). GLP-1 receptors are not present in bone cells [79]. GIP was first identified in the 1970's as a hormone secreted by cells in the enteric endocrine system (K cells) in the proximal small intestine. Because this 42-amino acid peptide was found to inhibit gastric acid secretion, it was initially named gastric inhibitory peptide (GIP) [80]. Subsequent studies demonstrated that GIP effects on inhibiting gastric acid secretion did not occur at physiological concentrations, in contrast to GIP effects on potentiating glucose-induced insulin secretion. Thus GIP's name was changed to glucose-dependent insulinotropic peptide. Our data and resulting publications demonstrate that GIP also serves as an important anabolic signal for bone, stimulating bone formation and inhibiting bone breakdown. To summarize our GIP data in vitro, (1) GIP receptors are present in both osteoblasts and osteoclasts [81, 82]; (2) in osteoblasts, GIP increases collagen type I synthesis and increases alkaline phosphatase activity [81]; (3) in osteoblasts, GIP stimulates proliferation [81]; and (4) in osteoclasts, GIP inhibits PTH-induced long bone resorption and decreases osteoclastic resorption pit depth [82]. In in vivo studies, (5) GIP receptor knockout mice have a lower bone mass, decreased serum markers of bone formation, and increased markers of bone breakdown [83], consistent with data

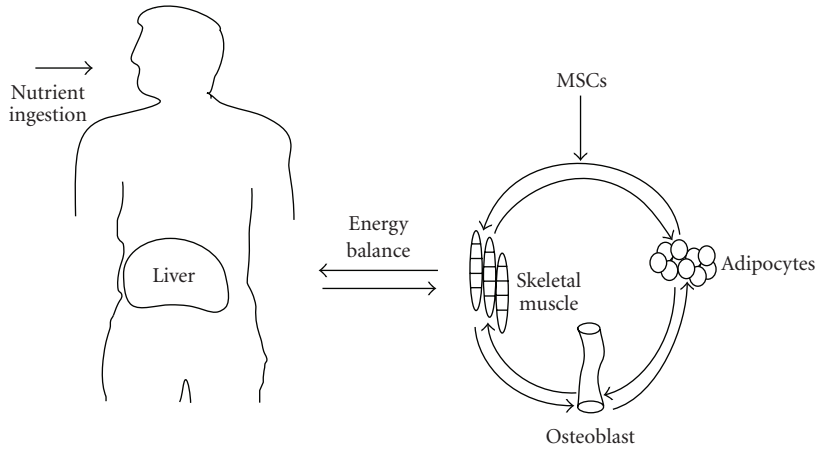

Figure 1: *Nutrition and tissue-generated hormonal signals modulate mesenchymal stem cell differentiation*. Hormonal signals generated upon nutrient ingestion impact the organism's energy balance, thus favoring anabolic versus catabolic activities. In turn, hormonal signals generated by target tissues such as muscle, bone, and fat modulate MSC differentiation into adipocytes or osteoblasts.

published by others, [84] and (6) GIP-overexpressing transgenic mice have increased bone mass, lower serum markers of bone breakdown, and increased markers of bone formation [85].

The GIP receptor knockout mouse was developed by Miyawaki and colleagues [86], and it is an interesting animal model linking nutritional hormones and bone formation. These knockout mice are not different from control mice in their weight, basal insulin, glucose levels, or in their insulin response to an intraperitoneal glucose tolerance test. However, blood glucose levels, in response to an oral glucose tolerance test or a high-fat diet, are higher in the knockout mice compared to controls, and the higher blood glucose levels in the knockout mice are associated with lower insulin levels. A subsequent report, also by Miyawaki et al. [87], demonstrates that although normal mice fed a high-fat diet gained weight, GIP receptor knockout mice were protected from the large weight gain associated with this diet. Interestingly, GIPR knockout mice fed a high-fat diet had lower leptin levels than control mice fed a high-fat diet (2-fold increase from basal in $GIPR^{-}/^{-}$ versus 4-fold increase in WT). Furthermore, if $GIPR^{-}/^{-}$ mice are crossed with the obese mouse model Lep^{ob}/Lep^{ob} to generate double homozygous mice, these double homozygous mice are partially protected from the weight gain seen in the Lep^{ob}/Lep^{ob} mice ($GIPR^{-}/^{-}/Lep^{ob}/Lep^{ob}$ had a 23% lower body weight). The authors conclude that under normal conditions an excessive amount of fat in the diet leads to GIP hypersecretion; this in turn leads to more adiposity, resulting in obesity and insulin resistance.

In our studies, we found that if the GIP receptor is downregulated, bone mass decreases and bone marrow adipocyte content increases [85]. These findings would suggest the possibility of direct GIP effects on MSCs. In fact, we have demonstrated that GIP receptors are present on MSCs and that stimulation of these cells with GIP promotes osteoblastic differentiation [88].

6. LEPTIN

The cytokine-like hormone leptin is recognized as a powerful regulator of appetite and energy balance [89]. Adipocytes are the primary source of leptin in the body and as such leptin plays an important role as a signal of energy status to the brain [90]. Leptin produced by peripheral body fat enters the circulation and crosses the blood-brain barrier to reach leptin receptors located in the hypothalamus. Leptin binding to the long form of the leptin receptor induces the expression of anorexigenic neuropeptides such as cocaine-amphetamine-related transcript (CART) and alpha-melanocyte-stimulating hormone (α-MSH), and suppresses the activity of orexigenic genes such as neuropeptide Y (NPY) and agouti-related peptide (AgRP), that are involved in regulating food intake [91].Leptin also regulates sympathetic outflows and functions as a beta-adrenergic agonist [92], and intrahypothalamic injections of leptin induce apoptosis of adipocytes in both peripheral fat and bone marrow [93]. Adipocytes express beta-adrenergic receptors, particularly beta 3 [94], and activation of these receptors can induce apoptosis through activation of a tyrosine kinase pathway [95].

Leptin also appears to regulate adipocyte populations in bone marrow directly, in addition to the central effects of leptin on adipocyte apoptosis. Leptin-deficient ob/ob mice show a significant increase in bone marrow adipocytes compared to lean mice [96], and peripheral leptin injections decrease the population of bone marrow adipocytes in ob/ob mice and increase bone formation [97]. As discussed above, the loss of bone marrow adipocytes with peripheral leptin treatment may be a centrally mediated effect, but the increased osteogenic differentiation and increased endocortical bone formation are more consistent with a direct effect of leptin on osteogenic differentiation [89]. Bone marrow stromal cells (BMSCs) express leptin receptors, and leptin binding increases the expression of osteogenic genes and directs

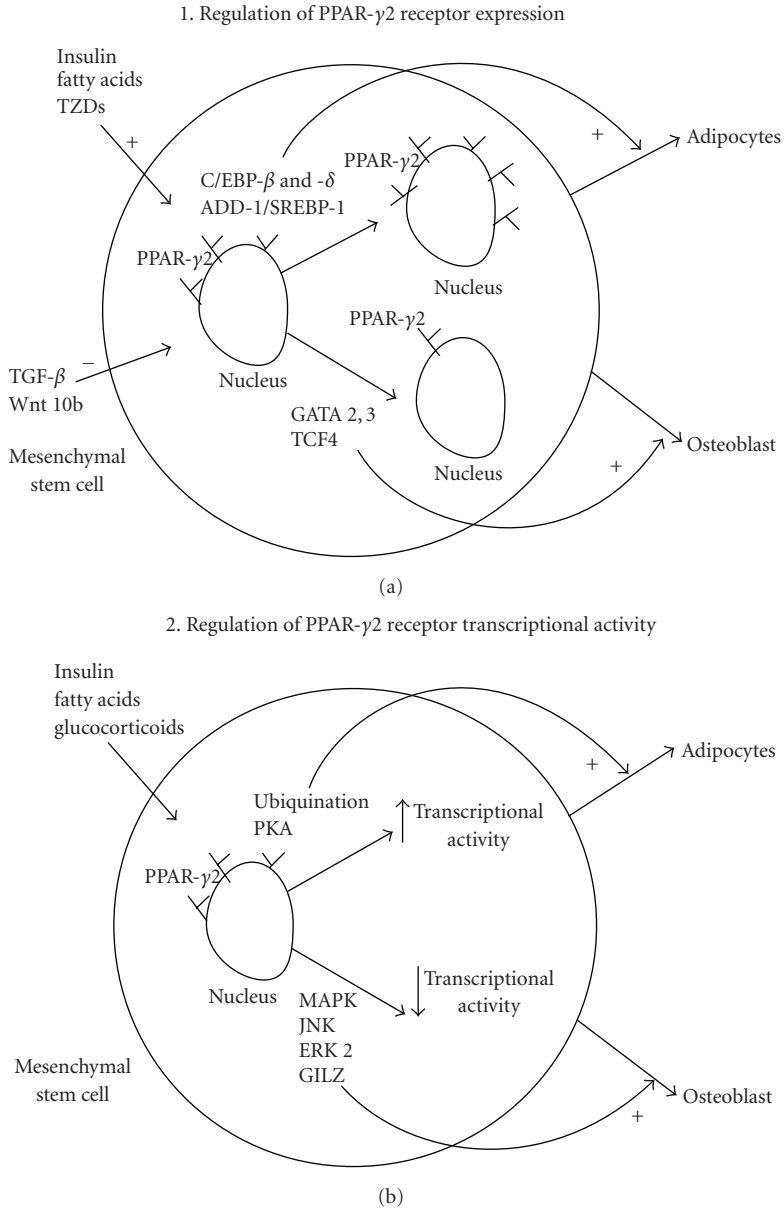

Figure 2: *PPAR-γ2 action is modulated by either changes in receptor expression or transcriptional activity*. (a) Natural (insulin, long-chain fatty acids, or eicosanoids) or synthetic ligands (TZDs) to the PPAR-γ2 receptor can either increase or decrease receptor expression resulting in increased adipocytic or increased osteoblastic differentiation, respectively. (b) It is also possible to stimulate or inhibit PPAR-γ2 transcriptional activity resulting in either increased adipocytic or osteoblastic differentiation, respectively.

BMSCs to the osteogenic rather than the adipogenic pathway [98]. In studies by Thomas et al. [98], leptin did not alter PPAR-γ or Cbfa-1 expression despite increasing osteogenesis and decreasing adipogenesis presumably by acting at a later stage in osteoblast differentiation. Bone marrow adipocytes secrete leptin themselves [99], raising the possibility that leptin may play a role in autocrine or paracrine signaling within the bone marrow microenvironment. We have found that age-associated bone loss in mice is associated with decreased serum leptin [100], and as noted earlier, aging is associated with bone loss and an increased accumulation of bone marrow adipocytes. These data suggest that leptin treatment, in conditions of increased leptin sensitivity (see below), may have significant potential for increasing bone formation and decreasing marrow adipogenesis with aging.

7. MYOSTATIN

Myostatin was initially identified as a factor regulating myogenic differentiation because its expression was localized to developing skeletal muscle, and because myostatin loss-of-function was observed to have dramatic effects on muscle mass in mice. It was, however, also noted that mice lacking myostatin showed decreased body fat [101, 102], and

myostatin deficiency decreased adiposity in leptin-deficient ob/ob mice [102]. This was thought to be an indirect effect of the increased muscle mass on metabolism. Since that time, we and others have found that myostatin deficiency inhibits adipogenesis in vivo, even when mice are fed a high-fat diet [103]. Transgenic overexpression of myostatin propeptide, which inhibits myostatin signaling, also inhibits body fat gain with a high-fat diet [104]. Similar alterations in myostatin signaling are associated with changes in body fat among humans. A child with a naturally occurring mutation in the myostatin gene was shown to have increased muscle mass as well as decreased subcutaneous fat [105]. Weight loss in morbidly obese subjects was associated with significant downregulation of myostatin mRNA in muscle biopsies, suggesting a role for myostatin in energy partitioning between protein and fat [106].

Although the in vivo data consistently show that myostatin has an adipogenic effect, and that myostatin deficiency has an anti-adipogenic effect, the in vitro data are less clear. Myostatin has been observed to promote adipogenesis in multipotential mouse C3H 10T (1/2) mesenchymal stem cells [107], but myostatin can also inhibit adipocyte differentiation in 3T3-L1 mouse preadipocytes [108] and inhibit BMP-7-mediated adipogenesis by binding to the same receptor as BMP-7, the activin IIB (ActRIIB) receptor [109]. These data suggest that the decreased fat mass of myostatin-deficient animals is simply an indirect effect of increased muscle mass since other mouse models showing increased muscle mass, such as transgenic mice overexpressing Akt [110] and Ski [111], also show decreased fat mass. However, we have recently identified expression of the myostatin receptor in bone marrow-derived mesenchymal stem cells (BMSCs), and found that BMSCs from myostatin-deficient mice demonstrate increased osteogenic differentiation and decreased adipogenic differentiation [112]. There are not many studies examining the effect of myostatin on PPAR-γ expression. A study by Artaza et al. [107] demonstrated that myostatin increased expression of C/EBP alpha and adipogenesis in mesenchymal stem cells, suggesting a myostatin effect on PPAR-γ, although they did not actually examine PPAR-γ expression. These data are consistent with previous reports showing increased bone mineral density in the bones of myostatin-deficient animals [113–115]. Furthermore, these data from bone marrow cells provide further evidence that myostatin is an adipogenic factor, as well as one that suppresses myogenesis and perhaps osteogenesis. In contrast, a study by Hirai et al. [116] found that myostatin inhibited PPAR-γ and C/EBP alpha expression in bovine preadipocytes. Thus, myostatin effects on PPAR-γ may be cell-type-dependent.

8. GLUCOCORTICOID-INDUCED LEUCINE ZIPPER

GILZ, which is also induced by estrogen and sonic hedgehog (Shh), is a new member of the leucine zipper protein [117, 118] and belongs to the TGF-β-stimulated clone-22 (TSC-22) family of transcription factors [119, 120]. Members of this family of proteins contain three distinct domains: an N-terminus TSC box, a middle leucine zipper domain, and a C-terminus polyproline-rich domain. GILZ was originally identified from dexamethasone-treated murine thymocytes [118]. Recent studies have shown that GILZ is also induced in many tissues (including lung, liver, brain, and kidney) and by the other glucocorticoids that are prescribed frequently in clinic such as methylprednisolone, fluticasone, and hydrocortisone, as well as anti-inflammatory cytokine interleukin-10 (IL-10) in human and murine macrophages [121–123]. Studies carried out in vitro have shown that overexpression of GILZ protected T cells from apoptosis induced by anti-CD3 antibody, but not other apoptosis-inducing agents such as dexamethasone, various doses of ultraviolet irradiation, starvation, or triggering induced by cross-linked anti-Fas monoclonal antibody [118]. However, T-cell-specific transgenic overexpression of GILZ resulted in thymocyte apoptosis ex vivo possibly through downregulation of Bcl-xL [124]. GILZ also inhibits interleukin-2 (IL2)/IL-2 receptor expression (63). This antiapoptotic function is mediated through direct protein-protein interactions between GILZ and NF-kB, and between GILZ and AP-1 (63–67). The direct interactions of GILZ with NF-kB, and GILZ with AP-1, block DNA binding and, therefore, the transcriptional activities of NF-kB and AP-1.

Studies by Shi et al. [125] found that GILZ is rapidly induced by dexamethasone in MSCs and a variety of cell lines, including osteoblasts (2T3), preadipocytes (3T3-L1), and a mesenchymal cell line (C3H10T1/2). It is interesting to note that the induction of GILZ in MSCs and C3H10T1/2 cells seems transient. GILZ can bind specifically toa 40-bp DNA fragment containing a unique tandemly repeated C/EBP-binding element present in the promoter of the PPAR-γ2 gene. Because glucocorticoids induce adipocyte differentiation, and GILZ is induced by glucocorticoids and binds to adipogenic PPAR-γ2 promoter, it was hypothesized that constitutive expression of GILZ would activate PPAR-γ2 expression and enhance adipogenesis. Contrary to expectations, overexpression of GILZ inhibited PPAR-γ2 transcription and blocked adipocyte differentiation of C3H10T1/2 mesenchymal cells and 3T3-L1 preadipocytes. These results demonstrated that GILZ functions as a transcriptional repressor of PPAR-γ2. Studies by Zhang et al. (unpublished data) show that overexpression of GILZ in mouse bone marrow MSCs can enhance MSC osteoblast differentiation. These data suggest that, by modulating PPAR-γ expression, GILZ may serve as an important regulator of the MSC lineage commitment between osteoblast and adipocyte. This role of GILZ may have potential clinic importance since as humans age, the number of adipocytes increase and the number of osteoblasts decrease resulting in weakened bone and age-related osteoporosis and fragility fractures. All these may have direct connection to the increased PPAR-γ expression and activity in aging bone marrow as it is known that aging activates marrow adipogenesis and fat secretes large amounts of cytokines that will, in turn, inhibit osteogenesis as mentioned earlier.

As previously mentioned, the transcriptional activity of PPAR-γ is regulated by phosphorylation (by kinases such as the mitogen-activated protein kinase (MAPK), which activate Jun N-terminal kinase, or JNK, and extracellular signal-regulated kinase 2, or ERK 2), and GILZ can directly interact

with Raf1, one of the MAPK members, resulting in the inhibition of Raf-1 phosphorylation and, subsequently, the suppression of both MEK/ERK-1/2 phosphorylation and AP-1-dependent transcription [119].

It has been a long standing paradox that glucocorticoids, while required for osteoblast differentiation of primary bone marrow stromal cells in vitro [126–128], induce bone loss in vivo. Since GILZ is induced by glucocorticoids and enhances MSC osteogenesis, we speculate that GILZ is the actual mediator of glucocorticoid action in this process. The possible pathways in which GILZ may convey therapeutic effects of glucocorticoids have been reviewed by Clark and Lasa [129].

Under normal conditions, GC levels fluctuate in response to environmental stressors (flight/fight, abrupt temperature changes, etc.). When the GC level is increased, GILZ is induced and prevents adipogenic differentiation. Under pathophysiological or pharmacological conditions, however, GC is elevated for a prolonged period of time and the negative feedback network is overwhelmed, resulting in harmful GC side effects, such as bone loss.

9. GIP, LEPTIN, MYOSTATIN, AND GILZ AS THERAPEUTIC TARGETS

Adequate nutrition and a positive energy balance are clearly important for bone growth. A reduction in caloric intake will retard growth plate expansion [130]. In addition, if the reduced caloric intake is accompanied by a reduced calcium intake, a shift in the balance between bone formation and resorption occurs, such that bone mass decreases over time [131]. In contrast, an increase in intake and gain in weight are associated with an increase in bone mass. Upon nutrient delivery to the intestine, there is a rapid rise in a number of enteric hormones that serve to inform the target tissues that nutrients are available for anabolic activity. One of these enteric hormones, GLP-2, has already been used to prevent bone loss in postmenopausal patients [62], although no data are available on marrow adiposity. Our data would suggest that GIP is another enteric hormone that can increase bone formation by promoting MSC differentiation into osteoblasts.

One of the challenges in using recombinant leptin therapy to either reduce body weight, suppress appetite, or stimulate bone formation is that most individuals are relatively resistant to exogenous leptin treatment due to relatively high levels of endogenous leptin [89, 96]. However, leptin may have significant effects on bone formation and appetite in conditions where leptin sensitivity is increased with energy deprivation. For example, leptin treatment has been observed to increase serum IGF-1 and serum osteocalcin in women with exercise-induced hypothalamic amenorrhea [132]. Anorexia nervosa is associated with markedly reduced leptin levels and osteoporosis [133–135] even if less severe, voluntary weight loss is associated with increased rates of bone loss in adults [136, 137]. Leptin treatment may have potential to reverse bone loss with weight loss, as well as maintenance of reduced weight following weight loss [138].

Treatment of normal rodents and dystrophin-deficient mdx mice with factors that block myostatin signaling, such as a soluble myostatin receptor, a propeptide, or follistatin, showed significant increases in muscle mass and improved muscle regeneration [139, 140]. The myostatin antibody MYO 029 is currently in Phase II clinical trials for treatment of Duchenne muscular dystrophy. To date, myostatin inhibitors have only been tested for their ability to improve muscle regeneration in cases of muscular dystrophy and acute injury, and their potential for inhibiting body fat gain and stimulating bone formation remains relatively unexplored. We expect that myostatin inhibitors have significant potential as novel therapies for decreasing adiposity and also improving bone formation and bone strength. Moreover, as noted earlier in this paper, glucorticoids play a major role in stimulating bone marrow adipogenesis, and the myostatin promoter is known to have a glucocorticoid response element [141]. Myostatin deficiency inhibits muscle atrophy with glucocorticoid treatment [142], and myostatin inhibitors may be useful for attenuating muscle atrophy and bone loss with prolonged use of glucocorticoids.

Bone cells are derived from marrow MSCs, and the best way to increase the number of bone-forming cells is to modulate differentiation pathways so that more MSCs are directed to the osteoblastogenesis pathway. The PPAR-γ pathway not only regulates adipocyte differentiation, but also inhibits osteoblast differentiation from mesenchymal progenitors [31], suggesting the possibility of interrupting the PPAR-γ pathway as a novel treatment of osteoporosis. GILZ, induced transiently by GC, is a sequence-specific transcriptional repressor of PPAR-γ [143]. No transcriptional repressors that can bind specifically to the promoter of PPAR-γ have been reported so far. Thus, GILZ may be a novel therapeutic target for drug development for a variety of conditions characterized by an altered adipocyte/osteoblast balance.

In summary, current therapeutic targets for prevention and treatment of osteoporosis involve anabolic agents stimulating osteoblastic activity or antiresorptive agents targeting the osteoclasts. Our data would suggest that modulating the MSC differentiation pathway, particularly via inhibition of the PPAR-γ2 receptor, thus favoring osteoblastic instead of adipocytic differentiation, might be an attractive therapeutic target for prevention and treatment of osteoporosis.

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Research Article

PPARγ2 Regulates a Molecular Signature of Marrow Mesenchymal Stem Cells

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Bone formation and hematopoiesis are anatomically juxtaposed and share common regulatory mechanisms. Bone marrow mesenchymal stromal/stem cells (MSC) contain a compartment that provides progeny with bone forming osteoblasts and fat laden adipocytes as well as fibroblasts, chondrocytes, and muscle cells. In addition, marrow MSC provide an environment for support of hematopoiesis, including the development of bone resorbing osteoclasts. The PPARγ2 nuclear receptor is an adipocyte-specific transcription factor that controls marrow MSC lineage allocation toward adipocytes and osteoblasts. Increased expression of PPARγ2 with aging correlates with changes in the MSC status in respect to both their intrinsic differentiation potential and production of signaling molecules that contribute to the formation of a specific marrow micro-environment. Here, we investigated the effect of PPARγ2 on MSC molecular signature in respect to the expression of gene markers associated exclusively with stem cell phenotype, as well as genes involved in the formation of a stem cell supporting marrow environment. We found that PPARγ2 is a powerful modulator of stem cell-related gene expression. In general, PPARγ2 affects the expression of genes specific for the maintenance of stem cell phenotype, including LIF, LIF receptor, Kit ligand, SDF-1, Rex-1/Zfp42, and Oct-4. Moreover, the antidiabetic PPARγ agonist TZD rosiglitazone specifically affects the expression of "stemness" genes, including ABCG2, Egfr, and CD44. Our data indicate that aging and anti-diabetic TZD therapy may affect mesenchymal stem cell phenotype through modulation of PPARγ2 activity. These observations may have important therapeutic consequences and indicate a need for more detailed studies of PPARγ2 role in stem cell biology.

1. INTRODUCTION

PPARγ, an essential regulator of lipid, glucose, and insulin metabolism [1], is expressed in bone marrow mesenchymal stem cells (MSC). PPARγ is expressed in mice and humans in two isoforms, PPARγ1 and PPARγ2, which originate from up to seven different transcripts due to alternative promoter usage and alternative splicing [2–5]. PPARγ2 differs from PPARγ1 by 30 additional amino acids on its N-terminus, which constitute AF-1 domain of ligand-independent gene-activating function [6]. While PPARγ1 is expressed in a variety of cell types, including osteoblasts, PPARγ2 is expressed in cells of adipocyte lineage and serves as an essential regulator of adipocyte differentiation and function [7, 8].

Osteoblasts and adipocytes are derived from a marrow mesenchymal cell compartment which also serves as a source of progenitors for marrow fibroblasts and cartilage cells and functions as hematopoiesis-supporting stroma [9, 10]. Commitment of marrow MSC toward adipocyte and osteoblast lineage occurs by a stochastic mechanism, in which lineage-specific transcription factors (such as Runx2 for osteoblasts and PPARγ2 for adipocytes) representing intrinsic determinants of this process are activated [8, 11]. Embryonic stem cells with a null mutation in PPARγ spontaneously differentiate to osteoblasts and are unable to differentiate to adipocytes [12]. In marrow MSC, PPARγ2 acts as a dominant negative regulator of osteoblast differentiation [8, 13]. Using a model of marrow MSC differentiation (U-33/γ2 cells), we have previously demonstrated that activation of the PPARγ2 isoform by the highly specific agonist and antidiabetic thiazolidinedione (TZD), rosiglitazone, converted cells of osteoblast lineage to terminally differentiated adipocytes

Table 1

(a) Genes expressed differently in P versus V.

Gene symbol	Probe ID[a]	FC[b]	Gene description	Biological process[c]
Cd3g	1419178_at	1.5	CD3 antigen, gamma polypeptide	Immune and hematopoietic system, cell surface receptor linked signal transduction
Cd3e	1445748_at	1.5	CD3 antigen, epsilon polypeptide	Cell surface receptor linked signal transduction, positive regulation of T cell proliferation and T cell receptor signaling pathway
Cd4	1419696_at	1.5	CD4 antigen	Immune response, cell adhesion, cell surface receptor linked signal transduction, positive regulation of T cell activation
Cd7	1419711_at	1.5	CD7 antigen	Immune response, myeloid cells antigen
Cd8a	1451673_at	1.7	CD8 antigen, alpha chain	Immune response, cell surface receptor linked signal transduction, cellular defense response, cytotoxic T cell differentiation
Cd19	1450570_a_at	1.9	CD19 antigen	Lymphocyte progenitors
Cd24a	1416034_at	9.8	CD24a antigen	Cell surface antigen expressed in T and B lymphocytes, macrophages, dendritic endothelial, and epithelial cells
Cd33	1450513_at	1.5	CD33 antigen	Myeloid cells antigen, cell adhesion
Cd37	1419206_at	1.7	CD37 antigen	B and T cell antigen
Cd96	1419226_at	1.5	CD96 antigen	T-cell activation, cell adhesion
Cd207	1425243_at	1.5	CD 207 antigen	Specific for Langerhans cell precursors
Cd209b	1426157_a_at	1.7	CD209b antigen	Dendritic cell-specific, positive regulation of tumor necrosis factor-alpha biosynthesis, positive regulation of phagocytosis
Cd209c	1421562_at	1.9	CD209c antigen	Dendritic cell specific
Cxcl9	1418652_at	1.6	Chemokine (C-X-C motif) ligand 9	Inflammatory response, immune response
Cxcl13	1448859_at	2.0	Chemokine (C-X-C motif) ligand 13	Chemotaxis, inflammatory response, immune response, lymph node development
Cxcl16	1418718_at	1.7	Chemokine (C-X-C motif) ligand 16	Chemotaxis, keratinocytes, released into the wound after injury
Fgf4	1450282_at	1.8	Fibroblast growth factor 4	Trophoblast proliferation and differentiation, regulation of progression through cell cycle, stem cell maintenance, embryonic limb and hindlimb morphogenesis, odontogenesis, negative regulation of apoptosis
Gata4	1441364_at	1.6	GATA binding protein 4	Embryonic development, regulation of transcription, heart development, embryonic gut morphogenesis
Gjb1	1448766_at	1.6	Gap junction membrane channel protein beta 1	Cell communication, cell-cell signaling
Kit/CD117	1452514_a_at	1.6	Kit oncogene	Germ cell development, transmembrane receptor protein tyrosine kinase signaling pathway, cell proliferation, cytokine and chemokine mediated signaling pathway, hematopoiesis, cell differentiation

(a) Continued.

Gene symbol	Probe ID[a]	FC[b]	Gene description	Biological process[c]
Kdr	1449379_at	1.6	Kinase insert domain protein receptor	Angiogenesis, vasculogenesis, transmembrane receptor protein tyrosine kinase signaling pathway, development, cell migration, hemopoiesis, cell differentiation, cell fate commitment, endothelial cell differentiation
Nkx2-5	1449566_at	1.9	NK2 transcription factor related, locus 5	Regulation of transcription, embryonic heart tube development
Psca	1451258_at	1.5	Prostate stem cell antigen	
Pou3f2	1450831_at	1.7	POU domain, class 3, transcription factor 2	Positive regulation of cell proliferation, regulation of transcription
Pou5f1/Oct-4	1417945_at	1.5	POU domain, class 5, transcription factor 1	Germ-line stem cell maintenance, expressed in mouse totipotent embryonic stem and germ cells, regulation of transcription
Sox10	1451689_a_at	2.3	SRY-box containing gene 10	Regulation of transcription, cell differentiation and maturation
Thy1/CD90	1423135_at	1.5	Thymus cell antigen 1, theta	MSC specific marker
Utf1	1416899_at	1.5	Undifferentiated embryonic cell transcription factor 1	Regulation of transcription
Col4a3bp	1420384_at	−1.6	Procollagen, type IV, alpha 3 binding protein	Goodpasture antigen binding protein
Egr2/Krox20	1427683_at	−3.9	Early growth response 2	Schwann cell differentiation, myelination, rhythmic behavior, regulates osteocalcin expression
Falz	1427310_at	−3.2	Fetal Alzheimer antigen	Negative regulation of transcription
H2-K1	1426324_at	−4.2	Histocompatibility 2, K1, K region	Immune response, antigen presentation, endogenous antigen via MHC class I
Lif	1421207_at	−8.7	Leukemia inhibitory factor (transient downregulation during cell growth)	Embryonic stem cell maintenance, immune response, tyrosine phosphorylation of Stat3 protein, muscle morphogenesis, neuron development
Lifr	1425107_a_at	−5.8	Leukemia inhibitory factor receptor	Positive regulation of cell proliferation
TNFRSF11b/OPG	1449033_aat	−34.6	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	Apoptosis, signal transduction, negative regulation of osteoclastogenesis
Zfp42/Rex-1	1451244_a_at	−1.9	Zinc finger protein 42	The putative human stem cell marker, Rex-1 (Zfp42): structural classification and expression in normal human epithelial and carcinoma cell cultures

[a] Affymetrix probe ID
[b] fold change
[c] gene ontology [28]

Table 1

(b) Genes expressed differently in PR versus P.

Gene symbol	Probe ID[a]	FC[b]	Gene Description	Biological Process[c]
Abcg2	1422906_at	−3.1	ATP-binding cassette, subfamily G, member 2	Stem cell marker, drug resistance
Cd9	1416066_at	−3.2	CD9 antigen	Stromal cell and adipose stem cell surface marker, tetraspan protein
Cd47	1419554_at	−2.4	CD47 antigen (Rh-related antigen,	Hematopoietic cells, membrane glycoprotein, the same as integrin-associated protein (IAP) and ovarian tumor marker OA3
Cd81	1416330_at	−1.6	CD 81 antigen	Cell adhesion, fertilization
Egfr	1424932_at	−1.8	Epidermal growth factor receptor	Active in early events of stem cells recruitment and differentiation
Gja7	1449094_at	−3.8	Gap junction membrane channel protein alpha 7	Cell communication, synaptic transmission, heart development, visual perception, cell development, cardiac muscle development
Il6st	1437303_at	−2.9	Interleukin 6 signal transducer	Signal transduction, positive regulation of cell proliferation, regulation of Notch signaling pathway
Lims1	1418231_at	−2.5	LIM and senescent cell antigen-like domains 1	Cell-matrix adhesion, establishment and/or maintenance of cell polarity, cell-cell adhesion, embryonic development
Cd36	1423166_at	178.8	CD36 antigen	Fatty acid transporter associated with adipogenesis
Cd200 (Ox2)	1448788_at	2.4	Cd200 antigen	Cell surface antigen of thymocytes, B cells, T cells, neurons, kidney glomeruli, tonsil follicles, the syncytiotrophoblast and endothelial cells
Cd5	1418353_at	1.6	CD5 antigen	B lymphocytes antigen
Cd63	1455777x_at	1.9	Cd63 antigen	Melanoma antigen
Vegfa	1451959_a_at	1.5	Vascular endothelial growth factor A	Regulation of progression through cell cycle, angiogenesis, development, cell proliferation, cell differentiation
Vegfb	1451803_a_at	2.6	Vascular endothelial growth factor A	Regulation of progression through cell cycle, angiogenesis, development, cell proliferation, cell differentiation

[a] Affymetrix probe ID
[b] fold change
[c] gene ontology [28]

and irreversibly suppressed both the osteoblast phenotype and the osteoblast-specific gene expression [8]. The expression of PPARγ2 in marrow MSC increases with aging [14]. Moreover, bone marrow derived from old animals produces unknown PPARγ activator(s) that stimulates adipocyte differentiation and suppresses osteoblast differentiation [14]. These changes cause alterations in the milieu of intrinsic and extrinsic signals that determine MSC lineage allocation. For instance, this contributes to the preferential MSC differentiation toward adipocytes and decreased differentiation toward osteoblasts that leads to the development of senile osteopenia.

PPARγ plays an important role in the maintenance of bone homeostasis as demonstrated in several animal models of either bone accrual or bone loss depending on the status of PPARγ activity [12, 15–19]. A decrease in PPARγ activity resulted in increased bone mass due to increased osteoblast number [12, 18], whereas increased PPARγ activity

Table 2: Genes regulated similarly in PR versus P and P versus V.

Gene symbol	Probe ID	FC		Gene description	Biological process
		PR versus P	P versus V		
Akp2	1423611_at	−11.5	−2.0	Alkaline phosphatase	Marker of osteoblasts
Cd2bp2	1417224_a_at	−1.9	−1.5	CD2 antigen binding protein 2	T cell activation
Cd29 (Itgb1)	1426918_at	−2.1	−1.5	Integrin beta 1 (fibronectin receptor beta)	Regulation of progression through cell cycle, G1/S transition of mitotic cell cycle, cell adhesion, cell-matrix adhesion, integrin-mediated signaling pathway, development, positive regulation of cell proliferation, negative regulation of cell differentiation
Cd44	1423760_at	−3.9	−5.6	CD44 antigen	Cell surface glycoprotein, cell adhesion, stem cells, implicated in tumor growth and dissemination
Cd105 (Eng)	1432176_a_at	−2.3	−2.0	Endoglin	Angiogenesis, cell adhesion, heart development, regulation of transforming growth factor beta receptor signaling pathway
Cd109	1425658_at	−2.8	−5.2	CD109 antigen	Membrane glycoprotein, elevated expression in variety of cancers
H2-D1	1451934_at	−3.2	−3.0	Histocompatibility 2, D region locus 1	Immune response, detected on surface of MSC and adipocyte stem cells at low levels and reduced with passage
H2-K1	1427746_x_at	−1.6	−1.5	Histocompatibility 2, K1, K region	Immune response, antigen presentation
Mki67	1426817_at	−4.3	−5.9	Antigen identified by monoclonal antibody Ki 67	Meiosis, cell proliferation
Pcna	1417947_at	−2.4	−1.7	Proliferating cell nuclear antigen	DNA replication
S100b	1434342_at	−4.2	−2.7	S100 protein, beta polypeptide, neural	Marker of differentiated neural cells
Spred1	1460116_s_at	−1.9	−2.1	Sprouty protein with EVH-1 domain 1, related sequence	Inhibition of MAP kinases, activated in hematopoietic cells, involved in mesoderm organization, inhibit Ras pathway (G protein)
Spred2	1434403_at	−2.3	−1.7	Sprouty protein with EVH-1 domain 2, related sequence	As above
Stag1	1434189_at	−1.5	−1.7	Stromal antigen 1	Key mediator of p53-dependent apoptotic pathway, cell cycle, chromosome segregation, mitosis, and cell division
Stag2	1421849_at	−1.6	−1.6	Stromal antigen 2	As above

Table 2: Continued.

Gene symbol	Probe ID[a]	FC[b] PR versus P	FC[b] P versus V	Gene description	Biological process[c]
Cd1d1	1449130_at	4.9	5.1	CD1d1 antigen	MHC class I-like glycoprotein, development and function of natural killer T lymphocytes
Cd151	1451232_at	1.9	1.5	CD151 antigen	PPARγ positively regulates it in squamous cell carcinoma, implicated in tumor invasiveness
Fabp4	1424155_at	69.6	1.7	Fatty acid binding protein 4	Marker of differentiated adipocytes

[a] Affymetrix probe ID
[b] fold change
[c] gene ontology [28]

due to TZD administration led to the bone loss [15–17, 19]. TZD-induced bone loss was accompanied with changes in the cellular composition of the bone marrow, such as decreased numbers of osteoblasts and increased numbers of adipocytes, and changes in the MSC phenotype characterized by a loss of MSC plasticity. These changes are characteristics for aging bone marrow [20]. Recently, several human studies have demonstrated that TZD use is associated with decreased bone mineral density and an increased risk of fractures in postmenopausal diabetic women [21–23]. This prompted US Food and Drug Administration to issue a warning of possible adverse effects of TZD on human bone.

The development of high throughput analysis of gene expression using microarrays has advanced studies on genes and signaling pathways controlled by a single gene product. The transcriptional role of PPARγ in either differentiated cells or functional tissues has been studied using DNA microarrays, mostly to determine its role in the physiology during disease and as a result of therapeutic treatment with TZDs of these target tissues [24–26]. None of these studies, however, were designed to test for the effect of the PPARγ2 isoform on the molecular signature of MSC. Using a model of marrow MSC differentiation under the control of the PPARγ2 transcription factor, we found that both the presence of PPARγ2 and its activation with the antidiabetic TZD, rosiglitazone, resulted in gene expression changes for multiple genes that characterize the stem cell phenotype and their phenotypic lineages. Even though our model was originally developed to study the mechanisms by which PPARγ2 suppressed osteoblastogenesis and promoted adipogenesis, our studies suggest that PPARγ2 has a profound effect on the expression of signature genes for cell "stemness."

2. MATERIAL AND METHODS

2.1. Cell cultures and RNA isolation

Murine marrow-derived U-33 (previously referred to as UAMS-33) cells represent a clonal cell line spontaneously immortalized in the long term bone marrow culture conditions. To study the effect of PPARγ2 on marrow mesenchymal stem cell differentiation, U-33 cells were stably transfected with either PPARγ2 expression construct (referred to as U-33/γ2 cells) or an empty vector control (referred to as U-33/c cells) as described previously [8]. Several independent clones were retrieved after transfection and carefully analyzed for their phenotype. Clone 28.6, representing U-33/γ2 cells, and clone γc2, representing U-33/c cells, were used in the experiments presented in this manuscript. Cells were maintained in αMEM supplemented with 10% FBS heat-inactivated (Hyclone, Logan, UT), 0.5 mg/ml G418 for positive selection of transfected cells, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin (sigma) at 37°C in a humidified atmosphere containing 5% CO_2. Media and additives were purchased from Life Technologies (Gaithersburg, MD).

Cells were propagated for one passage and than seeded at the density of 3×10^5 cells/cm^2. After 48 hours of growth, when cultures achieved approximately 80% confluency, cells were treated with either 1 μM rosiglitazone or the same volume of vehicle (DMSO) for 2, 24, and 72 hours, followed by RNA isolation using RNeasy kit (QIAGEN Inc., Valencia, CA). The replicate experiment was performed independently on a fresh batch of cells. Two replicates were used for microarray analysis. The factorial design of experiment was $2 \times 3 \times 2$ which corresponded to two cell lines (with and without PPARγ2), three time points (2, 24, 72 hours), and two treatment regiments (rosiglitazone and vehicle).

2.2. Microarray experiments

RNA quality was assessed using the Agilent Model 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Five micrograms of total RNA were processed for use on the microarray by using the Affymetrix GeneChip one-cycle target labeling kit (Affymetrix, Inc., Santa Clara, CA) according to the manufacturer's recommended protocols. The resultant biotinylated cRNA was fragmented then hybridized to the GeneChip Mouse Genome 430 2.0 Array (45,000 probe sets used to analyze over 39,000 mouse transcripts and variants from over 34,000 well-characterized mouse genes; Affymetrix, Inc.). The arrays were washed, stained,

Table 3: Genes regulated differently in PR versus P and P versus V conditions.

Gene symbol	Probe ID[a]	FC[b]		Gene description	Biological process[c]
		PR versus P	P versus V		
Actc1	1415927_at	−1.5	2.0	Actin, alpha, cardiac	Cytoskeleton organization and biogenesis, muscle development, regulation of heart and muscle contraction
Actg2	1422340_a_at	−4.7	2.3	Actin, gamma 2, smooth muscle, enteric	Cytoskeleton organization and biogenesis, muscle development
Cd97	1418394_a_at	2.3	−2.1	CD97 antigen	Cell adhesion, signal transduction, G-protein coupled receptor protein signaling pathway, neuropeptide signaling pathway
Cd166 (ALCAM)	1437466_at	2.1	−1.5	Activated leukocyte cell adhesion molecule	Cell adhesion, axon guidance, motor axon guidance
Cxcl1	1419209_at	−2.7	1.8	Chemokine (C-X-C motif) ligand 1	Regulation of progression through cell cycle, inflammatory response, immune response
Cxcl4	1448995_at	−2.1	2.8	Chemokine (C-X-C motif) ligand 4	Chemotaxis, immune response, negative regulation of angiogenesis, cytokine, and chemokine mediated signaling pathway, platelet activation, negative regulation of megakaryocyte differentiation
Cxcl12 (SDF-1)	1417574_at	−2.4	7.5	Chemokine (C-X-C motif) ligand 12 (stem cell differentiation factor)	Patterning of blood vessels, ameboidal cell migration, chemotaxis, immune response, germ cell development and migration, brain development, motor axon guidance, T cell proliferation, induction of positive chemotaxis
Cxcl16	1456428_at	−1.7	1.7	Chemokine (C-X-C motif) ligand 15	Chemotaxis, inflammatory response, immune response, signal transduction, hematopoiesis, neutrophil chemotaxis
Foxa1	1418496_at	−1.5	1.9	Forkhead box A1	Regulation of transcription, lung development, epithelial cell differentiation, branching morphogenesis of a tube
Kitl	1415854_at	−4.1	5.2	Kit ligand	Cell adhesion, germ cell development, positive regulation of peptidyl-tyrosine phosphorylation, cytokine product associated with MSC/stromal cells, stem cell factor
Ntf3	1450803_at	−1.5	1.9	Neurotrophin 3	Neuromuscular synaptic transmission, glial cell fate determination, axon guidance, brain and peripheral nervous system development, epidermis development, mechanoreceptor differentiation, regulation of neuron apoptosis

Table 3: Continued.

Gene symbol	Probe ID[a]	FC[b] PR versus P	FC[b] P versus V	Gene description	Biological process[c]
Pdgfα	1421916_at	−2.1	1.6	Platelet derived growth factor receptor, alpha polypeptide	Protein amino acid phosphorylation, transmembrane receptor protein tyrosine kinase signaling pathway, morphogenesis, organ morphogenesis, extracellular matrix organization and biogenesis, male genitalia development, odontogenesis
Tnfsf11 (RANKL)	1419083_at	−1.6	9.2	Tumor necrosis factor (ligand) superfamily, member 11	Positive regulation of osteoclast differentiation and bone resorption, immune response, lymph node development
Snai2	1418673_at	−6.4	1.9	Snail homolog 2 (Drosophila)	Development of human melanocytes, regulation of transcription, DNA dependent, development, response to radiation, regulation of survival gene product activity
Vegfc	1419417_at	−5.6	11.5	Vascular endothelial growth factor C	Regulation of progression through cell cycle, angiogenesis, positive regulation of neuroblast proliferation, development, positive regulation of cell proliferation, organ morphogenesis

[a] Affymetrix probe ID
[b] fold change
[c] gene ontology [28]

and scanned using the Affymetrix Model 450 Fluidics Station and Affymetrix Model 3000 scanner using the manufacturer's recommended protocols by the University of Iowa DNA Core Facility. Raw gene expression measurements were generated using the microarray suite (MAS) version 5.0 software (Affymetrix, Inc.). Statistical assessment of differential gene expression is described in Lecka-Czernik et al. [27].

3. RESULTS AND DISCUSSION

An essential role of PPARγ2 in the regulation of marrow MSC lineage allocation, together with the evidence of its increased activity in MSC with aging [14], prompted us to study the effect of PPARγ2 on the expression of stem cell gene markers. Two aspects were examined: the effect of the presence of PPARγ2 in U-33 stem cells and the effect of PPARγ2 activation with rosiglitazone on stem cell phenotype.

Here we used a model of marrow MSC differentiation under the exclusive control of a single protein, PPARγ2. This system allows for relatively unambiguous studies of the unique effects of PPARγ2 isoform on MSC phenotype. The model of PPARγ2-dependent MSC differentiation consists of two cell lines derived from the same parental cell line (U-33 cells), which either express the PPARγ2 protein (U-33/γ2 cells) or do not express the PPARγ2 protein (U-33/c cells) [8, 29]. To assess the effects of the presence of PPARγ2 on the phenotype of U-33 cells in nontreated conditions, we compared gene expression in U-33/γ2 and U-33/c cells maintained in basal growth conditions (this is referred to as the "P versus V" analysis). This comparison provides information about PPARγ2 activities, which are either ligand independent or acquired as a result of activation with natural ligands present in the growth media or endogenously produced by tested cells. The results of "P versus V" analysis may provide information on a role of PPARγ2 in a continuum of changes that occur in stem cells during aging. To assess an effect of rosiglitazone on the expression of stem cell-related genes, we compared gene expression in U-33/γ2 cells treated with rosiglitazone and nontreated U-33/γ2 cells (this is referred to as the "PR versus P" analysis). This analysis provides important information on the effects of rosiglitazone on the stem cell phenotype. Finally, comparison of the results of both analyzes provides information on differences between endogenous and artificially induced PPARγ2 activities in respect to stem cell gene expression.

To avoid differences in the cell phenotype due to different rates of cell growth, we chose the 72-hour time point for the analysis of gene expression (see Section 2). In basal growth conditions at this time point, cell cultures of U-33/γ2 and U-33/c were in state of confluence, cells acquired fibroblast-like appearance and cell cultures were indistinguishable morphologically from each other. In contrast, U-33/γ2 cells

Table 4: Genes whose expression was not affected in P versus V and PR versus P conditions.

Gene symbol	Probe ID[a]	Gene description
Afp	1416645_a_at	Alpha fetoprotein
Cd34	1416072_at	CD34 antigen
Cd3z	1438392_at	CD3 antigen, zeta polypeptide
Cd5l	1449193_at	CD5 antigen like
Cd6	1451910_a_at	CD6 antigen
Cd8b1	1448569_at	CD8 antigen, beta chain 1
Cd22	1419769_at	CD22 antigen
Cd53	1439589_at	CD53 antigen
Cd86	1420404_at	CD86 antigen
Cd164	1431527_at	CD164 antigen
Cd209e	1420582_at	Cd209e antigen
Cdh15	1418602_at	Protocadherin 15
Cer1	1450257_at	Cerberus 1 homolog
Col6a2	1452250_a_at	Procollagen, type VI, alpha 2
Erbb2ip	1439080_at	Erbb2 interacting protein
Erbb3	1452482_at	V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)
Fabp7	1450779_at	Fatty acid binding protein 7, brain
Fzd9	1427529_at	Frizzled homolog 9
Gata2	1450333_a_at	GATA binding protein 2
Gcg	1425952_a_at	Glucagon
Gcm2	1420455_at	Glial cells missing homolog 2
Gfap	1440142_s_at	Glial fibrillary acidic protein
Gjb3	1416715_at	Gap junction membrane channel protein beta 3
Gjb4	1422179_at	Gap junction membrane channel protein beta 4
Ina	1418178_at	Internexin neuronal intermediate filament protein, alpha
Ins1	1422447_at	Insulin I
Isl1	1444129_at	ISL1 transcription factor, LIM/homeodomain (islet 1)
Krt1-14	1460347_at	Keratin complex 1, acidic, gene 14
Krt1-17	1423227_at	Keratin complex 1, acidic, gene 17
Krt2-8	1435989_x_at	Keratin complex 2, basic, gene 8
Mbp	1454651_x_at	Myelin basic protein
Mtap1b	1450397_at	Microtubule-associated protein 1 B
Myh11	1448962_at	Myosin, heavy polypeptide 11, smooth muscle
Ncam1	1439556_at	Neural cell adhesion molecule 1
Ncam2	1425301_at	Neural cell adhesion molecule 2
Nes	1453997_a_at	Nestin
Ngfr	1421241_at	Nerve growth factor receptor (TNFR superfamily, member 16)
Nkx2-2	1421112_at	NK2 transcription factor related, locus 2 (Drosophila)
Numb	1425368_a_at	Numb gene homolog (Drosophila)
Olig1	1416149_at	Oligodendrocyte transcription factor 1
Pax6	1456342_at	Paired box gene 6
Pou3f3	1422331_at	POU domain, class 3, transcription factor 3
Pou6f1	1420749_a_at	POU domain, class 6, transcription factor 1
Prox1	1457432_at	Prospero-related homeobox 1
Ptprc	1440165_at	Protein tyrosine phosphatase, receptor type, C
Slc1a2	1451627_a_at	Solute carrier family 1 (glial high affinity glutamate transporter), member 2
Slc1a6	1418933_at	Solute carrier family 1 (high affinity aspartate/glutamate transporter), member 6
Sox1	1422205_at	SRY-box containing gene 1
Sox2	1416967_at	SRY-box containing gene 2
Syn1	1453467_s_at	Synapsin I
Tubb3	1415978_at	Tubulin, beta 3
Zfp110	1450998_at	Zinc finger protein 110

[a] Affymetrix probe ID

treated for 72 hours with rosiglitazone acquired adipocyte phenotype typified by large fat droplets. A morphological appearance of U-33/c cells treated with rosiglitazone was indistinguishable from nontreated U-33/c cells as well as nontreated U-33/γ2 cells.

There are no known exclusive markers for MSC. However, based on extensive work with MSCs and other stem cell populations, several proteins have emerged as candidate markers associated with a stem cell phenotype. These entities include ATP-binding cassette g2 (Abcg2), cell surface antigen CD44, stem cell factor or kit ligand (SCF/Kitl), epidermal growth factor receptor (Egfr), early growth response factor 2 (Egr2), leukemia inhibitory factor (Lif), leukemia inhibitory factor receptor (Lifr), and stromal-derived factor/CXC-chemokine ligand 12 (SDF-1/CXCL12). Based on the available published information for stem cell gene expression for the analysis, we arbitrarily chose 135 genes that represent markers of either early or lineage committed stem cells [9, 30–34]. The analysis showed that the expression of 38% of analyzed genes was not affected by activation state of PPARγ2 (see Table 4), the expression of 28% genes was exclusively affected by the presence of PPARγ2 ("P versus V" analysis) (see Table 1(a)), and the expression of 10% genes was exclusively affected by rosiglitazone-activated PPARγ2 ("PR versus P" analysis) (see Table 1(b)). The genes whose expression was affected by both rosiglitazone-activated and non-activated PPARγ2 constituted 24% of the total genes studied; their expression was affected in equal proportion either similarly (see Table 2) or in the opposite direction in these two conditions (see Table 3).

Comparison of the two cell lines indicates that a majority of analyzed genes are up-regulated in U-33/γ2 versus U-33/c cells (see Tables 1(a) and 3). Most of these genes are characteristic for stem cells of hematopoietic and neural lineages while some of them are expected to be up regulated in hematopoiesis supporting stromal cells (e.g. Kitl, RANKL (Table 1(a)), and the CXCL family (Tables 1(a) and 3)).

These interesting observations have at least two reasonable interpretations. The first interpretation suggests that observed differences are a reflection of different phenotypes of the two individual parental cells from which each of the two clones originated. Hence, differences in gene expression between both cell lines are PPARγ2-independent. The second possibility suggests that these differences are PPARγ2-dependent and result from either PPARγ2 ligand-independent activity or activity acquired from endogenous ligand. Several lines of evidence suggest a correlation between the adipocyte-like phenotype of marrow stroma cells and support for hematopoiesis [35, 36]. Hematopoiesis depends heavily on the microenvironment provided by mesenchymal cell compartment in the marrow and the ability of these cells to produce growth factors and cytokines that act in a paracrine fashion to influence the differentiation of hematopoietic progenitors. In the long term bone marrow cultures, an *in vitro* system of hematopoietic cell differentiation, stroma cell support for myelopoiesis, is provided by cultures consisting mostly of adipocytes [35, 37]. Similarly, *in vivo* studies in a model of SAMP6 mice that are characterized by senile osteopenia due to a diminished number of osteoblasts and increased myelopoiesis, correlates positively with an increased number of marrow adipocytes [38]. Interestingly, U-33/γ2 cells support osteoclastogenesis much better than U-33/c cells (unpublished observation), in part due to relatively higher RANKL (9-fold in "P versus V," Table 3) and lower OPG (−34.6-fold in "P versus V"; Table 1(a)) expression. Another important regulator of bone marrow hematopoiesis, including osteoclastogenesis, is represented by the chemokine CXCL12 or SDF-1 [39, 40]. Growing experimental evidence indicates that CXCL12 and its receptor CXCR4 axis is not only required for hematopoietic stem cell signaling but also has a crucial role in the formation of multiple organ systems during embryogenesis as well as adult nonhematopoietic tissue regeneration and tumorigenesis [39]. According to our analysis, an expression of CXCL12, but not CXCR4, is up regulated in U-33/γ2 cells ("P versus V") and suppressed by PPARγ2-activated with rosiglitazone ("PR versus P") (see Table 3). Thus, it is conceivable that mesenchymal cells which express PPARγ2 acquire the adipocyte-like phenotype typified by the production of number of cytokines and support hematopoietic stem cell differentiation.

While PPARγ2 has a positive effect on the stromal phenotype supporting hematopoiesis, it has a negative effect on the expression of "stemness" genes. The expression of LIF cytokine and its receptor, a regulatory system required for the stem cell self renewal, is significantly suppressed in U-33/γ2 cells as compared to U-33/c cells (see Table 1(a)). Interestingly, activation of PPARγ2 with rosiglitazone did not affect the expression of these genes. The presence of PPARγ2 in U-33/γ2 cells suppresses the expression of Egr2/Krox20, a stem cell-specific transcription factor with a role in the development of nervous system and endochondrial bone formation [41]. Egr2/Krox20 also regulates osteoblast differentiation and osteocalcin expression [42]. Again, rosiglitazone does not affect Egr2/Krox20 gene expression (see Table 1(a)). PPARγ2 cellular presence also affects expression of Zfp42 transcription factor, which is a marker of human and murine embryonic stem (ES) cells. Expression of Zfp42 is down regulated during ES cell differentiation [43]. An artificial knockdown of Zfp42 with RNAi resulted in spontaneous differentiation of ES cells toward endoderm and mesoderm lineages, whereas its overexpression led to the loss of self-renewal capacity of ES cells [44].

The expression of ABCG2, a well recognized stem cell marker [45], was down-regulated in "PR versus P" (−3.1 fold) (see Table 1(b)) and slightly in "P versus V" (−1.3 fold, $P < .01$) conditions (not shown). ABCG2 represents an ATP-binding cassette (ABC) transporter which serves to efflux certain xenobiotics (including anticancer drugs) that can lead to the development of multidrug resistance syndrome. This is a significant obstacle in cancer treatment [46]. This gene is also considered to be a marker of primitive pluripotent stem cells, termed "side population," which were identified based on their ability to exclude Hoest dye [45]. The ability to exclude a variety of substances may comprise a mechanism that protects stem cells from exogeneous and endogeneous toxins. Finding that ABCG2 expression is down regulated by PPARγ2, especially after activation with rosigli-

tazone, implicates PPARγ2 as a negative regulator of stem cell phenotype as well as a negative regulator of multidrug resistance. Similarly, Egfr a marker of early stem cells is down regulated by PPARγ2 when activated with rosiglitazone [47].

Interestingly, however, the expressions of Oct-4 (POU5f1) and FGF4, well recognized embryonic stem cell markers highly expressed in the totipotent and pluripotent ES cells [48, 49] are up regulated in U-33/γ2 cells compared to U-33/c cells and are not affected in U-33/γ2 cells treated with rosiglitazone (see Table 1(a)).

Another interesting grouping consists of genes whose expression is differentially regulated by both activated and nonactivated PPARγ2 (see Table 2). A number of genes implicated in early stem cell maintenance and recruitment, among them CD44, H2-D1, PCNA, CD109, Spred1 and 2, and Stag1 and 2, are down regulated in U-33/γ2 cells in both basal conditions and upon rosiglitazone treatment.

The last category represents gene markers specific for terminally-differentiated cells.Consistent with the proadipocytic and antiosteoblastic activities of PPARγ2 activated with rosiglitazone, the expression of the gene encoding FABP4 increases, whereas an expression of the gene underlying alkaline phosphatase decreases. Markers of the neuronal phenotype are either decreased (S100b, Table 2) or not affected (nestin and NCAMs, Table 4), and the expression of CD34, a bona fide marker for cells of hematopoietic lineage, is not affected (see Table 4). However, the expression patterns of gene markers characteristic for embryonic stem cells and a large number of markers that are associated with a nonmesenchymal phenotype, including markers of different hematopoietic and neuronal lineages, indicates that marrow mesenchymal U-33 cells possess a mixed phenotype with some characteristics of early primitive pluripotent stem cells and lineage oriented mesenchymal cells.

In conclusion, PPARγ2 is a powerful modulator of the stem cell phenotype and its activation with antidiabetic TZDs affect the expression of "stemness" genes. It is unclear at this time whether, and to what extent, PPARy2 is expressed in stem cells *in vivo* and whether this key transcription factor plays a significant role in stem cell biology. However, the findings presented here, together with previously published evidence of increased PPARγ2 expression in MSCs with aging [14] and a loss of marrow MSC plasticity or ability to convert between phenotypes as a result of aging and TZD therapy [20], suggest that aging and TZD therapy may affect stem cell phenotype through modulation of PPARγ2 activity. These observations may also have important therapeutic consequences and indicate a need for more detailed studies of PPARγ2 role in stem cell biology.

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Research Article

Inhibition of Protein Farnesylation Arrests Adipogenesis and Affects PPARγ Expression and Activation in Differentiating Mesenchymal Stem Cells

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Protein farnesylation is required for the activation of multiple proteins involved in cell differentiation and function. In white adipose tissue protein, farnesylation has shown to be essential for the successful differentiation of preadipocytes into adipocytes. We hypothesize that protein farnesylation is required for PPARγ2 expression and activation, and therefore for the differentiation of human mesenchymal stem cells (MSCs) into adipocytes. MSCs were plated and induced to differentiate into adipocytes for three weeks. Differentiating cells were treated with either an inhibitor of farnesylation (FTI-277) or vehicle alone. The effect of inhibition of farnesylation in differentiating adipocytes was determined by oil red O staining. Cell survival was quantified using MTS Formazan. Additionally, nuclear extracts were obtained and prelamin A, chaperon protein HDJ-2, PPARγ, and SREBP-1 were determined by western blot. Finally, DNA binding PPARγ activity was determined using an ELISA-based PPARγ activation quantification method. Treatment with an inhibitor of farnesylation (FTI-277) arrests adipogenesis without affecting cell survival. This effect was concomitant with lower levels of PPARγ expression and activity. Finally, accumulation of prelamin A induced an increased proportion of mature SREBP-1 which is known to affect PPARγ activity. In summary, inhibition of protein farnesylation arrests the adipogenic differentiation of MSCs and affects PPARγ expression and activity.

1. INTRODUCTION

A common phenomenon seen during the normal aging process is the redistribution of fat which accumulates in usually non-fat tissues [1–4]. Several hypotheses have been tested to explain age-related fat accumulation outside adipose tissue including a possible reduction in the capacity to metabolize fatty acids [5], a predominance of lipodystrophy [6], or finally a pure process of dedifferentiation of nonadipose mesenchymal stem cell (MSCs) into adipocytes-like cells [6–8].

Indeed, bone is not the exception to this phenomenon. One of the characteristics of senile osteoporosis is the predominance of adipose tissue within the bone marrow associated with a significant reduction in osteoblastogenesis and thus in bone formation [4, 9]. The predominance of adipogenesis seen in aging bone is the consequence of mesenchymal stem cells "dedifferentiation" which induces them to remain in a preadipocytic stage [7, 10].

There is evidence that among the multiple mechanisms involved in adipogenesis, protein farnesylation is essential for the differentiation of white fat precursors into mature adipocytes [11]. When human preadipocytes were induced to differentiate in the presence of insulin, addition of inhibitors of farnesylation affected their differentiation and decreased peroxisome proliferator activator gamma (PPARγ) expression [11]. Therefore, it is tempting to propose that, as in white fat, protein farnesylation could be necessary for the successful differentiation of MSCs into adipocytes within the bone marrow.

Lamin A is an example of a protein that not only requires farnesylation to be activated [12] but also plays an important role in adipogenesis [13]. Lamin A belongs to the group of proteins that form the lamina which keeps the nuclear envelope playing a role in a number of nuclear processes including DNA replication and cell differentiation [12, 14]. Alterations in lamin A activation as well as mutations in the

lamin A encoding gene are known as "laminopathies." In humans, lamins have been linked to Familial partial lipodystrophy (FPLD) a disease that is characterized by adipose tissue repartitioning with multiple metabolic disturbances, including insulin resistance and dyslipidemia [15]. Lamins have also been associated with other type of lipodystrophies such as Dunnigan-type Familial partial lipodystrophy [13]. Due to the fact that all these models of lamin A mutations affect adipogenesis and in some cases PPARγ expression and activity [13, 15], we hypothesize that protein farnesylation in general and lamin A farnesylation in particular could be required for adipogenesis in a model of adipogenic differentiating mesenchymal stem cells (MSCs). In summary, the determination of the potential effect that protein farnesylation has on adipogenesis and PPARγ expression in the bone marrow could offer a new approach to the understanding of the pathophysiology and treatment of senile osteoporosis.

2. MATERIALS AND METHODS

Reagents

FTase inhibitor-277 (FTI-277) was purchased from Sigma-Aldrich Corporation (St. Louis, Mo, USA). FTI-277 was dissolved in Dimethyl sulphoxide and then filter-sterilized using a 0.2 μm filter. Other reagents were from Sigma-Aldrich Corporation unless stated otherwise.

In vitro Differentiation of MSCs

Human MSCs (BioWhittaker, Walkersville, Md, USA) were induced to differentiate into adipocytes as previously described [16]. Briefly, MSCs were plated at a density of 5×10^5 cells per well in 100 cm^2 dishes containing MSCs growth media (BioWhittaker, Walkersville, Md, USA) with 10% fetal calf serum (FCS) and incubated at 37°C for 24 hours. After the cells reached 60% confluence, media were replaced with MSCs growth media or induced to differentiate into adipocytes using adipogenesis induction media (AIM) (prepared with DMEM, 4.5 g/L glucose, 1 μM dexamethasone, 0.2 mM indomethacin, 1.7 μM insulin, 0.5 mM 3-isobutyl-1-methylxanthine,10% FCS, 0.05 U/mL penicillin, and 0.05 μg/mL streptomycin) for 3 days, incubated 3 days in adipogenesis maintenance medium (DMEM, 4.5 g/L glucose, 1.7 μM insulin, 10% FBS, 0.05 U/mL penicillin, and 0.05 μg/mL streptomycin), and then switched to induction media again to promote adipogenic phenotype as previously described [16]. In all experiments, media were changed every three days.

Identification of the effect of FTI-277 on adipocyte differentiation

MSCs were plated in 4 cm^2 dishes in a density of 4×10^4 cells per dish. At 60% confluence, media were replaced with AIM containing either FTI-277 (5–10 μM) or vehicle alone. At timed intervals (weeks 1, 2, and 3), media were aspirated and cells were stained for oil red O and counterstained with hematoxylin. Differentiated adipocytes were considered those polygonal in shape, with eccentrically located nuclei, considerable cytoplasm, and lipid droplets scattered throughout.

Identification of nuclear blebbing using Propidium Iodide Staining

Cells were plated in 6-well plates, induced to differentiate, and treated as previously described. After 2 weeks of differentiation and treatment, cells were fixed using 70% ethanol for 20 minutes. After thorough washing in PBS, cells were stained for nuclear red fluorescence using propidium iodide. Nuclei were then observed via UV lightusing an Olympus IX-70 microscope (Olympus, London, UK). Cells showing deformities in the nuclear shape or vacuolization were considered positive for blebbing as previously described [17].

Measurement of viable cells after treatment with FTI-277

MSCs were seeded at a density of 4×10^2 cells/well in 96-well cluster plates (Falcon, Becton-Dickinson, NJ, USA). At 60% confluence, cells were committed to differentiate into adipocytes as previously described. Cells were treated with increasing concentrations of FTI-277 (5–10 μM) or with vehicle alone. Cell viability was assessed using MTS Formazan before induction (time 0) and 48–72 hours after differentiation was induced. MTS Formazan assesses mitochondrial function by the ability of viable cells to convert soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTS) into an insoluble dark blue Formazan reaction product measured photometrically as previously described [18]. A stock solution of MTS was dissolved in PBS at a concentration of 5 mg/mL and was added in a 1 : 10 ratio (MTS/DMEM) to each well incubated at 37°C for 4 hours and the optical density determined at a wavelength of 570–630 nm on a microplate reader model 3550 (Biorad, Hercules, Calif, USA). In preliminary experiments, the absorbance was found to be directly proportional to the number of cells over a wide range ($2 \times 10^2 - 5 \times 10^4$ cells/well). The percent survival was defined as $[(\text{experimental}_{\text{absorbance}} - \text{blank}_{\text{absorbance}})/\text{control}_{\text{absorbance}} - \text{blank}_{\text{absorbance}})] \times 100$, where the $\text{control}_{\text{absorbance}}$ is the optical density obtained for 1×10^4 cells/well (number of cells plated at the start of the experiment), and $\text{blank}_{\text{absorbance}}$ is the optical density determined in wells containing medium and MTS alone.

Western blot analysis

MSCs were treated as previously described and then lysed in 20 mM tris-HCl, pH 7, 5, 200 mM DTT, 200 mM KCl, 0.5 ml glycerol and protease inhibitor tablets (Roche Diagnostics Canada, Laval, QC, Canada), freeze-thawed 3 times in a dry ice-ethanol bath and centrifuged at 11,500 rcf for 15 minutes to remove insoluble material. Lysates were dissolved in SDS electrophoresis buffer (Bio-Rad, Hercules, Calif, USA) and proteins separated on SDS-polyacrylamide gels and subsequently electrotransfered to polyvinylidene difluoride membranes. After membrane blocking with PBS containing 0.1%

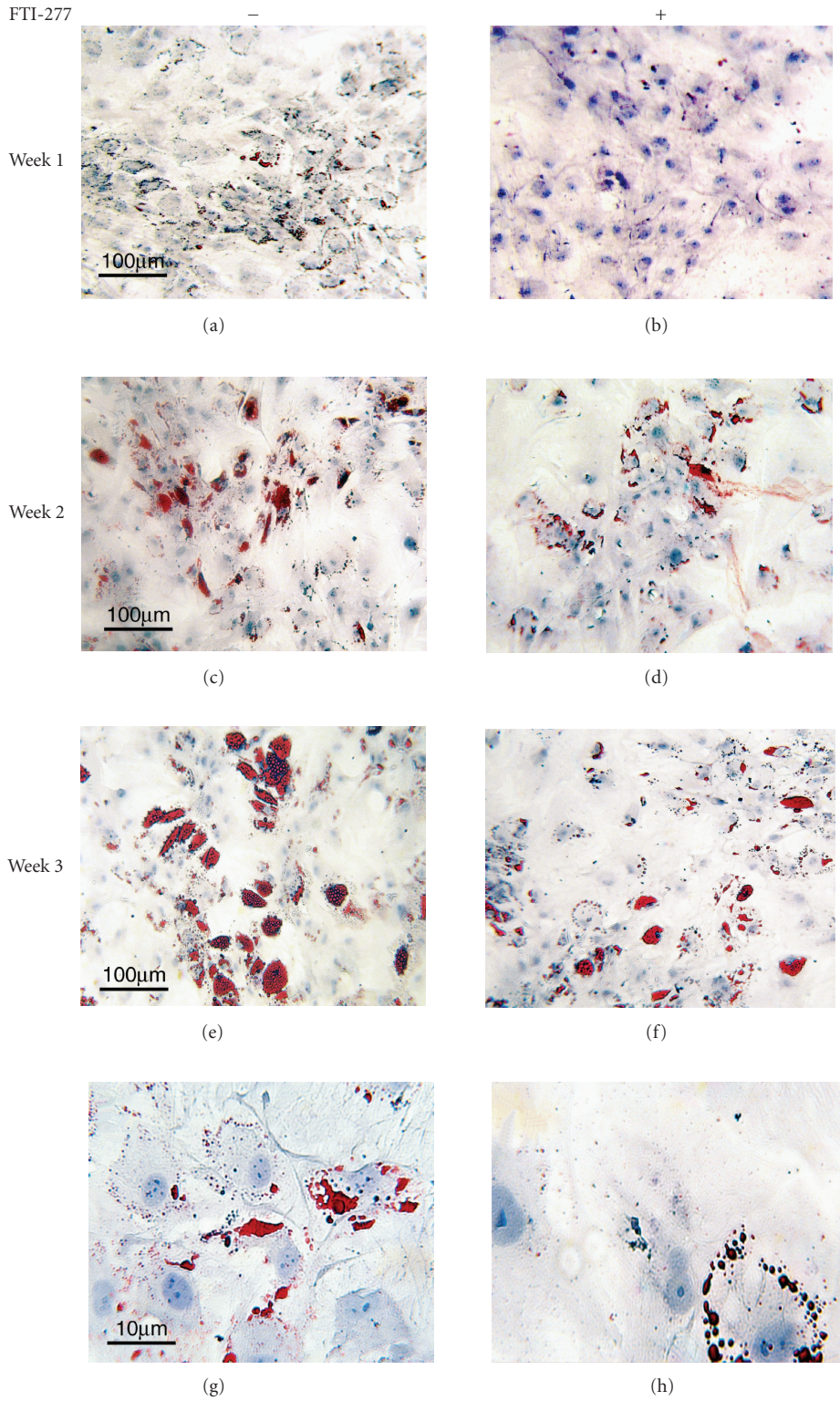
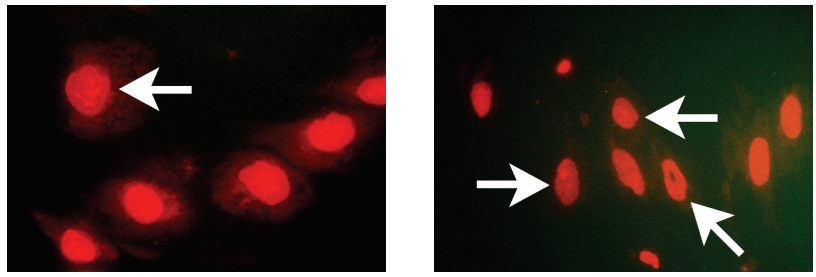


Figure 1: Effect of FTI-277on adipogenesis: human MSCs were committed to differentiate into adipocytes and treated for three weeks with either FTI-277 (5 μM) (b, d, f, and h) or vehicle alone (a, c, e, and g). At timed intervals (week 1 (a and b), week 2 (c and d), and week 3 (e and f)), cells were fixed, stained with oil red o, and counterstained with hematoxylin to assess adipocyte differentiation. Lower magnification (10×) shows higher amount of fat droplets (red) and differentiated adipocytes in untreated cells at all time points (a, c, and e) as compared with FTI-277-treated cells (b, d, and f). At higher magnification (100 ×), the amount and distribution of fat droplets is highly affected by treatment (h) where lipid droplets (red) are unable to reach confluence as compared with untreated cells (g). Note the changes in the cytoplasm after treatment (h) including vacuolization, irregular nuclei, and "mega" cytoplasm.



(a) (b)

Figure 2: Nuclear changes in differentiating MSCs after inhibition of protein farnesylation: cells were plated and induced to differentiate as previously described. At week 2 of differentiation, cells were fixed and stained using propidium iodide to identify nuclear changes (blebbing and vacuolization). The figure shows the changes in nuclear morphology compatible with blebbing (white arrows) in most of the cells after treatment with FTI-277 (5 μM) (b). In contrast, untreated cells (a) showed fewer changes compatible with blebbing. Morphologically, cells treated with FTI-277 showed smaller nuclei than AIM-treated cells. Photomicrographs were taken at $\times$100 magnification and represent three different experiments.

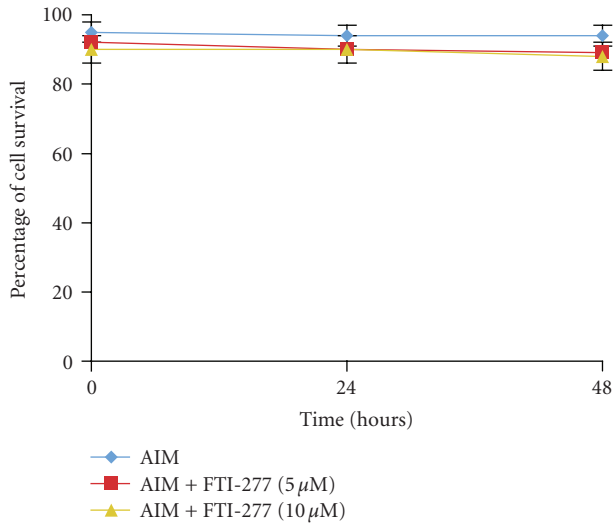


Figure 3: Effect of FTI-277 on survival of adipogenic differentiating MSCs: MSCs were plated 96-well plates and induced to differentiate into adipocytes. Cells were treated with either FTI-277 (5–10 μM) or vehicle alone. After 24 and 48 hours, cell survival was assessed by MTS Formazan as described in methods. There was no difference between treated and nontreated cells at both time intervals. This experiment was repeated three times.

Tween 20 and 10% non-fat dry milk, membranes were incubated overnight at 4°C using an antibody directed against prelamin A (which crossreacts with lamin C), PPARγ, sterol regulatory element binding protein 1 (SREBP-1), lamin B, and the chaperon protein HDJ-2 (Santa Cruz Biotechnology, Santa Cruz, Calif, USA). The bound antibodies were detected with the corresponding secondary antibodies conjugated with horseradish peroxidase (HRP). Blots were developed by enhanced chemiluminescence using Lumi-GLO reagents (Kirkegoard & Perry, Gaithensburg, Mass, USA).

PPARγ activity measurement

DNA binding PPARγ activity was determined using the ELISA-based PPARγ activation TransAM kit (Active Motif, Rixensart, Belgium) as previously described [16]. The Trans-AM PPAR-Kit contains a 96-well plate on which an oligonucleotide containing a peroxisome proliferator response element (PPRE) (5′-AACTAGGTCAAAGGTCA-3′) has been immobilized. PPAR-contained in nuclear extract specifically binds to this oligonucleotide. The primary antibody used in the Trans-AM PPAR-Kit recognizes an accessible epitope on PPAR-protein upon DNA binding. Addition of a secondary horseradish peroxidase (HRP)-conjugated antibody provides a sensitive colorimetric readout easily quantified by spectrophotometry (450 nm). To quantify PPAR-activation, 20 μg of nuclear extract was measured using the Trans-AM PPAR Kit according to the manufacturer's instructions (Active Motif, Carlsbad, Calif, USA).

Statistical analysis

All results are expressed as mean $\pm$ standard error of the median (SEM) of 3 replicate determinations. Statistical comparisons are based on oneway analysis of variance (ANOVA) for different time intervals or Student's t-test. A probability value of $P < .05$ was considered significant.

3. RESULTS AND DISCUSSION

The progression of MSCs differentiation entails the up and down regulation of multiple genes that will induce a change in cell phenotype as well as cell function [19]. This process has been widely described and involves a three-week exposure to differentiation media in which cells exposed to insulin-containing adipogenesis induction media become preadipocytes at week 2 and mature adipocytes at week 3 [20, 21]. The widely reported gene changes, occurring both in vitro [22] and in vivo [19], have provided to the field of bone research an armamentarium to potential therapeutic targets for senile osteoporosis [8, 20].

With aging, there is a predominant adipogenic differentiation of bone marrow MSCs which is mostly associated to high expression of PPARγ2 [23, 24]. This factor determines the commitment of MSCs into adipocytes at the expense of

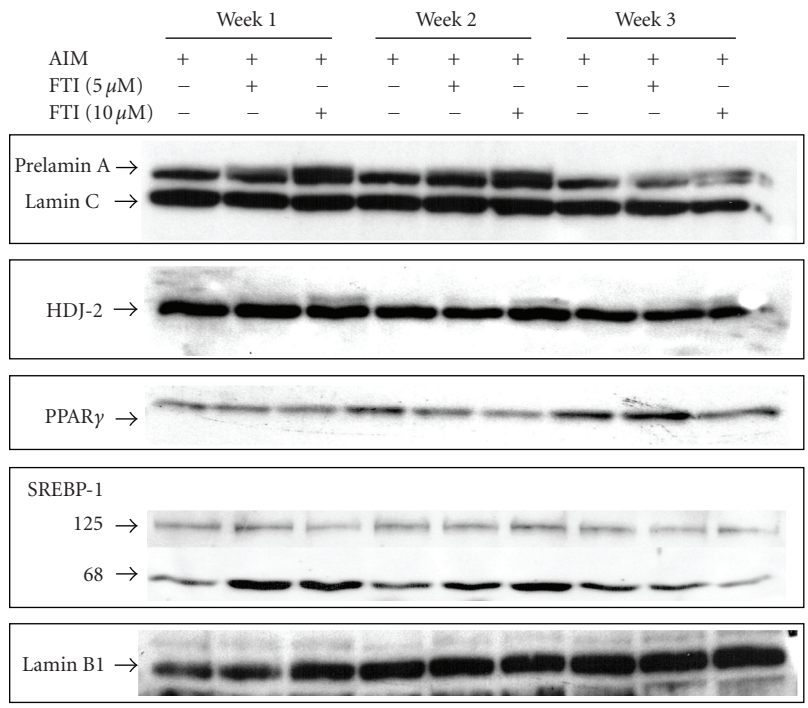


Figure 4: Effect of FTI-277 on protein farnesylation and transcription factors for adipogenesis in differentiating MSCs: human MSCs were plated in 6-well plates as previously described. After confluence, media were replaced with AIM with FTI-277 (5 and 10 μM) or vehicle alone. Media were replaced every three days for three weeks. Nuclear extracts were obtained at weeks 1, 2, and 3 of differentiation and treated as described in Materials and Methods. Membranes were incubated overnight at 4°C using an antibody directed against either prelamin A, HDJ-2, PPARγ, SREBP-1, and lamin B1. The bound antibodies were detected with the corresponding secondary antibodies conjugated with horseradish peroxidase. Blots were developed by enhanced chemiluminescence using Perkin-Elmer reagents. Treatment with increasing doses of FTI-277 induced an increase in both, prelamin A and unfarnesylated HDJ-2 expression (second upper band) suggesting that FTI-277 was effective on inhibiting farnesylation in this model of MSCs differentiation. Although a lower expression of both prelamin A and HDJ-2 at week 3 of differentiation was found, the presence of an upper band in the treated cells suggests that inhibition of farnesylation by FTI-277 was still effective. Furthermore, inhibition of farnesylation correlates with lower levels of PPARγ. Finally, at weeks 1 and 2, a sharp SREBP-1 68-kDa band (mature) correlates with higher levels of prelamin A expression whereas the 125-kDa precursor proteins is much less intensely stained. These results suggest that inhibition of farnesylation affects adipogenesis due to reduced expression of PPARγ which correlate with higher levels of mature SREBP-1. Membranes were stripped and immunoblotted for lamin B1 levels to demonstrate equal loading of proteins. The images are representative of three different experiments.

their differentiation into osteoblast with a subsequent decline in bone formation [5, 8].

Overall, although there is a correlation between aging and the transcription factors for bone marrow adipogenesis [23], the link between them and the wholesome aging process remains unclear.

Protein farnesylation is an essential step required for the activation of several proteins involved in adipogenesis (i.e., GLUT-4, CREB, p21) [11]. Farnesylation is activated by a protein farnesyltransferase (FTase) which adds a 15-carbon farnesyl group to the cystein found within the Caa*X* motif [25, 26]. This addition will induce the activation of multiple proteins such as p21, HDJ-2, and lamins (A/C and B) [26]. Protein farnesylation could be inhibited using inhibitors of FTase.

In the case of fat, insulin-stimulated prenylation of the Ras family GTPases triggers the intrinsic cascade of adipogenesis [11]. This effect is inhibited by FTI-277 in subcutaneous fat cells thus affecting adipocyte differentiation of preadipocytes [11, 15]. In contrast, the effect of inhibition of protein farnesylation in human MSCs committed to differentiate into adipocytes remains unknown.

Among the proteins that require farnesylation to be activated, lamin A seems to play an important role in adipogenic differentiation of MSCs. In fact, two studies have found changes in lamin A expression in normal models of adipocyte differentiation [27, 28]. The first one identified lamin expression in human adipose cells both in relation to anatomical site and differentiation state finding that lamin A and B1, but not B2, were expressed in mature human adipocytes whereas preadipocytes expressed all four lamins [27]. A second study looked at proteomic changes in adipocyte differentiation of cells obtained from subcutaneous fat. Amongst the 170 protein features found in their study at day 9 of differentiation, lamin A expression was included in the group of proteins of the cytoskeleton with >3-fold reduction in its expression [28].

Recent evidence looking at the role of lamins in adipogenesis has demonstrated that overexpression of lamin A inhibits adipogenic differentiation of 3T3 preadipocytes [12].

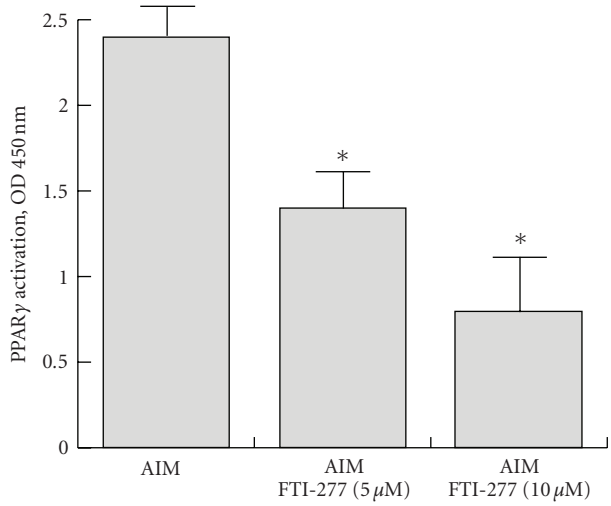


Figure 5: Effect of FTI-277 on PPARγ2 activity: PPARγ DNA binding activity was determined using ELISA-based PPARγ activation kit and quantified by colorimetry. The levels of activity after treatment with either AIM or AIM + FTI-277 (5–10 μM) are shown. Both dosages (5 and 10 μM) significantly reduced the activity of the PPARγ complex in the nuclei. Values are mean ± SEM of 6 wells per group in three independent experiments; $^*P < .01$ versus matched untreated cells.

This effect was associated with inhibition of expression of PPARγ2. In contrast, fibroblasts obtained from mice lacking lamin A showed higher potential to differentiate into adipocytes. This evidence suggests that a reduction in lamin A expression, which may happen with aging, would facilitate the differentiation of MSCs into adipocytes.

Indeed, Young et al [25] have suggested that neither the presence nor the absence of lamin A explains by itself the physiologic role of lamin A in cell function and differentiation. They demonstrated that lamin A could be negligible without affecting cell function and differentiation [25]. Therefore, they propose that it is farnesylation and not lamin A itself that could be important for disease pathogenesis.

In fact, both the absence of lamin A and the presence of high levels of prelamin A seem to play opposing roles in adipogenesis in several models of subcutaneous fat. Total absence of lamin A would stimulate adipogenesis [12] whereas increased levels of prelamin A due to lack of farnesylation inhibit adipogenesis through to the inhibition of PPARγ activity [15].

Since subcutaneous and bone marrow fat could have significant physiological differences, in this study we decided to test if inhibition of lamin A farnesylation has similar effect on human MSCs than the effect seen in subcutaneous fat.

Differentiating MSCs were treated with an inhibitor of protein farnesylation and the changes in their phenotype and capacity to produce fat droplets assessed. As shown in Figure 1, cells treated with FTI-277 showed changes in their phenotype which include cytoplasm vacuolization, big nuclei, and decreased capacity to produce fat. Furthermore, in agreement with previous reports on nuclear changes induced by lack of lamin A activity, treated cells showed nuclear changes compatible with nuclear blebbing and vacuolization (see Figure 2) [18]. These changes did not have an effect of cell survival (see Figure 3).

To test if in effect there was an inhibition in protein farnesylation, we assessed the expression of two proteins that require farnesylation to be activated, lamin A and the chaperon protein HDJ-2. These two proteins are considered as key markers of effective inhibition of farnesylation [29]. As shown in Figure 4, the presence of a double upper band demonstrates the presence of prelamin A and unfarnesylated HDJ-2 probing that FTI-277 inhibits farnesylation in this model in a dose-dependent manner. This effect was more significant at week 1 and 2 of differentiation suggesting that the effect was more significant during the preadipocyte stages. However, although there is a reduction in both HDJ-2 and prelamin A at week 3 of differentiation, the double upper band remains visible (see Figure 4).

Furthermore, we were interested in looking at the effect that inhibition of farnesylation has on PPARγ2 expression and activity. A previous study using subcutaneous fat has demonstrated that accumulation of prelamin A induced a reduction in the levels of PPARγ expression [15]. In agreement with their results, our study using human MSCs shows a reduction in the levels of PPARγ expression (see Figure 4) at all time intervals (weeks 1, 2 and 3). Furthermore, at weeks 1 and 2, the lower expression of PPARγ correlates with a significantly increased proportion of mature SREBP-1. The fact that a higher proportion of mature SREBP-1 is found in FTI-treated cells is also in agreement with previous reports which suggest that sequestration of SREBP-1 by prelamin A has an inhibitory effect on PPARγ activity [15, 30]. This effect was predominantly found during the preadipocyte stages.

Finally, from a mechanistic approach, we looked at the PPARγ2 nuclear complex activity in order to identify if protein farnesylation is required for effective activation of this complex. We found that treatment with FTI-277 affects the PPARγ2 nuclear complex in a dose-dependent manner (see Figure 5).

Overall, in this model of human MSCs differentiation, we have found that inhibition of farnesylation has an effect on adipogenesis simultaneously affecting PPARγ2 expression and activity more markedly during the preadipocyte stages of differentiation (week 1 and 2). A potential limitation of our study is that pharmacological inhibition of farnesylation could affect many of the proteins that are required in adipogenesis. Therefore, further studies looking at farnesyltransferase knockdown in this model should be pursued.

In summary, our results outline the role of protein farnesylation in bone marrow adipogenesis and more specifically in the activation of PPARγ in a model of insulin-induced bone marrow adipogenesis.

ACKNOWLEDGMENTS

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Research Article

PPARs Expression in Adult Mouse Neural Stem Cells: Modulation of PPARs during Astroglial Differentiaton of NSC

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PPAR isotypes are involved in the regulation of cell proliferation, death, and differentiation, with different roles and mechanisms depending on the specific isotype and ligand and on the differentiated, undifferentiated, or transformed status of the cell. Differentiation stimuli are integrated by key transcription factors which regulate specific sets of specialized genes to allow proliferative cells to exit the cell cycle and acquire specialized functions. The main differentiation programs known to be controlled by PPARs both during development and in the adult are placental differentiation, adipogenesis, osteoblast differentiation, skin differentiation, and gut differentiation. PPARs may also be involved in the differentiation of macrophages, brain, and breast. However, their functions in this cell type and organs still awaits further elucidation. PPARs may be involved in cell proliferation and differentiation processes of neural stem cells (NSC). To this aim, in this work the expression of the three PPAR isotypes and RXRs in NSC has been investigated.

1. INTRODUCTION

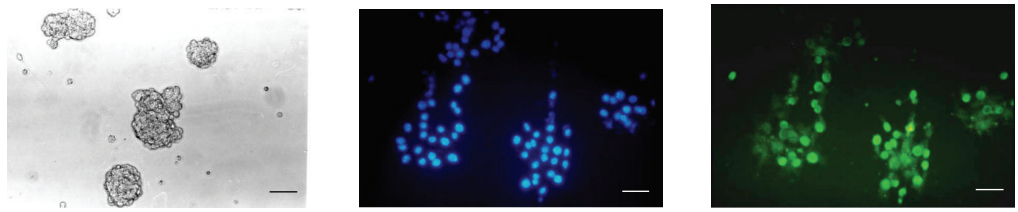
Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear hormone receptor superfamily [1]. After the isolation of PPARα (NR1C1) as the receptor mediating peroxisome proliferation in rodent hepatocytes in 1990 [2], two related isotypes, PPARβ/δ (NR1C2; referred to as PPARβ herein) and PPARγ (NR1C3), have been characterized [3]. PPARs exhibit a broad but isotype-specific tissue expression pattern which can account for the variety of cellular functions they regulate. PPARα is expressed in tissues with high fatty acid catabolism such as the liver, the heart, the brown adipose tissue, the kidney, and the intestine. The two PPARγ isoforms γ1 and γ2 act in the white and brown adipose tissues to promote adipocyte differentiation and lipid storage [4] while only the expression of PPARγ1 extends to other tissues such as the gut or immune cells. PPARβ has a broad expression being detected in all tested tissues but important functions have been assigned to this isotype in the skeletal muscle, the adipose tissue, the skin, the gut, and the brain.

PPARs are sensors capable of adapting gene expression to integrate various lipid signals. The diversity of functions in which they are implicated is also reflected by the diversity of ligands that can be accommodated within their ligand binding pocket. Indeed, PPARs are activated by a wide range of naturally occurring or metabolized lipids derived from the diet or from intracellular signaling pathways, which include saturated and unsaturated fatty acids and fatty acid derivatives such as prostaglandins and leukotriens [5, 6].

In contrast to steroid hormone receptors which act as homodimers, PPARs activate the transcription of their target genes as heterodimers with retinoid X receptors (RXR, NR2B) [7, 8]. The three RXR isotypes (α, β, and γ) can dimerize with PPARs, and specific association with each isotype seems to influence the recognition of target gene promoters [9]. However, very little is known on the specificity of RXR isotype utilized by the different PPARs in vivo. The observation that 9-*cis* retinoic acid and synthetic RXR agonists can promote the transcription of PPAR target genes leads to a model of permissive transcriptional activation where PPAR/RXR heterodimers can induce transcription in response to PPAR or RXR activation [10, 11]. Moreover, concomitant treatment with both PPAR and RXR agonists potentiates the effects observed with each ligand alone. However, the molecular mechanisms underlying transcriptional permissivity and synergy are not well understood in terms of cofactor recruitment by each partner of the heterodimer.

Table 1: Primers and PCR cycling. The adopted sequences of specific primers and relative cycling conditions of each RT-PCR are indicated.

Gene	Gene bank number	Size (bp)	Sequence	Annealing (°C)	Cicles
PPAR α	Gazouli et al., 2002	741	F 5′ggtcaaggcccgggtcatactcgcagg3′ R 5′tcagtacatgtctctgtagatctct3′	69	40
PPAR β	Gazouli et al., 2002	130	F 5′gtcatggaacagccacaggaggagacccct3′ R 5′gggaggaattctgggagaggtctgcacagc3′	69	40
PPAR δ	Gazouli et al., 2002	421	F 5′gagatgccattctggcccaccaacttcgg3′ R 5′tatcataaataagcttcaatcggatggttc3′	69	40
β -Actin	NM_031144	661	F 5′tgacggggtcacccacactgtgcccatcta3′ R 5′ctagaagcattgcggtggacgatggaggg3′	65	28



(a) (b) (c)

Figure 1: Contrast phase microscopy of neural stem cells growing in neurospheres (a). In (c), BrdU incorporation is shown. Hoechst nuclear staining of the same field is shown in (b). Bar = 40 μm.

Finally, the interplay between PPAR and RXR pathways is further illustrated by PPAR target gene activation in response to RXR homodimers [12].

Cellular proliferation allows the renewal of tissues by providing a pool of undifferentiated cells or progenitors from stem cells. All three PPAR isotypes are involved in the regulation of cell proliferation, death, and differentiation, with different roles and mechanisms depending on the specific isotype and ligand and on the differentiated, undifferentiated, or transformed status of the cell. Thus, proliferative and antiapoptotic or antiproliferative, prodifferentiating and proapoptotic effects, and even procarcinogenic effects have been reported for PPARs [13].

Differentiation stimuli are integrated by key transcription factors which regulate specific sets of specialized genes to allow proliferative cells to exit the cell cycle and acquire specialized functions. The main differentiation programs known to be controlled by PPARs both during development and in the adult are placental differentiation, adipogenesis, osteoblast differentiation, skin differentiation, and gut differentiation. PPARs may also be involved in the differentiation of macrophages, brain, and breast [14]. However, their functions in this cell type and organs still await further elucidation.

In astroglial cells, we have demonstrated the involvement of PPARα in astrocytic differentiation [14]. The expression of PPARβ in the brain peaks between days 13.5 and 15.5 of rat embryonic development [15]. The role of PPARβ in the development of the central nervous system is further illustrated by the myelination defects of the corpus callosum observed in PPARβ null mice [16]. However, the outputs in terms of brain development and the mechanisms regulating the potential implication of PPARβ in the differentiation of cerebral cells are unknown. Recently we have demonstrated that PPARβ expression and activation are increased during neuronal in vitro maturation, thus suggesting a role for this transcription factor in this process [17]. Moreover, we have demonstrated that PPARβ agonists trigger neuronal differentiation in a human neuroblastoma cell line [18]. Very recently we found that PPARβ activation by the synthetic agonist GW0742 leads to early neuronal maturation and BDNF increase, thus suggesting a role for PPARβ in neuronal plasticity (Benedetti et al., manuscript in preparation).

On the basis of the previous evidences, we hypothesize that PPARs may be involved in cell proliferation and differentiation processes of neural stem cells (NSC). To this aim, the expression of the three PPAR isotypes and RXRs in NSC has been investigated.

2. MATERIALS AND METHODS

2.1. Materials

CD1 mice were from Charles River (Harlan, Lecco, Italy); fetal bovine serum (FBS) and Earl's balanced salt solution (EBSS) were obtained from Invitrogen SRL (Milan, Italy); papain was from Worthington Biochemical (Lakewood, NJ, USA); the culture media was a kind gift of Dr Rosella Galli SCRI-DIBIT (Milan, Italy); EGF and bFGF were from Peprotech (Rocky Hill, NJ, USA); matrigel basement membrane matrix-GFR was from Becton Dickinson (Lincoln Park, NJ, USA); BCA protein detection kit from Pierce (Rockford,

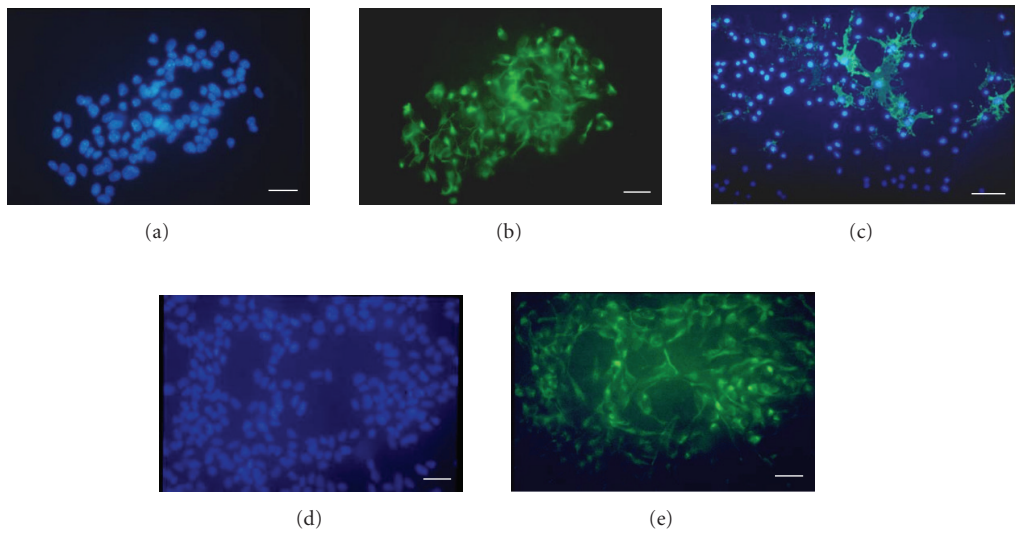


Figure 2: Immunolocalization in S0 neurospheres of nestin (b) and PLP (e). Nuclear staining of the same field is shown in (a) and (d), respectively. Double A2B5/Hoechst immunostaining is shown in (c). Bar = 70 μm

Ill, USA); antinestin (RAT 401) antibody was from Developmental Studies Hybridoma Bank (DSHB) (University of Iowa, Iowa City, Iowa, USA); mouse anti-PLP and-A2B5 antibodies were from Chemicon International Inc. (Temecula, Calif, USA); mouse anti-β-tubulin III antibody was from Promega (Mannheim, Germany); rabbit polyclonal anti-PPAR α, β/δ, γ antibodies were both from Affinity Bioreagents Inc. (Golden, Colo, USA) and from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif, USA); ECL kit was from Amersham Life Sciences (Little Chalfont, Buckinghamshire, UK); vectashield mounting medium from Vector Laboratories (Burlingame, Calif, USA); trizol reagent and platinum Taq DNA polymerase were from Invitrogen. Kit Gene Specific Relative RT-PCR was from Ambion (Austin, Tex, USA). All other chemicals were from Sigma Aldrich (St. Louis, Mo, USA).

2.2. Primary culture and culture propagation differentiation

Adult CD1 Swiss-Albino mice were killed by cervical dislocation and their brains removed and placed into PBS with penicillin and streptomycin (0.1 mg/mL). The tissues containing the forebrain periventricular region SVZ were dissected and incubated in Earl's balanced salt solution (EBSS) containing papain (1 mg/mL), EDTA (0.2 mg/mL), and cystein (0.2 mg/mL) at 37°C for 1 hour. The pieces of tissue were collected by centrifugation at 200 g for 5 minutes and resuspended in 1 mL of the DMEM/ F12 containing 0.7 mg of ovomucoid inhibitor. The cells were dissociated using a fire-polished Pasteur pipette and were collected by centrifugation at 300 g for 5 minutes. The cellular pellets were resuspended in DMEM/F12 containing HEPES buffer (5 mM), glucose (0.6%), sodium bicarbonate (3 mM), L-glutamine (2 mM), insulin (25 mg/mL), putrescine (60 μM), apotransferrin (100 μM), progesterone (6.3 ng/mL), sodium selenite (5.2 ng/mL), heparin (2 μg/mL), EGF (20 ng/mL), and bFGF (10 ng/mL), counted and plated in uncoated 25 cm^2 flask at 8×10^3 cells/cm^2.

Neurospheres were passaged by harvesting them by centrifugation (200 g for 5 minutes) and triturating them in 200 μL of medium with an automatic pipetter (P200 Gilson).

2.3. Differentiation of stem cell progeny and immunofluorescence

For differentiation, neurospheres were plated onto Matrigel basement membrane matrix-coated (100 μg/mL) well in the medium described above with addition of FBS (10%) without EGF and bFGF for 5 days (S10).

Indifferentiated (S0) and differentiated (S10) neurospheres grown on Matrigel GFR glass coverslips were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 minutes at room temperature (RT) and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at RT. Nonspecific binding sites were blocked with 10% bovine serum albumin (BSA); in PBS, for 10 minutes at RT. This procedure was performed prior to incubation with primary antibodies, except when the A2B5 or the O4 mouse monoclonal antibodies were used. In this case, fixation followed incubation.

For single immunofluorescent staining, cells were incubated with either of the following primary antibodies: 1:5 mouse monoclonal antinestin, 1:200 mouse monoclonal antiglial fibrillary acidic protein (GFAP), 1:300 mouse monoclonal anti-β-tubulin III, 1:30 mouse monoclonal PLP, 1:100 rabbit polyclonal anti-PPARα, β/δ , γ, and with 1:200 antimouse monoclonal A2B5 and O4 overnight at 4°C. All the slides were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat antirabbit IgG, antimouse IgG, or antimouse IgM antibodies (1:100), for 30 minutes at RT.

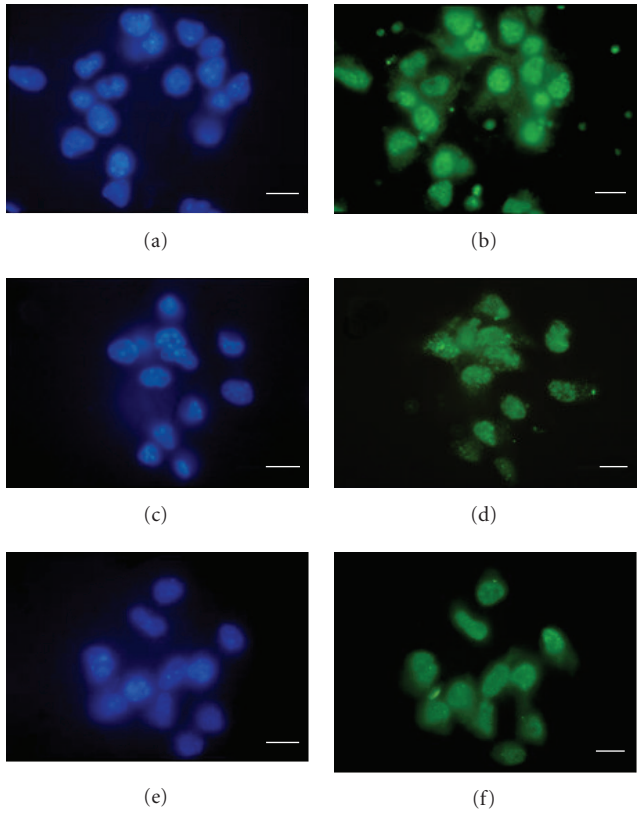


Figure 3: PPARs immunolocalization in S0 neurospheres. (b) PPARα, (d) PPARβ, (f) PPARγ. Hoechst nuclear staining is shown in (a), (b), and (c), respectively. Bar = 20 μm.

Both primary and secondary antibodies were diluted with PBS containing 10% BSA. Controls were performed by substituting the primary antibody with PBS-BSA, containing or not rabbit nonimmune serum.

Double immunofluorescence with anti-A2B5 and anti-GFAP antibodies was performed as described. Briefly, cells were first incubated with 1:100 anti-A2B5, then fixed with 4% paraformaldehyde in phosphate buffered saline (PBS), and incubated with 1:100 secondary FITC-conjugated goat anti-IgM antibodies. Subsequently, the cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at RT and incubated with 1:200 mouse monoclonal antiglial fibrillary acidic protein (GFAP), followed by 1:100 secondary tetramethylrhodamine isothiocyanate (TRITC)-conjugated antirabbit IgG. The nuclei were stained with 0.5 μm/mL Hoechst 33258 diluted in each secondary antibodies mixture.

Coverslips were mounted with Vectashield mounting medium and examined in a Zeiss Axioplan 2 fluorescence microscope.

2.4. Immunocytochemistry oil red O staining

Indifferentiated (S0) and differentiated (S10) neurospheres grown on Matrigel GFR glass coverslips were fixed with 10% formaline in PBS for 10 minutes at room temperature (RT) and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at RT. Nonspecific binding sites were blocked with PBS containing 10% BSA for 30 minutes at RT. Immunocytochemistry staining was performed with mouse antinestin (1:5) and anti-GFAP 1:2000 in PBS containing 10% BSA for 1 hour at RT and then with peroxidase-conjugated antimouse IgG secondary antibodies (1:200 in PBS containing 10% BSA) for 30 minutes at RT; the immunoreactivity was detected with the 3,3′diaminobenzidine (DAB) reaction. Subsequently, the oil red O staining was performed by the method of Diascro et al. (1998), with minor modifications. Briefly, the cells were stained with 0.35% oil red O, for 1 hour at RT. The working solution of oil red O was prepared as described by Ramirez-Zacarias et al. [19].

After washing with distilled water, cells were counterstained with Mayer's hematoxylin and allowed to air dry. Coverslips were mounted with Kaiser's glycerol gelatin and observed with a Leitz Wetzlar Ortholux light microscope.

2.5. Protein detection

For cell lysis, 10^7 cells were suspended in 150 μL of RIPA lysis buffer containing NaF [100 mM], $Na_4P_2O_7$ [2 mM], Na_3VO_4 [2 mM], NP-40 [1%], SDS [0.1%], EDTA [5 mM], DOC [0.5%], protease inhibitor cocktail, in PBS 1x solution. The lysates were cleared by centrifugation at 12000 rpm for 20 minutes.

Protein concentration was determined by BCA protein assay kit, using bovine serum albumin as a standard. Samples (20/50 μg protein) were run on 10%–15% polyacrylamide denaturing gels according to Laemmli [20]. Protein bands were transferred on polyvinylidene difluoride (PVDF) sheets by wet electrophoretic transfer according to Towbin et al. [21]. Nonspecific binding sites were blocked for 1 hour at room temperature with 5% nonfat dry milk in Tris-buffered saline containing 0.25% Tween 20 (TBS-T). Membranes were incubated with the primary antibody at the appropriate dilutions [1:50 for mouse antinestin, 1:1000 mouse anti-GFAP, 1:2000 rabbit antiactin, rabbit anti-PPARα, β, γ] overnight at +4°C in blocking solution, followed by incubation with HP-conjugated secondary antibody (antirabbit; antimouse), at the appropriate dilution (1:2000 in blocking solution), for 1 hour at 4°C. After rinsing, the specific immune complexes were detected by ECL method. Band relative densities were determined and normalized using a semiquantitative densitometric analysis and values are given as relative units.

2.6. RT-PCR

Total cellular RNA was extracted by trizol reagent (Invitrogen) according to the manufacturer's instructions. The total RNA concentration was determined spectrophotometrically in RNAase-free water and 1 μg aliquots of total RNA were reverse transcribed into cDNA using Kit Gene Specific Relative RT-PCR. After RT 2 μL of the cDNA was used as template in 20 μL of PCR mixture and *Taq* platinum. The number of cycles was obtained empirically by sampling the PCR amplification of positive control between 22 and 40 cycles and selecting the approximate midpoint of a linear

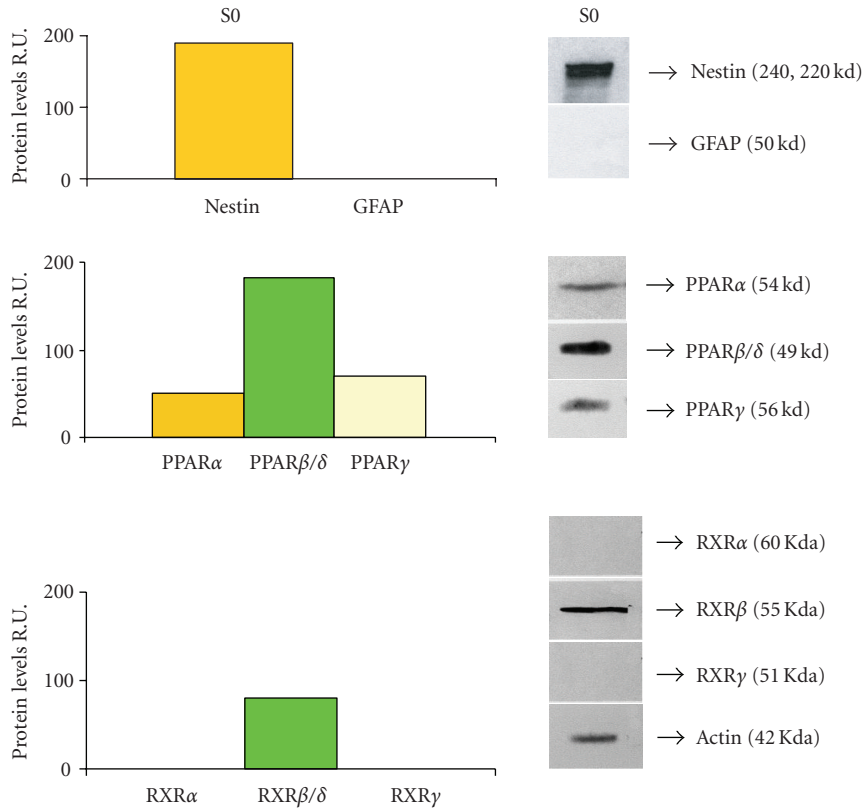


Figure 4: Western blotting and relative densitometric analysis in S0 neurosphere cell lysates. An example of western blotting is shown. Densitometric data are means ± SD of 5 different experiments.

amplification. Table 1 reports primers sequences and amplification conditions for each gene studied. β-Actin was used as internal control and used for normalization. PCR products were separated by electrophoresis on 2% agarose gels containing ethidium bromide (0.5 μg/mL) in Tris-borate EDTA buffer. A molecular weight marker was run in parallel and bands of the expected molecular size were detected under UV light. The relative densities of the PCR fragments were determined and normalized using a semiquantitative densitometric analysis and values are given as relative units.

2.7. Statistics

Statistical analysis for multiple comparisons was performed by one-way ANOVA followed by Scheffe's post hoc test. All statistical calculations were performed using SPSS software. P values <.05 were considered statistically significant.

3. RESULTS

In Figure 1, contrast phase microscopy of neural stem cells growing in neurospheres (Figure 1(a)) and after BrdU incorporation (Figure 1(c)) are shown. Nuclear staining with Hoechst 33258 (Figure 1(b)) clearly shows that almost all cells appear positive for BrdU indicating that they are mitotic in our experimental conditions. Since the proliferation ability is not only exclusive of stem cells, but is shared with progenitors of different lineages, markers of indifferentiated status have also been investigated.

The immunolocalization of nestin (Figure 2(b)) as compared with Hoechst nuclear staining (Figure 2(a)) shows that almost all cells are immunopositive for nestin, which is asymmetrically concentrated in the perinuclear region. Proteolipid protein (PLP) immunolocalization Figure 2(e), membrane protein of indifferentiated status, shows that almost all cells appear immunopositive for PLP (compare with Figure 2(d)). Only few cells are immunopositive for A2B5, marker of astroglial restricted precursors (Figure 2(c)). GFAP, β tubulin III, and O4, markers of astrocytes, neurons, and oligodendrocytes, respectively, are not expressed (not shown).

Figure 3 shows the immunolocalization of the three PPAR isotypes in neurospheres. Nuclear staining of the same fields is shown in Figures 3(a), 3(b), and 3(c). All the three PPARs are present, almost exclusively localized in the nuclei. See Figures (3(b), 3(d), 3(f)).

Western blotting analysis for nestin, GFAP, PPARα, β, and γ, and RXRs in neurosphere cell lysates confirms the presence of the three PPARs and shows that the only RXR isotype detectable in these cells is the RXRβ (Figure 4).

To assess the possible quantitative/qualitative variations of the receptors during differentiation, neurospheres were cultured in absence of growth factors and in the presence of 10% FBS for 5 days (S10). Figure 5 shows the

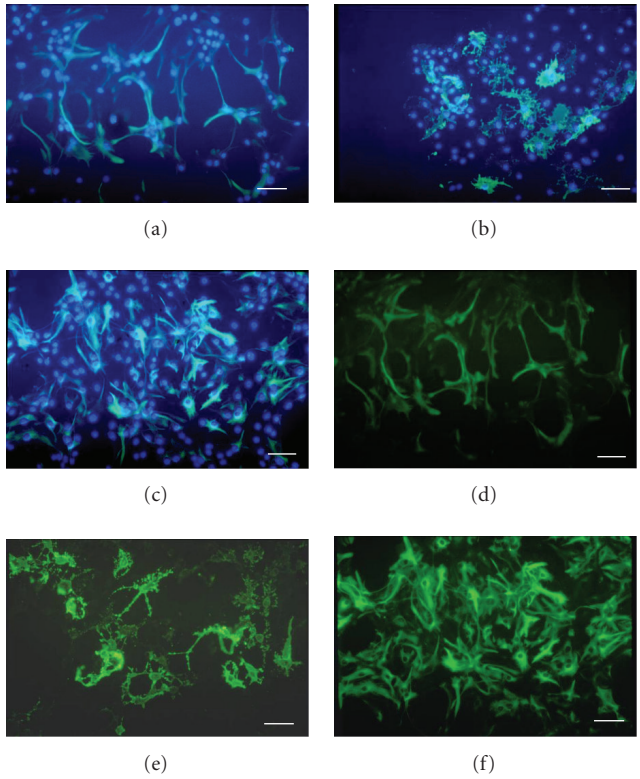


Figure 5: Immunolocalization of nestin, A2B5, and GFAP in S10 neurospheres. In (a), (b), and (c), double immunostaining of nestin/Hoechst, A2B5/Hoechst, and GFAP/Hoechst is shown, respectively. In (d), (e), and (f), the single immunostaining is shown. Bar = 40 μm.

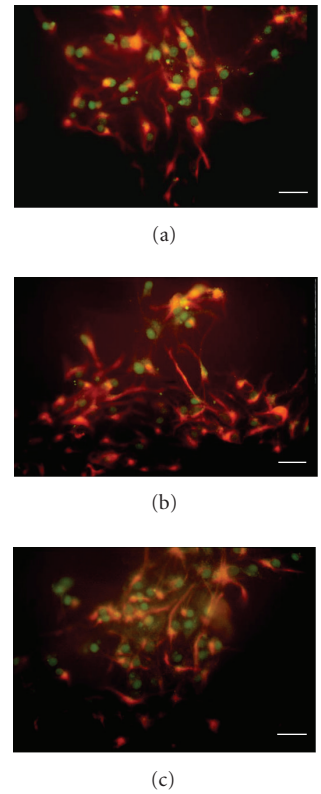


Figure 6: Double immunofluorescence staining for GFAP/PPAR in S10 neurospheres is shown. (a) PPARα, (b) PPARβ, (c) PPARγ. Bar = 30 μm.

immunolocalization of the above-mentioned differentiation markers in S10 neurospheres. Nestin is still expressed, but with lower Fuorescence intensity (Figures 5(a) and 5(b)). Moreover, the protein is no more concentrated in the perinuclear region, but unifromely localized throughout the cytoplasm, including the cellular processes; the number of A2B5 immunopositive cells appears slightly increased (Figures 5(b) and 5(e)), while a clear immunofluorescence for GFAP (Figures 5(c) and 5(f)) is observed in many S10 cells. β-Tubulin III and O4 are absent (not shown).

These results demonstrated that, in our differentiating conditions, S10 neurospheres are mainly composed by differentiated astrocytes and their A2B5 precursors.

In Figure 6, double immunofluorescence staining for GFAP and PPARs in S10 neurospheres is shown. In these cells the PPARs are still present but with different fluorescence intensity. In particular, PPARα immunostaining (Figure 6(a)) is stronger, while PPARβ appears weaker than in S0 neurospheres (Figure 6(b)); PPARγ appears unchanged (Figure 6(c)).

Figure 7 shows the western blotting analysis for nestin, GFAP, PPARs, and RXRs in S0 and S10 neurosphere cell lysates. In S10 cells, nestin is significantly decreased, while GFAP is strongly expressed. Interestingly, RXRα, not present in S0 neurospheres, is now detected while RXRβ is unchanged. In agreement with the immunofluorescence data, PPARβ is strongly decreased and PPARγ appears unchanged; concerning PPARα, no significant quantitative differences are observed.

The RT-PCR analysis of PPAR mRNAs in S0 and S10 neurospheres (Figure 8) shows that, during astroglial differentiation, PPARα is significantly increased while PPARβ expression is significantly decreased. PPARγ appears unchanged.

Figure 9 shows the double staining of oil red positive lipid droplets and nestin in S0 (Figure 9(a)) and oil red/GFAP in S10 (Figure 9(b)) neurospheres. Nuclei were counterstained with Mayer heamallume. In S0 neurospheres, almost all immunoreactive nestin cells show several lipid droplets in their cytoplasm, some of which being very large. In S10 GFAP-positive cells, lipid droplets are no more observed.

4. DISCUSSION

In this paper, the presence of all three isotypes of PPARs in mouse adult neural stem cells has been established for the first time. Moreover, we demonstrated that PPARs are subjected to both quantitative and qualitative variations during astroglial differentiation.

The proliferative and undifferentiated status has been demonstrated by immunofluorescence and western blotting.

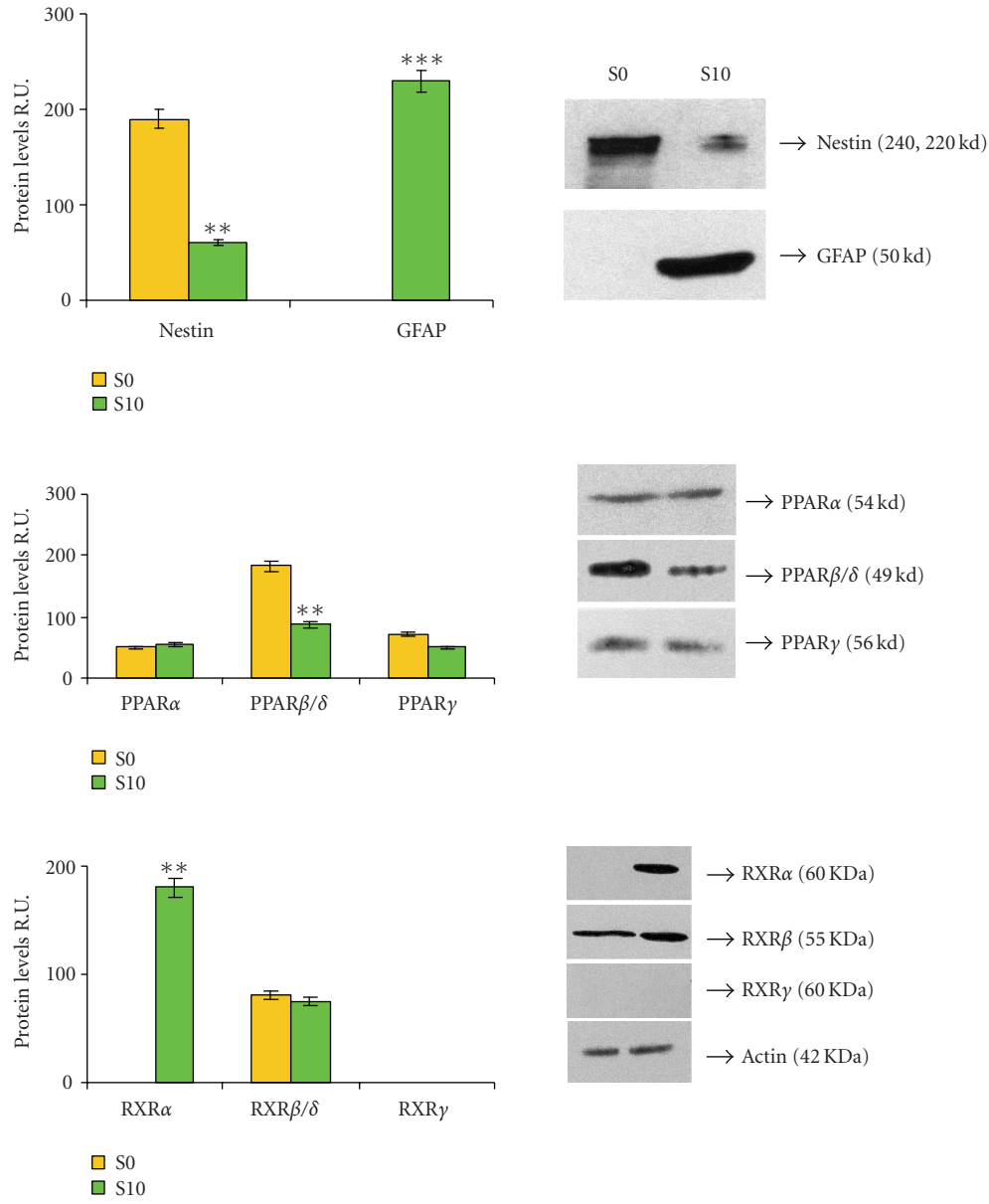


FIGURE 7: Western blotting and relative densitometric analysis in S10 neurosphere cell lysates. An example of western blotting is shown. Densitometric data are means ± SD of 5 different experiments. *, $P < .05$; **, $P < .001$.

BrdU incorporation demonstrates that almost all cells of the neurospheres are proliferative and the presence of nestin and PLP, in the absence of markers of differentiation such as GFAP, β-tubulin III, and O4, is cosistent with the undifferentiated status and allows to conclude that the cellular population of our neurospheres is constituted by undifferentiated cells [22].

The strongly polarized immunolocalization of nestin suggests that the cells are dividing by asymmetric divisions. In fact, recent studies have demonstrated that, in stem cells, some proteins exhibit different distribution according to their division modality [23, 24].

The result that neural stem cells possess all three PPAR isotypes is new and unexpected. In fact, one would have hypothesized that PPARβ could be the most abundant owing to its relevant presence and early expression during brain development [15] and owing to its involvement in cell proliferation and in the first stages of cellular differentiation [25–27]. Our results demonstrate that all three PPARs are expressed and that they have a nuclear localization in agreement with their function as transcription factors.

It is known that PPARs act in heterodimeric form with RXRs. The immunoblotting data reveal that in neural stem cells only RXRβ is present. This finding is in agreement with previous results demonstrating this isotype as the mainly present in rodent brain [28, 29] and suggests that one or more PPAR isotypes may heterodimerize with RXRβ.

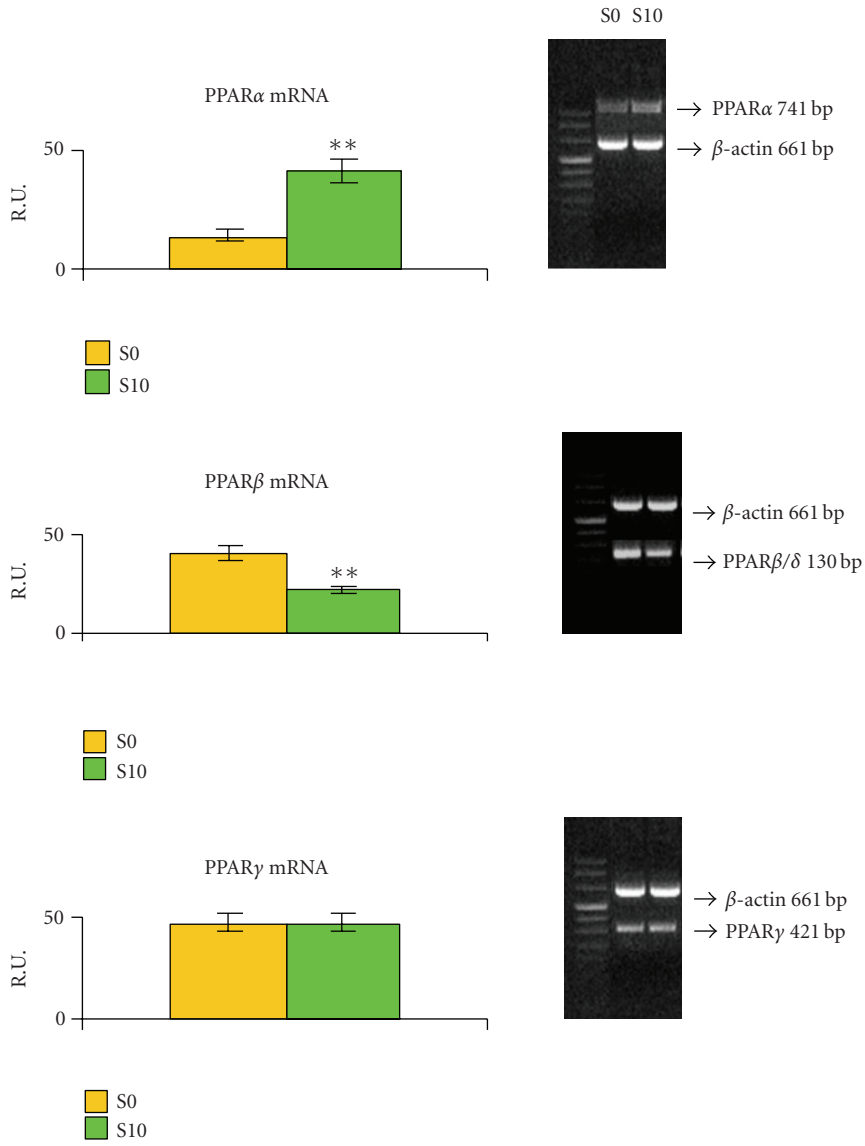
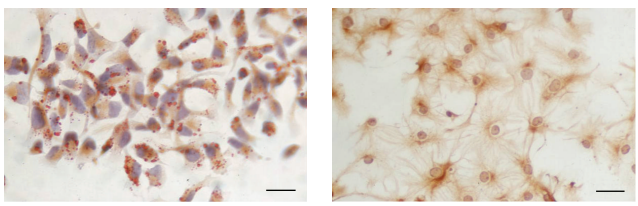


Figure 8: RT-PCR analysis in S0 and S10 neurospheres. An example of RT-PCR is shown. Densitometric data are means ± SD of 5 different experiments. Semiquantification has been performed against the housekeeping gene β-actin. **, $P < .001$.



(a) (b)

Figure 9: Double oil red/nestin in S0 neurospheres (a) and oil red/GFAP in S10 (b) neurospheres. Bar = 20 μm.

The simultaneous presence of the three PPARs in the nucleus does not indicate that they are all transcriptionally active; in fact it has been proposed that unliganded PPARβ may act as potent inhibitor of the transcriptional activity of the α and γ isotypes [30]. It is possible to hypothesize that in neural stem cells PPARβ contributes to the maintenance of the undifferentiated, proliferative status, by regulating both genes involved in cell cycle control, as observed in other cell types [18, 31, 32], and inhibiting the activity of the other PPARs, which may be, in turn, involved in cellular differentiation [13, 14].

The finding of large lipid droplets in the cytoplasm of NSC is new and suggests a role for PPARγ in this phenomenon. In fact, the importance of this transcription factor is well known in adipocyte differentiation as well as in cellular types where lipidogenesis occurs, such as oligodendrocytes and macrophages [33, 34]. In agreement with this hypothesis, the PPARγ appears to be strongly expressed both at mRNA level and at protein level in undifferentiated NSC.

When NSC were subjected to astroglial differentiation, as expected, GFAP was highly expressed and the nestin was significantly decreased. Moreover, its intracellular distribution is completely different from S0 neurospheres, with the asymmetrical concentration of the protein in the juxtanuclear region being no more observed. The persistance of nestin in these differentiated cells is consistent with data from other authors that have reported a coexpression of GFAP and nestin in astrocytes in culture from postnatal animals; this coexpression, which is not observed in vivo, is induced by in vitro conditions and in vivo during astrogliosis [14, 35].

In the S10 cells, PPARs undergo quantitative modifications. A modulation of PPARs both at protein and mRNA levels is observed. The observed strong decrease of PPARβ is particularly interesting, since it could indicate the removal or reduction of its inhibitory effect on the other PPARs [30]. In this respect, PPARβ might be considered as inhibitor of astroglial differentiation [30, 36]. PPARγ does not vary, both at mRNA and protein levels, while PPARα is significantly increased only at mRNA level. This might be due to the fact that the RT-PCR and western blotting analyses were performed after 5 days of differentiation in vitro. Probably, to observe a significant increase of the protein, a longer time should be tested. However, the increase of PPARα suggests a role for this transcription factor in astroglial differentiation, supported by our previous findings on astrocyte in in vitro differentiation [14]. Moreover, the appearance of RXRα, its heterodimeric pattern [29], is in agreement with this suggestion. As regards RXRs, during NSC astroglial differentiation, the data obtained demonstrate that RXRγ is never expressed, in agreement with its restricted localization in adult brain [29, 37], RXRβ remains unchanged, while RXRα is expressed de novo by differentiated cells. Thus, a downregulation of PPARβ, accompanied by PPARα and RXRα increase may be a condition for the differentiation toward astroglial lineage.

As regards PPARγ, the fact that this receptor is not modified may indicate that it is not crucial for astrocyte differentiation, at least concerning the differentiation of type I astrocytes. However, the presence of some A2B5/GFAP immunopositive cells may indicate that, in our experimental conditions, differentiation toward type II astrocytes may also occur. Since type II astrocytes share a common progenitor with oligodendrocytes, the O2A cells, the persistence of PPARγ in differentiating neurospheres could indicate that it may be involved in the oligodendrocyte differentiation pathway.

Regarding the presence of lipid droplets in undifferentiated cells, their disappearance during differentiation may be in agreement with the hypothesis that in our experimental conditions, the differentiation toward type I astrocytes is preferred. In fact, differentiated astrocytes are able to utilize lipids as energy fuel [38] through catabolic lipid pathways requiring PPARα and not PPARγ activity, involved instead in lipidogenesis.

Overall, the data presented in this work indicate that the decrease of PPARβ and the concomitant increase and/or activation of PPARα together with RXRα are involved in astroglial differentiation of NSC.

In our opinion, however, it should be underlined that the regulation of different differentiation pathways and/or the maintenance of undifferentiated status are more affected by the quantitative ratios existing among the receptors isotypes (both PPARs and RXRs) rather than by the absolute amounts of each one of them.

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