

Apoptosis and Metabolism of Mesenchymal Stem Cells during Chondrogenic Differentiation *In Vitro*

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Abstract : Transplantation of mesenchymal stem cells (MSCs) is often used to treat cartilage defects, due to the lack of intrinsic self-healing capacity of this non-vascularized tissue. Nevertheless, it is extremely challenging to fully differentiate MSCs to chondrocytes, in particular the articular chondrocyte lineage, as well as maintain stable chondrogenic phenotype during *ex vivo* expansion. Cellular apoptosis and metabolism are important factors that influence the process of chondrogenesis, which have largely been overlooked. For example, lowered metabolism as a result of hypoxia enhances chondrogenic differentiation of MSCs, whilst inhibiting osteogenesis. It is also known that the regulation of apoptosis has a profound influence on chondrocyte hypertrophy during chondrogenesis. The focus of this review is therefore to critically examine the influence of apoptosis and cellular metabolism on chondrogenic differentiation of MSCs.

Key words: *Apoptosis, Chondrogenic differentiation, Mesenchymal stem cells, Metabolism*

1. Introduction

Cartilage defects lack intrinsic self-healing capacity due to absence of tissue vascularization.¹ Recruitment or seeding of either chondrocytes or mesenchymal stem cells is often necessary to achieve cartilage regeneration. Although autologous chondrocyte implantation has achieved limited success, their availability and *ex vivo* culture poses major challenges. Chondrocytes usually lose their cartilaginous phenotype during expansion in monolayer culture.² Mesenchymal stem cells (MSCs) can be harvested from the bone marrow, the superficial zone of cartilage, periosteum, synovium, muscle and fat.³ The ability of these cells to differentiate into multiple lineages, including chondrocytes, osteoblasts, adipose and muscle cells, make them an attractive cell source for musculoskeletal regeneration.^{4,5}

Although intensive research has been carried out on chondrogenic differentiation of MSCs, it is still challenging to

fully differentiate MSCs to chondrocytes, in particular articular chondrocytes, and maintain stable chondrogenic phenotype during *ex vivo* expansion.^{6,7} Studies have shown that cartilage regeneration from induced pluripotent stem cells (iPSC) can be achieved with type II collagen up-regulated cells.⁸ A small molecule kartogenin can promote chondrocyte differentiation by disrupting the interaction between filamin A and transcription factor core-binding factor β subunit *in vitro*.⁹ Cellular apoptosis and metabolism during chondrogenic differentiation are important, but seldom discussed. The focus of this review is on apoptosis and metabolism of MSCs during chondrogenic differentiation.

2. Embryonic Differentiation of MSCs to Chondrocytes

MSC can differentiate to bone and cartilage during embryonic development, and are responsible for maintenance and repair of these tissues in the adult body.¹⁰ MSCs condense and differentiate to chondrogenic lineages, either within the growth plate or articular cartilage during embryonic

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development.¹¹ Chondrocytes within the growth plate undergo endochondral ossification, unlike their counterparts in articular cartilage, and are programmed for hypertrophy and apoptosis.¹²

MSCs differentiate into interzonal chondrocytes before committing into articular chondrocytes. Aggregation of MSCs initiates formation of the interzone through a series of molecular events. Wnt-14, expressed fairly early during interzone formation, upregulate gene expression of GDF-5, an interzone marker and expressed in the early interzone of mouse and chick embryo limb joints.¹³ With further development a process of cavitation proceeds within the interzone leads to formation of a liquid-filled space sandwiched with two opposing sides of the developing joint. Cell death triggered by accumulation of hyaluronic acid in the interzone leads to cavitation.¹⁴ After cavitation, one of the opposing sides of the joints begins a process of morphogenesis through which the concave and the convex surfaces are formed.¹¹

The two different differentiation pathways of MSC to chondrocytes converge at interzone formation. Integrin $\alpha 5\beta 1$ is vital in deciding whether MSC become either interzone joint-forming cells or pre-hypertrophic chondrocytes that will eventually undergo hypertrophy.¹⁵ Blocking of $\alpha 5\beta 1$ integrin can inhibit the differentiation of pre-hypertrophic chondrocytes. However, mis-expression of $\alpha 5\beta 1$ integrin leads to fusion of joints and formation of pre-hypertrophic chondrocytes. Therefore down-regulation of $\alpha 5\beta 1$ integrin can reprogram diaphyseal MSC to become joint-forming cells, instead of forming pre-hypertrophic chondrocytes.

3. Metabolism of MSC during Chondrogenic Differentiation *In Vitro*

3.1 Importance of Metabolism

Metabolism involves both anabolic reactions and catabolic reactions, which constitutes an entire network of chemical reactions carried out by living cells. Anabolic reactions are responsible for the synthesis of all compounds needed for cell maintenance, growth and reproduction, while catabolic reactions degrade large molecules to liberate smaller molecules and provide energy.¹⁶ Both external and internal stimuli can affect metabolism, such as changes in the energy demand and supply of metabolic molecules.¹⁷ Energy is essential for most cellular processes. Hence, we focus on carbohydrate metabolism and energy generation in this review.

3.2 Carbohydrate Metabolism of MSCs during *In Vitro* Chondrogenic Differentiation

Most of the energy of cells is produced through catabolism of

D-glucose, either through glycolysis or oxidative phosphorylation.¹⁸ The metabolic phenotype of MSCs is a combination of the above two pathways. Mitochondria is the center of energy metabolism in mammalian cells where adenosine triphosphates (ATPs) are generated and several vital biosynthetic pathways take place.¹⁹ Rotary turbine-like ATP synthase machinery localized in the inner membrane of mitochondria drives phosphorylation of ADP to ATP, with the proton gradient being generated by the electron transport chain (ETC).¹⁹ The density and activity of proteins related to oxidative phosphorylation and mitochondria vary in different cell types according to their different energy demands.²⁰ Additionally, ATP can also be produced through glycolysis. D-glucose is broken down to pyruvate in the cytoplasm, along with the release of ATP.

The metabolic phenotype of MSCs shifts to predominantly glycolytic metabolism during chondrogenic differentiation.⁶ The oxygen consumption of MSCs during chondrogenesis drops by approximately 70% during the initial 24 h in pellet culture. Only at most 13% of the total ATP is being provided by oxidative phosphorylation.⁶ Hypoxia enhances chondrogenic differentiation of MSCs, whilst inhibiting osteogenic differentiation of MSC. Hypoxia promotes chondrogenic differentiation of MSCs through activation of Sox9 via a hypoxia-inducible factor -1 α (HIF-1 α)-dependent mechanism.^{6,21-23} HIF-1 α binds to specific hypoxia responsive elements (HRE), which then initiate the transcription of Sox9 and subsequently enhance gene expression of collagen II and aggrecan.³ Mitochondrial activity is maintained at a relatively low level and glycolysis is the main energy source for undifferentiated MSCs.¹⁸ However, both cell oxygen consumption and oxidative phosphorylation progressively decrease during chondrogenic differentiation, which lead to a significantly decreased respiratory rate of MSCs.^{6,20}

3.3 Regulation of Metabolism to Promote Differentiation of MSCs to Cartilage

Culture medium, growth factors and microenvironment are crucial for MSC differentiation.^{3,22} To enhance chondrogenesis of MSCs *in vitro*, close cell-to-cell contact and the addition of chondrogenic bioactive factors are essential. Dexamethasone, TGF- β , fibroblast growth factor (FGF), BMPs and insulin-like growth factor (IGF) can induce chondrogenic differentiation to different extents.²² In addition, many approaches are applied to modulate microenvironment of MSCs for efficient chondrogenesis. Hypoxia (5% O₂) promotes chondrogenesis of many types of MSCs more efficiently than normal conditions (20% O₂).^{21,24} The low oxygen concentration in the above culture conditions results in high glycolysis levels of cells, which may in turn

enhance the chondrogenesis of MSCs.²⁵ Although the low respiratory rate of MSCs during chondrogenic differentiation and the beneficial effects of hypoxia on chondrogenesis have already been extensively studied, it is still unclear whether chondrogenic differentiation of MSCs can be enhanced by reduced metabolism.

4. Apoptosis of MSC

4.1 Apoptosis of MSC

Apoptosis is essential and tightly regulated during development, particularly in arthrogenesis and endochondral ossification of growth plate. Apoptosis is mediated by three major pathways, including mitochondria dependent pathway, endoplasmic reticulum stress pathway and death receptor mediated pathway. The process of apoptosis involves: activation of caspase-3, loss of lysosomal integrity, increase in pro-apoptotic protein, Bax and cleavage of PARP. Cell condensation (pellet, micromass, or others) is essential for chondrogenic differentiation of MSCs. Hypertrophic changes of differentiated chondrocytes inevitably ensue with enhanced expression of collagen type X, alkaline phosphatase and other ossification markers. The current strategy for chondrogenic differentiation is heavily based on endochondral ossification rather than articular cartilage formation. Significant levels of apoptosis inevitably occur during chondrogenesis within pellet cultures, as demonstrated by Annexin V, TUNEL staining and lysosomal labeling.²⁴ However, while there was no obvious apoptosis in MSCs grown in a monolayer, the chondrocytes grown in monolayer culture undergoes apoptosis, and the degree of apoptosis is proportional to cell density.²⁶ Hence, the differentiation status may induce apoptosis in chondrogenically differentiated MSCs. Additionally, pellet cultures may induce hypoxia which is detrimental for survival of MSCs. Hypoxia can also induce apoptosis in MSCs.²⁷

Apoptosis can be inhibited by serum free medium supplemented with ITS, which is also associated with an increase in the expression of type II collagen, and a decrease in the expression of type X collagen, Runx2, and other osteogenic genes. By contrast, the supplementation of TGF- β 1 increased the expression of Sox9, type II and type X collagen and decreased the expression of osteogenic genes.²⁶

4.2 Apoptosis of Mesenchymal Stem Cells during *In Vitro* Chondrogenic Differentiation

During embryonic development of articular cartilage, the cells in the middle of the interzone undergo hypertrophy and

apoptosis, leading to formation of cavitations.²⁸ Once chondrocytes become hypertrophic, their genetic expression profile is altered and they begin synthesizing type X collagen. Hypertrophic chondrocytes produce vascular endothelial growth factor that induces blood vessels to migrate. Hypertrophic chondrocytes also mineralize their surrounding matrix, prior to undergoing apoptosis. The cartilage matrix will provide a scaffold for osteoblasts to infiltrate.²⁹ The apoptosis of chondrocytes also gives rise to osteoarthritis. During chondrogenic induction of MSCs *in vitro*, the cell numbers would diminish as a result of cellular apoptosis.

4.3 Regulation of Apoptosis to Promote Differentiation of MSCs to Cartilage

It is a good strategy to differentiate MSC to cartilage through micromass or pellet culture. However, this type of cell mass culture tends to be hypoxic and lack nutrition, which can accelerate apoptosis of MSC.²⁷ Exogenous reactive oxygen species (ROS) are one of the important factors that induce apoptosis of MSCs. Hypoxia can activate caspase-8 and p38 expression by MSCs. Hydrogen peroxide can induce the apoptosis of MSC via the mitochondrial death pathway and endoplasmic reticulum stress.³⁰ Suppressing expression of p38 can promote survival of MSC after transplantation.

Apoptosis can decrease the efficiency of differentiation. On the other hand, apoptosis may also occur in undifferentiated cells. Studies have shown that the differentiation status of cells influences apoptosis in chondrocytes and MSCs undergoing chondrogenic differentiation.²⁶ So it is credible to consider that differentiated cells are prone to apoptosis. Additionally, the chondrocytes differentiated from MSCs *in vitro*, tend to be more like growth plate rather than articular cartilage. Hence, these tissue-engineered chondrocytes will undergo apoptosis after hypertrophy.¹² During chondrogenesis *in vitro*, the cells cultured within the centre of the pellet may be more prone to apoptosis, as compared to cells at the periphery because of hypoxia and lack of nutrition. The apoptotic body of MSC may also influence the efficiency of differentiation of MSC. Hence, it is vital to explore the molecular pathway leading to apoptosis of MSCs during induction to chondrocytes *in vitro*. Appropriate suppression of this molecular pathway will slow down the apoptosis of MSC, as well as improve the efficiency of differentiation to cartilage *in vitro*.²⁷

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