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MINI REVIEW ARTICLE

Polyamine delivery as a tool to modulate stem cell differentiation in skeletal tissue engineering

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Abstract The first step in skeleton development is the condensation of mesenchymal precursors followed by any of two different types of ossification, depending on the type of bone segment: in intramembranous ossification, the bone is deposed directly in the mesenchymal anlagen, whereas in endochondral ossification, the bone is deposed onto a template of cartilage that is subsequently substituted by bone. Polyamines and polyamine-related enzymes have been implicated in bone development as global regulators of the transcriptional and translational activity of stem cells and pivotal transcription factors. Therefore, it is tempting to investigate their use as a tool to improve regenerative medicine strategies in orthopedics. Growing evidence in vitro suggests a role for polyamines in enhancing differentiation in both adult stem cells and differentiated chondrocytes. Adipose-derived stem cells have recently proved to be a convenient alternative to bone marrow stromal cells, due to their easy accessibility and the high frequency of stem cell precursors per volume unit. State-of-the-art "prolotherapy" approaches for skeleton regeneration include the use of adipose-derived stem cells and platelet concentrates, such as platelet-rich plasma (PRP). Besides several growth factors, PRP also contains polyamines in the micromolar range, which may also exert an anti-apoptotic effect, thus helping to explain the efficacy of PRP in enhancing osteogenesis in vitro and in vivo. On the other hand, spermidine and spermine are both able to enhance hypertrophy and terminal differentiation of chondrocytes and therefore appear to be inducers of endochondral ossification. Finally, the peculiar activity of spermidine as an inducer of autophagy suggests the possibility of exploiting its use to enhance this cytoprotective mechanism to counteract the degenerative changes underlying either the aging or degenerative diseases that affect bone or cartilage.

Keywords Polyamines · Adipose-derived stem cells · Skeleton development · Osteogenesis · Apoptosis

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Introduction

Polyamines are essential for cell proliferation and normal cell growth during development and remodeling of mammalian tissues (Pendeville et al. 2001). Polyamine levels and their metabolic pathways are closely regulated in cells, given their potential role in determining cell fate and differentiation via their involvement in epigenetic tuning of signaling pathways and modulation of transcription and translation. Ornithine decarboxylase (ODC), the key biosynthetic enzyme in polyamine metabolism, is up-regulated upon condensation of mesenchymal stem cells, and the intracellular concentration of these polycations can be subsequently finely modulated by intervening in both transport systems and tuning of anabolic and catabolic pathways. Evidence from literature indicates that polyamine levels and their biosynthetic enzymes are significantly modulated on differentiation (Rath and Reddi 1981; Vittur et al. 1986; Bargoni and Tazartes 1988), and conversely from differentiated to induced pluripotent stem cells (Meissen et al. 2012; Zhao et al. 2012), thus suggesting that this class of molecules is a powerful tool to obtain an array of different cell lineages for musculoskeletal regeneration starting from stem cells. Furthermore, growing evidence indicates that polyamines also provide protection against DNA oxidative damage (Rider et al. 2007) and/or promote cytoprotective processes such as autophagy (Bennetzen et al. 2012; Marino et al. 2011); these observations further increase their appeal in regenerative medicine.

The metabolic pathways of these polycations (Casero and Marton 2007) are briefly summarized in Fig. 1. The key biosynthetic enzyme is ODC, which is required to produce putrescine, the simplest polyamine, via decarboxylation of ornithine. A parallel pathway is then engaged, controlled by another decarboxylase, *S*-adenosylmethionine decarboxylase (SAM DC), which delivers decarboxylated *S*-adenosylmethionine (dcSAM). The latter is required to produce the other members of the polyamine family: spermidine via the catalytic activity of spermidine synthase (SPDS), which joins an aminopropylic group from dcSAM to putrescine, and finally spermine via the catalytic activity of spermine synthase (SPMS), which joins an aminopropylic group from dcSAM to spermidine. Catabolism is manly controlled by spermidine/spermine N^1 acetyltransferase (SSAT) which intervenes to regulate the cellular content of polyamines via their acetylation. Acetylated products are either excreted from the cells or oxidized by acetylpolyamine oxidase (PAO) that causes the interconversion back to the lower polyamine. A spermine

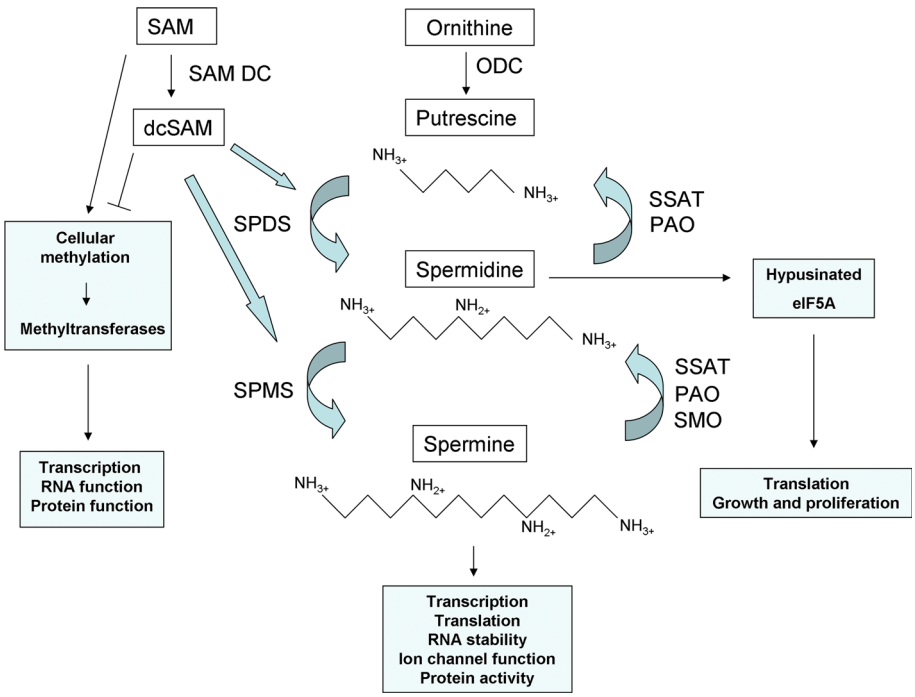


Fig. 1 Polyamine metabolism and cellular functions. The scheme is adapted from Zhao et al. (2012) and Childs et al. (2003). ODC and SAM DC are the two rate-limiting enzymes for biosynthesis. ODC decarboxylates ornithine to putrescine, which is converted into spermidine and then spermine by SPDS and SPMS, which use dcSAM as the aminopropyl donor. SSAT, PAO and SMO are catabolic enzymes. Interconnections between polyamines pathways and cellular functions are indicated in *boxes*. SAM, *S*-adenosylmethionine; dcSAM, decarboxylated *S*-adenosylmethionine; SAM DC, *S*-adenosylmethionine decarboxylase; ODC, ornithine decarboxylase; SPDS, spermidine synthase; SPMS, spermine synthase; SSAT, spermidine/spermine N^1-acetyltransferase; PAO, acetylpolyamine oxidase; SMO, spermine oxidase

oxidase (SMO) has been described that is able to directly oxidize spermine producing spermidine (Cervelli et al. 2012).

Polyamines in skeletal tissue differentiation: role in chondrogenesis and endochondral ossification

During development, the skeleton is formed through two different types of ossification depending on the type of bone: intramembranous ossification (the skull bones and the lateral halves of the clavicles) and endochondral ossification (long bones). In both cases the first step is condensation of mesenchymal stem cells, but different phenomena occur downstream: in "intramembranous ossification" the bone is deposed directly in the mesenchymal anlagen by the newly differentiated osteoblasts; in "endochondral ossification", the bone is deposed on a cartilage template originating from the condensed mesenchyma through a process termed chondrogenesis (Goldring et al. 2006). Therefore, from a developmental point of view there is both "permanent" and "temporary" cartilage, the first destined to cover the surface of the joints and the latter representing the template for endochondral ossification, a process which, besides morphogenesis of long bones, is also involved in fracture healing or growth plate closure. In "temporary" cartilage, chondrocyte differentiation occurs across a timely and spatially tightly regulated pattern with mesenchymal cell condensation, chondroprogenitor proliferation and differentiation down to terminal differentiation and cartilage matrix calcification (Goldring et al. 2006). The cartilage template is called "temporary" because its default fate is terminal differentiation and matrix mineralization, and differs from articular cartilage which is programmed to remain "permanent" and where calcification occurs only pathologically as the result of a "loss of maturational arrest". Chondrogenesis and endochondral ossification as well as cartilage derangement in osteoarthritis (OA) are orchestrated by pivotal transcription factors (TFs): RUNX2, SOX-9 and β-catenin (Fig. 2). The transcription factors RUNX2 and SOX-9 not only determine, respectively, the osteoblastic or chondroblastic fate of the staminal precursor, but are also involved in the progression of the differentiative programs. Wnt pathway and β-catenin, which acts as transcriptional co-activator in complex with TCF/LEF transcription factor, are essential in both intramembranous and endochondral bone formation (Hartmann 2006). The canonical Wnt signaling pathway is evolutionarily conserved and is activated by the binding of a Wnt-protein ligand to a Frizzled family receptor, which in turn triggers the phosphoprotein Dishevelled inside the cell, leading to the activation of β-catenin necessary for transcriptional activity (Hartmann 2006).

The involvement of the polyamine pathway in stem cell chondrogenesis has been long known. In an in vivo model of endochondral bone development, the kinetics of the induction of polyamines and their biosynthetic enzymes were analyzed (Rath and Reddi 1981). Both ODC and SAM DC were strongly up-regulated in stem cells before chondrogenesis, which was marked by a nearly twofold increase of the intracellular polyamines. Notably, these molecules markedly grew with a peak more than fivefold the initial concentration coincident with extracellular matrix (ECM) remodeling, preosteoblast differentiation and early osteogenesis. Other studies showed that the addition of exogenous polyamines to the cultures of growing rabbit costal chondrocytes promoted glycosaminoglycan (GAG) production, a marker of the differentiated chondrocyte phenotype (Takano et al. 1981). ODC induction as a critical step in the establishment of a differentiated chondrocyte phenotype was also confirmed by other studies showing that parathormone (PTH) was able to induce ODC activity in quiescent chondrocytes prior to GAG synthesis, which in turn was prevented by an ODC inhibitor (Takigawa et al. 1981; Takano et al. 1983). We also previously reported that IKKα, a critical inducer of chondrocyte differentiation (Olivotto et al. 2008), is able to regulate both ODC mRNA expression and activity (Facchini et al. 2012). In the "temporary" cartilage template, ODC has only been immunolocalized in resting chondrocytes (Vittur et al. 1986) and, therefore, this is the area where polyamine biosynthesis takes place. However, polyamines and particularly spermidine are mainly detected extracellularly at the level of the ossifying zone. This is where spermidine plays a crucial role in ECM remodeling preceding its calcification and contributing to dissociation and partial removal of proteoglycans from collagen (Vittur et al. 1986). A high expression of polyamines and their key metabolic enzymes (ODC, SAM DC and SSAT) has also been confirmed in the cartilage of chicken embryo during development (Bargoni and Tazartes 1988).

The expression of tissue transglutaminase (also known as transglutaminase 2, TG2), which can use polyamines as a substrate in skeletal tissues, is strictly regulated and correlates with chondrocyte differentiation, cross-linking of the mineralizing matrix and cartilage calcification. Notably, whereas TG is absent in normal healthy articular cartilage (Aeschlimann et al. 1993), increased transglutaminases-mediated activity is reported in OA (Orlandi et al. 2009). In endochondral ossification, TG externalization occurs before matrix mineralization preceding bone formation, and this sequence of events is recapitulated in OA development as described below. According to the Evo-Devo pathogenic model of OA (Aigner et al. 2007), articular cartilage degeneration underlying OA can be considered as the result of chondrocyte "loss of

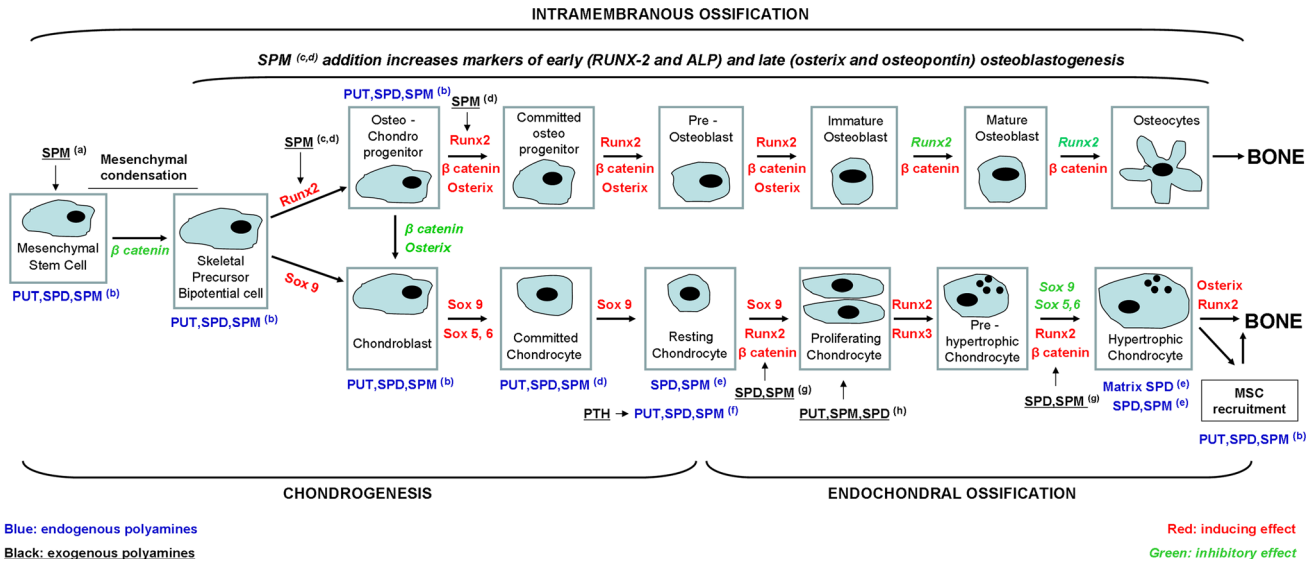


Fig. 2 Diagram illustrating the two pathways of ossifications, i.e., intramembranous and endochondral, with reference to the transcription factors involved. References for the figure are derived from Goldring et al. (2006), Hartmann (2006), Hu et al. (2005) Kobayashi and Kronenberg (2005), Komori (2006), Mackie et al. (2008), Nakashima and de Crombrugghe (2003), Wagner and Karsenty (2001). *PUT* putrescine, *SPD* spermidine, *SPM* spermine. References to the presence of endogenous polyamines are indicated in *blue*, whereas references to the exogenous addition of polyamines to boost differentiation are indicated in *black*. Along the differentiation pathways, transcription factors with an inducing effect are indicated in *red*, while those with an inhibiting effect are indicated in *green*. *Mesenchymal precursor recruitment and condensation* At the stage of precursors, exogenous SPM increases SSAT and polyamine modulating factor-1 (PMF-1) gene expression in human adipose-derived mesenchymal stem cells [**a**, (Tjabringa et al. 2006)]. In an in vivo model of endochondral ossification, during MSC condensation and proliferation and before chondroblast differentiation, a peak in ODC and SAM DC activity was found. This resulted in an increase in polyamines, particularly PUT, concomitant with mesenchymal cell proliferation and chondrogenesis [**b**, (Rath and Reddi 1981)]. *Intramembranous ossification* At the level of skeletal precursors (goat adipose-derived stem cells), SPM addition determines an increase in RUNX-2, an early marker of osteoblastogenesis and osteopontin, a marker of late differentiation. SPM induces SSAT catabolic enzyme, together with an increase of alkaline phosphatase activity, a marker of early osteoblastogenesis [**c**, (Tjabringa et al. 2008)]. In human adipose-derived stem cells grown in 3D culture, SPM induces RUNX-2, β-catenin and Osterix, the three principal transcription factors in osteoblastogenesis. SPM induces RUNX-2 and β-catenin expression and increases their nuclear translocation and activation. SPM also increases alkaline phosphatase expression. Both SPM and SPD induce calcium deposition [**d**, (Guidotti et al. 2013)]. *Chondrogenesis and endochondral ossification* As stated above, initial chondrogenesis is associated with increased PUT, SPD and SPM levels. Notably, a high polyamine peak was found during osteogenesis and osteoprogenitor differentiation of mesenchymal cells recruited at the site of bone formation [**b**, (Rath and Reddi 1981)]. In resting chondrocytes, PUT was undetectable, whereas SPD and SPM were immunodetected, with a loss of staining in proliferating and pre-hypertrophic chondrocytes, in which only SPD is present in the extracellular matrix. SPD and SPM increase the activity of alkaline phosphatase that is essential for matrix remodeling. Particularly, SPD interacts with and dissociates proteoglycans from collagen in matrix remodeling. Therefore, polyamines are involved in pre-osseous cartilage calcification [**e**, (Vittur et al. 1986)]. In confluent quiescent cultures of chondrocytes, PTH addition increases ODC activity and polyamine synthesis, which precedes synthesis of GAG, a marker of chondrocyte differentiation [**f**, (Takigawa et al. 1981)]. In the 3D culture of osteoarthritic human chondrocytes, SPD and SPM addition induces chondrocyte terminal differentiation promoting SOX-9, β-catenin and RUNX-2 expression, together with MMP-13 and collagen X, markers of the chondrocyte hypertrophic maturation. Moreover, SPD increases calcium deposition [**g**, (Facchini et al. 2012)]. In log phase chondrocyte cultures, the addition of PUT, SPD and SPM induced differentiation (GAG synthesis) without affecting proliferation. This suggests that these molecules are essential for the expression of the differentiated phenotype [**h**, (Takano et al. 1981)] (color figure online)

maturational arrest" (Drissi et al. 2005) and downstream progression along the normal chondrocyte differentiation pathway to hypertrophy and terminal differentiation. Accordingly, osteoarthritic "hypertrophic" articular chondrocytes acquire the expression of TG2 (Tarantino et al. 2013). It has been reported that the CXC chemokines IL-8/CXCL8 and GROα/CXCL1, particularly abundant in the OA chondrocyte inflammatory environment (Attur et al. 2002; Borzi et al. 1999; Pulsatelli et al. 1999), are responsible for the p38 MAPK-mediated stimulation of TG2 activity (Merz et al. 2003). TG2 is then in turn responsible for the elicitation of the hypertrophic phenotype and particularly for the induction of collagen X and MMP-13 (Merz et al. 2003), the latter being the most active collagenase for proteolytic cleavage of collagen 2 and release of bioactive neoepitopes (C1, 2C) able to further boost the differentiation process (Dejica et al. 2012; Gauci et al. 2008). TG2 has been indicated as being important in mediating cartilage remodeling, in accordance with the observation of reduced cartilage destruction in TG2 KD

mice undergoing OA inductive surgery (Orlandi et al. 2009). TG2 is essential to promote calcification of the surrounding matrix in hypertrophic chondrocytes (Johnson and Terkeltaub 2005) and osteoblasts (Heath et al. 2001), and is pivotal in supporting osteoblast maturation (Nurminskaya and Kaartinen 2006). Moreover, by acting as substrates for TG2-mediated post-translational modifications, polyamines may increase the activity of proteins, such as phospholipase A2, which can further boost inflammation in OA (Cordella-Miele et al. 1993).

The importance of the polyamine metabolic pathway and particularly the pivotal role of spermine in osteogenesis are also supported by the phenotype of a naturally occurring genetic defect of spermine synthase, the Snyder–Robinson syndrome. This disease is responsible for multiple skeletal defects and osteoporosis (Cason et al. 2003; Janne et al. 2004). It is therefore likely that spermine plays a unique role in bone development, which cannot be compensated by spermidine. In this perspective, our recent report indicates that at equimolar concentrations, spermine proved to be more efficient than spermidine in inducing RUNX2 and MMP13 mRNA expression, RUNX2 nuclear localization and ECM remodeling in 3D chondrocyte cultures (Facchini et al. 2012). The activity of spermine in cartilage might be potentiated compared to the other polyamines because of its peculiar facilitated transport via glypican 1-mediated endocytosis (as reviewed by (Poulin et al. 2012). It is interesting to note here that glypican belongs to a family of proteoglycans specifically expressed during chondrogenesis (Knudson and Knudson 2001) and that there is a positive feedback loop between the early osteoblast commitment factor RUNX-2 and glypican 1 expression (Teplyuk et al. 2009). These findings are highly suggestive of a relevant role of spermine in osteogenesis and in osteoblast maturation of committed osteoprogenitors.

Polyamines as pleiotropic regulators

The progression of stem cells across toti-, pluri-, multi-, oligo-, and uni-potency ultimately leading to somatic cell differentiation occurs through dramatic changes of the transcriptome as a result of the recruitment of selected TFs, whose activity is made possible by specific chromatin modifiers, which intervene in opening specific portions of the chromatin while others are kept inaccessible by virtue of histone post-translational modifications and DNA methylation (Tollervey and Lunyak 2012). Some recent literature suggests that polyamines are able to epigenetically affect the level of cell activation: increased polyamine metabolism is associated with a decreased level of S-adenosylmethionine (SAM), causing global DNA hypomethylation and therefore increased activity of transcription factors (Karouzakis et al. 2012). Besides, polyamines are able to bind directly to DNA and affect the histone acetylation status, by stabilizing nucleosomes and influencing gene expression and chromatin remodeling. These direct and indirect polyamine effects on chromatin remodeling have consequences on DNA structure, function and stability, since these molecules are also required for the activity of DNA topoisomerase II (Alm et al. 1999).

An increasing number of literature reports highlight the ability of spermidine to favor autophagy via effects on both the acetylproteome (Marino et al. 2011) and the phosphoproteome (Bennetzen et al. 2012). With regard to acetylproteome, converging and biphasic activities of spermidine and resveratrol are described, with a fast autophagic response mediated by deacetylation of cytoplasmic protein followed by a delayed, protein synthesis-mediated sustained autophagic response, dependent on transcriptional reprogramming. This occurs via the inhibition of histone H3 acetylation and therefore global gene silencing, but at the same time with the acetylation of selected nuclear proteins. More in detail, spermidine and resveratrol similarly affect the acetylation pattern of 170 proteins belonging to the recently elucidated human autophagy protein network (Marino et al. 2011). The evaluation of the spermidine autophagy promoting activity in cartilage and the assessment of the critical spermidine concentration able to exert these effects would be very interesting, since autophagy, a fundamental homeostatic mechanism for removal of dysfunctional cell components, which is compromised in aging, has recently been recognized as a key cytoprotective activity in articular cartilage (Lotz and Carames 2011). With regard to phosphoproteome, spermidine has also proven able to drive the post-translational modification of essential intermediates of the apoptotic signaling pathway, thus suggesting a molecular mechanism for the crosstalk between autophagy and apoptosis (Bennetzen et al. 2012).

However, throughout development, the heterogeneity of different tissues might be at least in part dependent on fine tuning of the polyamine levels and their ratios at selected time windows to obtain a given cell type with a peculiar polyamine signature, by virtue of the ability of these molecules to interact with RNA, DNA and protein, including the capacity to modulate the activity of specific protein kinases. Evidence of a polyamine role in cellular phosphoproteome and kinase activity is given by studies about the serine/threonine protein kinase casein kinase 2, whose activity is stimulated by polyamines through interaction with the enzyme and induction of a change in structure into the catalytically active form (Childs et al. 2003). It has recently become clear that from simpler organisms to mammals, polyamines can co-ordinately

promote the translation of a given set of proteins called the "polyamine modulon" (Igarashi and Kashiwagi 2011) by altering RNA structures and favoring the start of translation. This modulon also includes TFs and kinases. Moreover, polyamines strongly influence some RNA-binding proteins such as the HUR family (Xiao and Wang 2011).

Besides the post-translational modification of the histones and modulation of chromatin accessibility, polyamines are also capable of influencing post-translational modification and location of the TFs themselves. This is in keeping with our previous observation that, conversely, decreasing the rate of polyamine biosynthesis by the specific ODC inhibitor DFMO impairs the release of inflammatory mediators from cells stimulated with inflammatory cytokines (Facchini et al. 2005) via effects on both NF-κB nuclear translocation and binding activity.

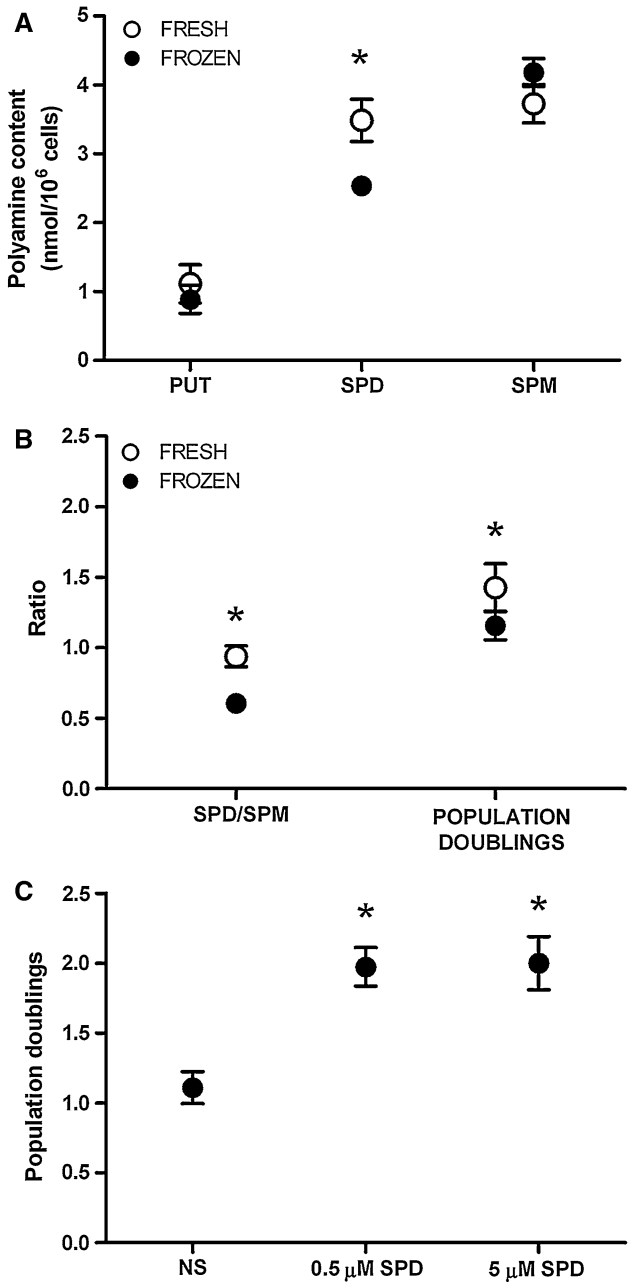
Polyamine metabolism also plays a pivotal role in the choice between proliferation and differentiation. In this perspective, a very recent paper highlighted both ODC and SAM DC, the two rate-limiting enzymes in the polyamine biosynthetic pathway, as responsible for embryonic stem cell (ESC) self-renewal, possibly via sustained polyamine biosynthesis and polyamine-dependent stabilization of the MYC protein (Zhao et al. 2012). The two decarboxylases are responsible for the delivery of putrescine and dcSAM, respectively, the precursors of both spermidine and spermine. At the same time, SAM DC reduces the availability of SAM for methylation reactions. Noteworthy, microarray analyses of ESC have indicated that SAM DC is selectively down-regulated upon differentiation, suggesting that polyamine anabolism and DNA demethylation are critically associated with totipotency. Conversely, the overexpression of SAM DC or the addition of spermine resulted in inhibition of neural progenitor cell differentiation (Zhao et al. 2012).

Spermine and spermidine are readily interconverted within the cell and therefore studies designed to determine the selective functions of each of these molecules can be biased by a certain degree of interconversion between the two molecules. This notwithstanding, polyamines have demonstrated differential activities with regard to the control of selected cell functions. The induction of autophagy has only been reported to spermidine, which also represents an absolute requirement to generate hypusine, an integral component of the eIF5A translation factor, essential for cell growth and protein synthesis. To this purpose, we have collected data suggesting that the loss of intracellular spermidine can contribute to the known vulnerability of adipose-derived stem cells (ASC) upon viable freezing (James et al. 2011). Figure 3a shows the polyamine content of ASC from three different subjects comparing cultures established with cells either with or without viable freezing. After viable freezing, the cultures show a reduction in intracellular spermidine as well as spermidine/spermine ratio and a comparable reduction of the population doublings (Fig. 3b).

To demonstrate the critical requirement of spermidine for ASC proliferation, we performed a rescue experiment using viably frozen ASC derived from three different patients, thawed and plated at 10,000 cell per cm^2 and cultured in control conditions or in the presence of 0.5 and 5 μM spermidine. As shown in Fig. 3c, spermidine at both 0.5 and 5 μM concentrations significantly increased ASC proliferation rate.

Spermine and spermidine have been found to protect cells from hydrogen peroxide-induced oxidative damage (Rider et al. 2007) with a higher antioxidant activity displayed by spermine as found with the use of cells pharmacologically depleted of either spermine or spermidine. This antioxidant activity prevents DNA damage response and is particularly effective, since spermine is intimately associated with DNA and is the most effective polyamine in DNA duplex formation and DNA protection from double-strand breaks following exposure to reactive oxygen species (Childs et al. 2003). Prevention of DNA damage corresponds to prevention of apoptosis and cellular senescence and, therefore from a regenerative point of view, to an increase in the pool of precursors which better tolerate the apoptotic pressure due to the process of asymmetric division associated with differentiation. We have recently reported the anti-apoptotic activity exerted by spermine in the osteogenic differentiation of adipose-derived stem cells in 3D culture (Guidotti et al. 2013). These results are in accordance with increased DNA damage in mouse ESC engineered to lack spermine synthase gene and therefore endowed with a normal content of putrescine and spermidine, but devoid of spermine, which is a more active ROS scavenger at low, physiological concentration (Korhonen et al. 2001).

However, the abundant literature available on the effect of polyamines on apoptosis shows contradictory results and apoptosis has been described following either higher or reduced polyamine levels (reviewed in Minois et al. (2011)). We have also previously reported the ability of DFMO, a specific ODC inhibitor, to inhibit apoptosis in rat H9c2 cardiac cells, primary or immortalized human chondrocytes and rat bone marrow stem cells (reviewed in Flamigni et al. (2007)). However, DFMO can reduce or enhance the susceptibility to apoptosis even in the same cell type, depending on the specific death stimulus and thus on the death pathway (Stefanelli et al. 2001). Moreover, DFMO treatment usually depletes cells of putrescine and spermidine, but hardly affects spermine content. Exogenous spermine at micromolar concentrations has been found to exert cytoprotective effects in some experimental models, such as in Zhao et al. (2007). In isolated rat hearts



◀**Fig. 3** Viable freezing affects both ASC intracellular polyamine content and cell culture growth, while spermidine delivery rescues the proliferation rate. ASC were obtained as indicated in (Guidotti et al. 2013) from three subjects (2 men and 1 woman, aged 53, 45 and 27). Cells at p0 were grown to confluence. Upon confluence, a part of the cells was immediately subcultured, whereas some other cells were viably frozen in DMSO, and subsequently thawed and plated. In both cases, plating density was 10,000 cells per cm^2. After the same time interval (5–11 days) for fresh or frozen culture derived from a given patient, cells were collected, counted and pelleted for HPLC analysis (Guidotti et al. 2013). **a** Polyamine content in fresh (*open circle*) or frozen (*dark circle*) cultures, as assessed by HPLC and expressed as nmol per million cells. **b** *Left* spermidine/spermine ratio of fresh and frozen cultures and *right* population doublings (ratio between the final and the starting count) of the subcultures preceding HPLC analysis for fresh (*open circle*) and frozen (*dark circle*) cells. Differences in spermidine content, spermidine/spermine ratio and population doublings of fresh versus frozen cells were statistically significant as assessed by Student's *t* test at $p < 0.05$, as indicated by *asterisk*. **c** Population doublings after 72 h of spermidine treatment of viably frozen ASC derived from three different patients (2 men and 1 woman, aged 45, 44 and 27), thawed and plated in six-well plates at 10,000 cells per cm^2. The cells were cultured in control conditions (*NS* not stimulated) or in the presence of 0.5 and 5 μM spermidine. Aminoguanidine (1 mM) was present in all the conditions. After 72 h, the cells were collected and counted. Spermidine at both 0.5 and 5 μM increased ASC proliferation rate. Differences in population doublings of ASC cultured with and without spermidine were statistically significant as assessed by Student's *t* test at $p < 0.05$, as indicated by *asterisk*

exposed to ischemia–reperfusion injury, increased activity of ODC and SSAT, leading to putrescine accumulation, was detected after acute ischemia; however, a loss of spermine associated with reduced myocardial cell viability was observed after reperfusion (Han et al. 2007). In mouse fibroblasts lacking spermine synthase activity, specific spermine deficiency did not significantly influence caspase activity after some apoptotic stimuli, i.e., etoposide, cycloheximide or staurosporine, but sensitized these cells to UV irradiation (Stefanelli et al. 2001). Therefore, the relationship between polyamines and apoptosis appears to be complex and dependent on the death stimulus and cell context, as well as on the actual level of single polyamines and the rate of polyamine pathways occurring in a particular model.

Quite recently, we have reported the dose-dependent ability of spermidine and spermine to induce hypertrophy and terminal differentiation of osteoarthritic chondrocytes (Facchini et al. 2012). This action can be interpreted as an acceleration of the differentiation process with differential activities between the two molecules: spermine was more active in inducing the osteogenic marker RUNX2, and spermidine more active in inducing the chondrogenic marker SOX-9. At the same time, increased availability of spermine and spermidine might readily induce the expression of SSAT through a recently reported mechanism of polyamine-dependent removal of translational repressor (Perez-Leal et al. 2012). It should be noted that the putrescine content depends not only on the biosynthetic ODC activity, but also on the level of the catabolic enzyme SSAT, which is low in stem cells, but increases across differentiation, such as following 1,25-dihydroxyvitamin-D3-mediated osteogenesis (Tjabringa et al. 2008). Reduction of the putrescine content has been indeed recently reported to be pivotal in cellular reprogramming of mouse fibroblasts to induced pluripotent stem cells (Meissen et al. 2012). Actually, SSAT has been indicated as a key metabolic regulator, able not only to regulate polyamine content, but also to directly affect HIF-1, NF-κB and integrin-mediated signaling (Pegg 2008).

Polyamines and stem cells in skeletal engineering

The past few years have seen the upsurge of ASC as a more convenient alternative to mesenchymal stem cells (MSC) derived from bone marrow in regenerative medicine targeted at bone or cartilage and this has already generated several clinical trials (Gimble et al. 2012). For both types of adult stem cells, the current state of the art of the literature includes studies undertaken with cultured expanded stem cells, as well as cells belonging to the original niche, i.e., bone marrow concentrate in the case of bone marrow and the stromal vascular fraction (SVF) in the case of adipose tissue. The main advantages of ASC over MSC are a lower morbidity of the harvesting procedure and a 500-fold higher rates of precursors compared to the bone marrow biopsies when normalized to the sample volume. Moreover, an increasing body of evidence shows the ability of ASC to exert a unique paracrine (Lee et al. 2011) and immunomodulatory activity (Mariani and Facchini 2012), whose importance perhaps exceeds their "plastic" function based on direct lineage differentiation.

With regard to cartilage regeneration, the two sources of cells present peculiar features and different culture requirements: MSC chondrogenesis requires TGF-β3, whereas ASC are more sensitive to BMP-6 (Hildner et al. 2011). Among ASC, there are also differences relating to the anatomical site of origin: subcutaneous ASC need to be expanded to exert a higher chondrogenic potential (Estes et al. 2006), whereas infrapatellar fat cells are already chondrogenic (Jurgens et al. 2009). Furthermore, ASC express less HLA-ABC compared to MSC and therefore are amenable to allogeneic transplantations.

Bone regeneration may be theoretically achieved via either *intramembranous* or *endochondral ossification*. With regard to "intramembranous ossification", the ASC fraction contains heterogeneous precursors of both the osteoblast and the endothelial lineages, which is a premise for a fast and successful vascularized bone engineering that requires both vasculogenesis and osteogenesis (Valenzuela et al. 2013). However, rather than exploiting the ability of performing intramembranous ossification, the advantage of pursuing an approach mimicking the process of endochondral ossification, i.e., via remodeling of a cartilaginous template (Scotti et al. 2010, 2013) to generate "bone organs" with functionality similar to that of native bones has recently been suggested.

Adipose tissue is very attractive for regenerative purposes, because it can yield both a heterogeneous SVF or culture expanded, homogeneous and adherent stem cells. Despite their regenerative potential, ASC have entered the scene of regenerative medicine with some years of delay compared to MSC and therefore disparity in their popularity compared to MSC. At the time of writing, browsing the NIH website which registers both US and non-US clinical trials (http://www.ClinicalTrials.gov) and searching with the term "adipose-derived stromal cells" we found one clinical trial aimed at cartilage regeneration (20 with MSC), four focused on bone regeneration (171 with MSC) and one focused on the regeneration of intervertebral disc (5 with MSC). Instead, searching with "stromal vascular fraction" and bone, we found one study aimed at exploiting the whole niche of adipose tissue to bone regeneration (33 with bone marrow concentrate), given the high abundance of endothelial precursors.

A blood-derived product that has recently become the focus of intense clinical interest is PRP (Amini et al. 2012; Santo et al. 2013; Sanchez-Gonzalez et al. 2012). Firstly used by dentists for bone augmentation, it has recently entered orthopedic practice. PRP derives from the platelet concentration achieved at the patients' bedside through a number of different devices. It represents a safe, easily available and cheap drug delivery system to release in situ mitogenic and chemotactic substances and bioactive peptides (Sanchez-Gonzalez et al. 2012) and can be used to locally immobilize stem cells at the site of injury. The composition of the PRPs with regard to the content of platelets, plasma, erythrocytes and white blood cells varies, depending on the device and procedure used (Tschon et al. 2011; Kon et al. 2011). The Rizzoli Orthopeadic Institute has selected a two-step procedure which yields at least fivefold platelet concentration and an enrichment in leukocytes.

We have recently analyzed the polyamine content of PRP obtained from five different subjects (age range 31–65) and found that this blood derivative presents a significant polyamine content, particularly of spermine and spermidine (Guidotti et al. 2013). Mean ± SEM of concentrations expressed as pmol/mg protein were: putrescine 7.98 ± 5.09; spermidine 172.38 ± 25.54; and spermine 155.66 ± 34.76. A preliminary evaluation performed on these same samples suggests that age affects PRP polyamine content: young individuals (30 years) presented a spermine/spermidine ratio of about 1.2, while older subjects (54–65 years) had a ratio of about 0.8. Indeed, spermine has been recently shown to be selectively reduced across age in the whole blood of healthy subjects, with a nearly ninefold decrease in people aged 60–80 versus 31–56-year-olds (Pucciarelli et al. 2012). Noteworthy, healthy nonagenarians/centenarians instead present only a slightly reduced (1.4-fold) spermine concentration compared to young people, but a higher relative percentage content suggesting that spermine can contribute to longevity and protection from degenerative disease and cancer (Pucciarelli et al. 2012). In blood, polyamine contents are highest in leukocytes and several orders higher than erythrocytes, whereas platelets contain lower levels

(Cooper et al. 1976). With regard to this, it is interesting to note that besides platelets, PRP also contains red blood cells and leukocytes, which should be considered as the major constituents responsible for the polyamine content of PRP. Indeed, polyamine content is 10^2 to 10^3-fold higher in nucleated blood cells, since most polyamines are associated with RNA, ribosomes and DNA, the latter only found in nucleated cells. Hence, PRP obtained at the Rizzoli Institute (with the two-step procedure that is associated with an enrichment in leukocytes compared to the one-step procedure) yields polyamine concentrations in the micromolar range.

Because of the higher stem cell concentration, the ease and safety of the access and the potential to transfer the intact niche, now recognized as an added value in regenerative medicine, ASC are at present the focus of much interest. Their use within the SVF in conjunction with autologous PRP is now considered as the current challenge of "prolotherapy" (or "proliferation therapy"), i.e., a method of regenerative injection treatments able to stimulate healing. ASC have also been reported to be less affected than MSC by in vitro and in vivo senescence and related loss of differentiation potential (Chen et al. 2012). However, ASC, particularly those belonging to the visceral fat of obese patients, are severely affected by an increased level of oxidative damage and senescence as a function of the body mass index of the patient, since obesity is now recognized as a true pro-inflammatory condition (Roldan et al. 2011) and ASC derived from obese patients a source of microRNAs responsible for an overall impairment of stemness and cell differentiation potential and increased senescence.

The connection between spermine and regenerative potential is in keeping with recent literature reports, which have highlighted the ability of this polyamine to boost osteogenesis in vitro starting from ASC. The first report was that of Tjabringa (2008), who described the osteogenic promoting ability of spermine during in vitro osteogenic differentiation of goat-derived ASC as assessed with induction of early (RUNX-2 and alkaline phosphatase) and late (osteopontin) markers. Spermine was also responsible for the modulation of transcription of spermine metabolic genes, particularly of SSAT. The same authors recently included spermine addition among various differentiation protocols available for osteogenic differentiation of ASC (Kroeze et al. 2011). We have recently investigated the molecular mechanisms underlying the osteogenic ability of spermine and found that it has a dual activity. Spermine addition promotes osteogenic differentiation facilitating the progression from the early to the mature osteoblast differentiation phase: it initially promotes gene and protein expression of RUNX2, an early marker of the osteoblast lineage; then, increases β-catenin expression and activation, leading to the induction of Osterix gene expression, the mature osteoblast commitment factor. Therefore, enhancement of the β-catenin signaling pathway reinforces osteoblast lineage decision at the expense of the adipogenic lineage. This has recently been confirmed even in MSC undergoing mechanical stimulation, thus providing a molecular explanation for the beneficial effects of physical exercise in terms of decreased adipose tissue and increased bone mass (Sen et al. 2008). Besides its osteogenic promoting activity, the addition of spermine attenuated the level of apoptosis associated with the process of asymmetric cell division underlying differentiation, increasing at the same time the anabolic activity of the cells (Guidotti et al. 2013) in terms of deposition of ECM proteins. Based on our evaluation of polyamine content of PRP, it is possible that some of the beneficial effects of this blood derivative originate from a combination of the anti-apoptotic effect of spermine together with the autophagy sustaining activity of spermidine, and therefore our study contributes to increasing the knowledge of the mechanisms whereby this increasingly popular prolotherapy tool exerts its activity.

Conclusion

Gathering findings from the study of normal skeleton development and regenerative biology in orthopedics, together with the expanding knowledge of the multiple effects of polyamines on most cell functions, suggest that this class of molecules is a powerful tool to improve the outcome of musculoskeletal regenerative medicine.

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Conflict of interest The authors declare that they have no conflict of interest.

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