Rescue of the senescence phenotype of AD MSCs by autophagy activation in 3D spheroids.

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Human MSCs (hMSCs) are cells capable of **self-renewal and multi-lineage differentiation** into various tissues of mesodermal origin. These cells can be easily isolated and expanded from the **stroma of virtually all organs, although the preferred sources are bone marrow and subcutaneous fat.**

MSCs have been broadly applied in the treatment of various diseases, including graft-versus-host disease (GVHD), Crohn's disease (CD), diabetes mellitus (DM), multiple sclerosis (MS), myocardial infarction (MI), liver failure, and rejection after liver transplant.

Upon isolation, hMSCs are characterized by their capability to develop as fibroblast colony-forming-units, and differentiate into **osteocytes, chondrocytes, and adipocytes**.

hMSCs **are positive for CD73, CD90, CD105, CD106, CD29, CD166**, and negative for **CD11b**, **CD14, CD34, CD45, HLA-DR, CD79α and CD19**.

The function of MSCs is known to **decline with age,** a process that may be implicated in the loss of maintenance of tissue homeostasis leading to organ failure and diseases of aging

Cultured primary cells do not grown infinitely, but undergo only a limited number of cell division, in a process called **cellular senescence**. Cell therapy protocols generally require hundreds of million hMSCs per treatment and, consequently, these cells need to be expanded in vitro for about 10 weeks before implantation. Notably**, patient's clinical history, age, and genetic makeup strongly influence the length of this expansion period and the quality of the obtained cells. Aged MSCs** generally perform less well than their younger counterparts in various disease models and mounting evidence strongly suggests that cellular senescence contribute to aging and age-related diseases.

It would, thus, be of great significance to monitor the occurrence of a senescent phenotype in hMSCs addressed to clinical uses and to evaluate the functional consequences of senescence in hMSCs which could affect their clinical therapeutic potential, taking into account their **paracrine effects, immunomodulatory activity, differentiation potential, and cell migration ability.**

The term senescence was applied to cells that ceased to divide in culture, based on the speculation that their behaviour recapitulated organismal ageing. Consequently, cellular senescence is sometimes termed cellular ageing or replicative senescence

MARKERS of SENESCENCE

Telomere shortening provided the first molecular explanation for why many cells cease to divide in culture. Dysfunctional telomeres trigger senescence through the p53 pathway.
This response is often termed telomere-initiated cellular senescence. Some cells undergo replicative senescence independently of telomere shortening.

Resistance to apoptosis might partly explain why senescent cells are so stable in culture. This attribute might also explain why the number of senescent cells increases with age.

Changes in cell cycle inhibitors: p21Cip1 and p16InK4a. These CDKIs are components of tumour suppressor pathways that are governed by the p53 and retinoblastoma pRB proteins.

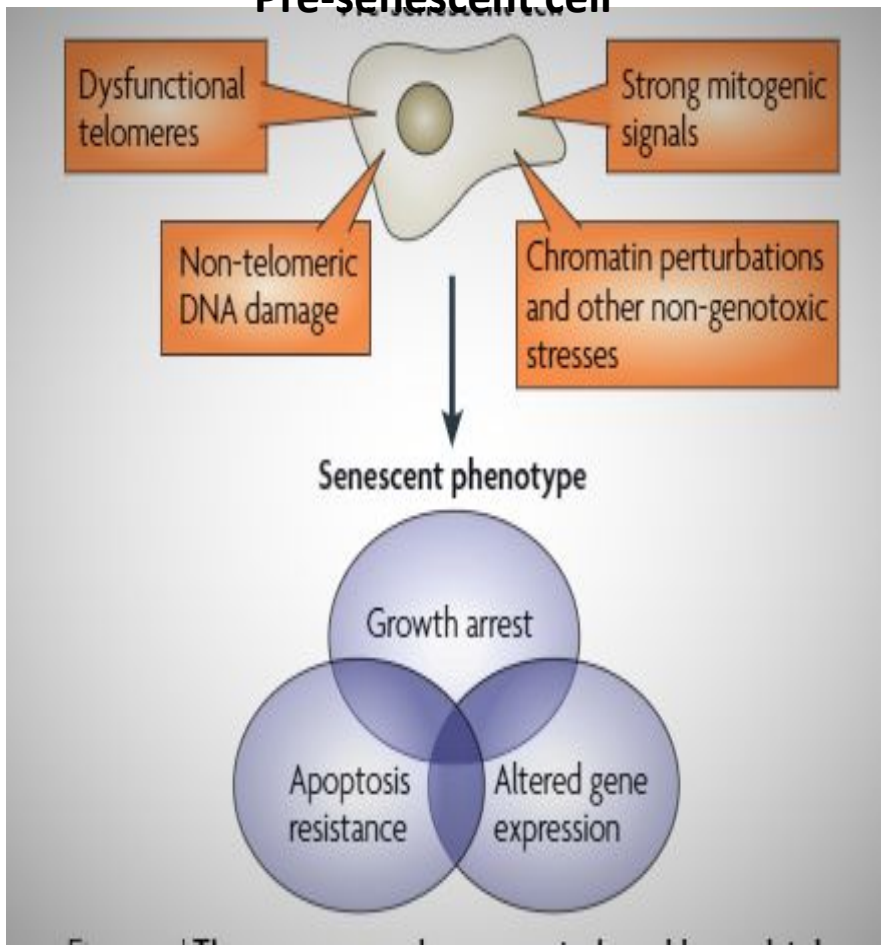
New markers in Oncogene induced senescence: DEC1 (differentiated embryo chondrocyte expressed1), p15 (a CDKI) and DCR2 (decoy death receptor2). The specificity and significance of these proteins for senescent cells are not yet clear, but they are promising additional markers.

Dramatic structural changes of chromatin in senescent cells- Lamin B1. Presence of certain heterochromatin associated histone modifications (H3 lys9 methylation) and heterochromatin protein1 (Hp1)). In some cases - global heterochromatin loss, characterized by markers H3K9me3 and H3K27me3. Predominantly during OIS in vitro, heterochromatin is redistributed into 30–50 punctate DNA-dense senescence-associated heterochromatin foci (SAHF). SAHF are silent domains that co-localize with H3K9me3 and heterochromatin protein 1 (HP1) and may lock cells in a senescent state by transcriptionally repressing genes involved in cell proliferation.

DNA methyltransferases (DNMTs)

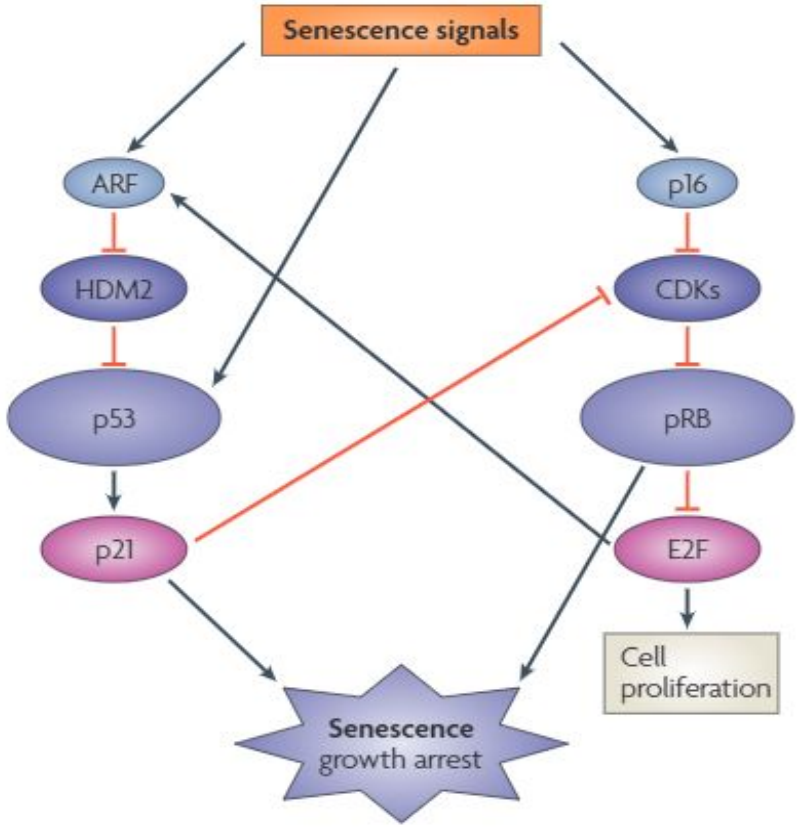
Expression levels of DNMT1 and DNMT3B are significantly decreased during the replicative senescence of MSCs, leading to a decrease in the DNA methylation level, called hypomethylation, which is a distinct feature of senescent cells.
In contrast, DNMT3a expression was found to be increased during replicative senescence, participating in the new methylation associated with senescence. DNMT inhibitors, such as 5-azacytidine, can upregulate p16INK4a/CDKN2A, p21CIP1/WAF1 and miRNAs targeting EZH1, and the induction of cellular senescence in MSCs

Pre-senescent cell



Senescence markers

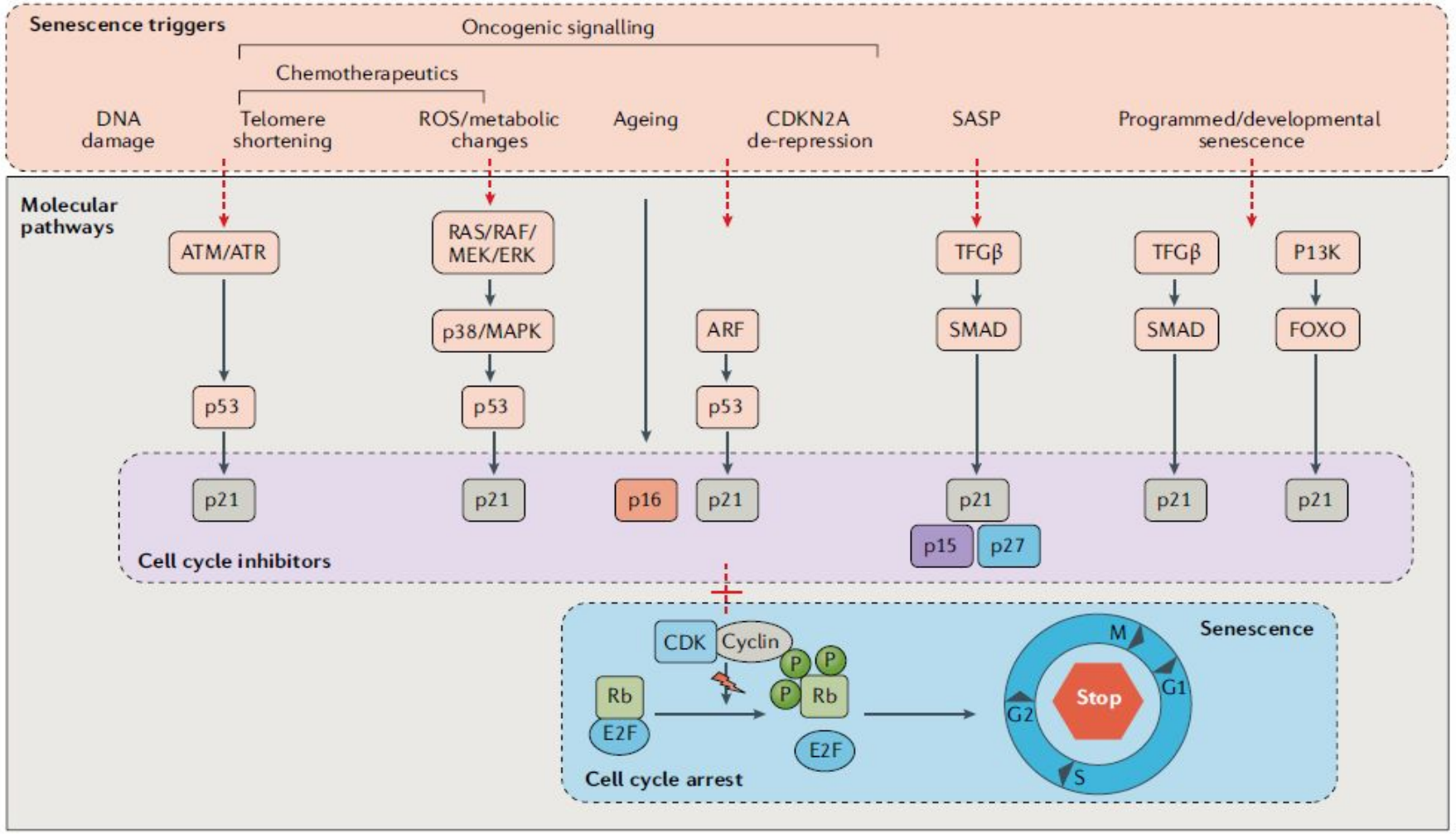
The hallmark of cellular senescence is an inability to progress through the cell cycle. **G1 cell cycle arrest**, yet they **remain metabolically active**. This growth arrest is established and maintained by the p53 and p16–pRB tumour suppressor pathways. In contrast to quiescence, the senescence growth arrest is essentially permanent.



Stimuli that generate a DDR (IR and telomere dysfunction) induce senescence primarily through the p53 pathway. p21 is a crucial transcriptional target of p53 and mediator of p53-dependent senescence.

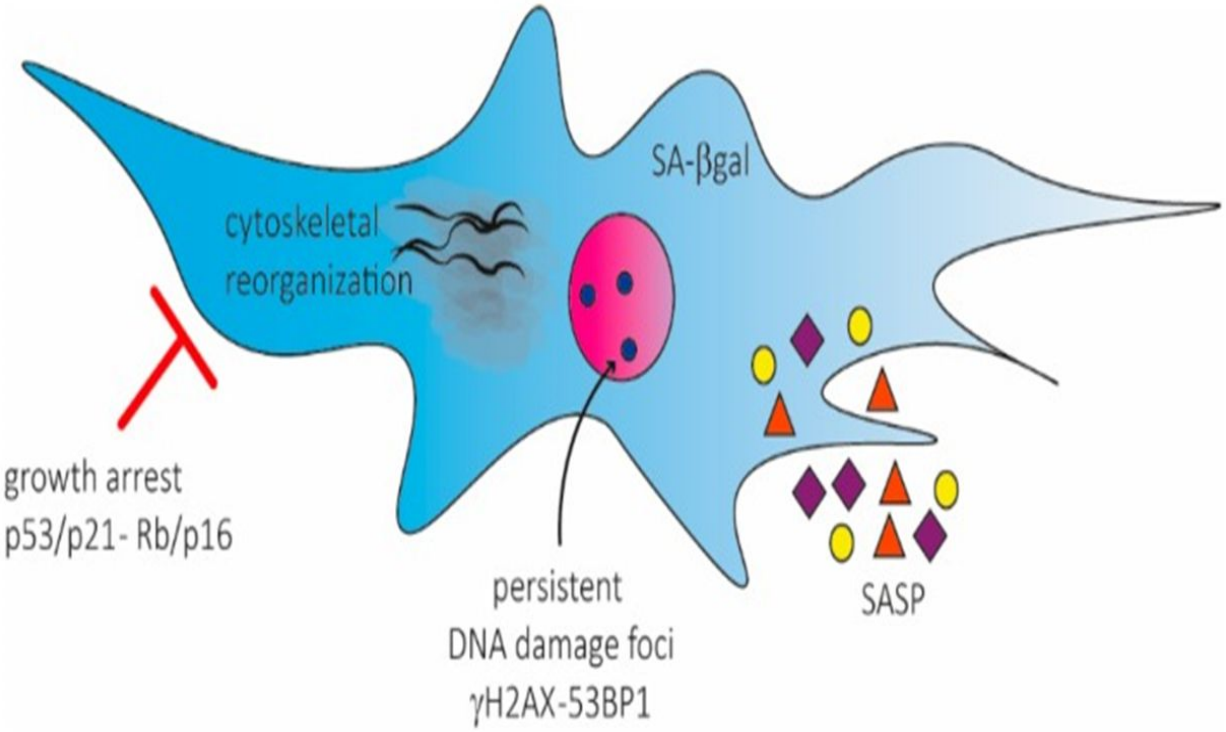
p16–pRB pathway can establish self-maintaining senescence associated heterochromatin. This activity may be due to the ability of pRB to complex with histone modifying enzymes that form repressive chromatin.

Campisi J, d'Adda di Fagagna F., 2007. Cellular senescence: When bad things happen to good cells. Nat Rev Mol Cell Biol



Drugs that cause overexpression of p53, Rb, p21 or p16Ink4a or activate these proteins can induce senescence by activating the p53–p21 and/or p16Ink4a–Rb tumour suppressor pathways. Activators of p53, such as nutlin-3a (an MDM2 antagonist), can induce senescence.

Lozano-Torres B., et al., 2019. The chemistry of senescence . NATuRe RevIewS volume 3; 427



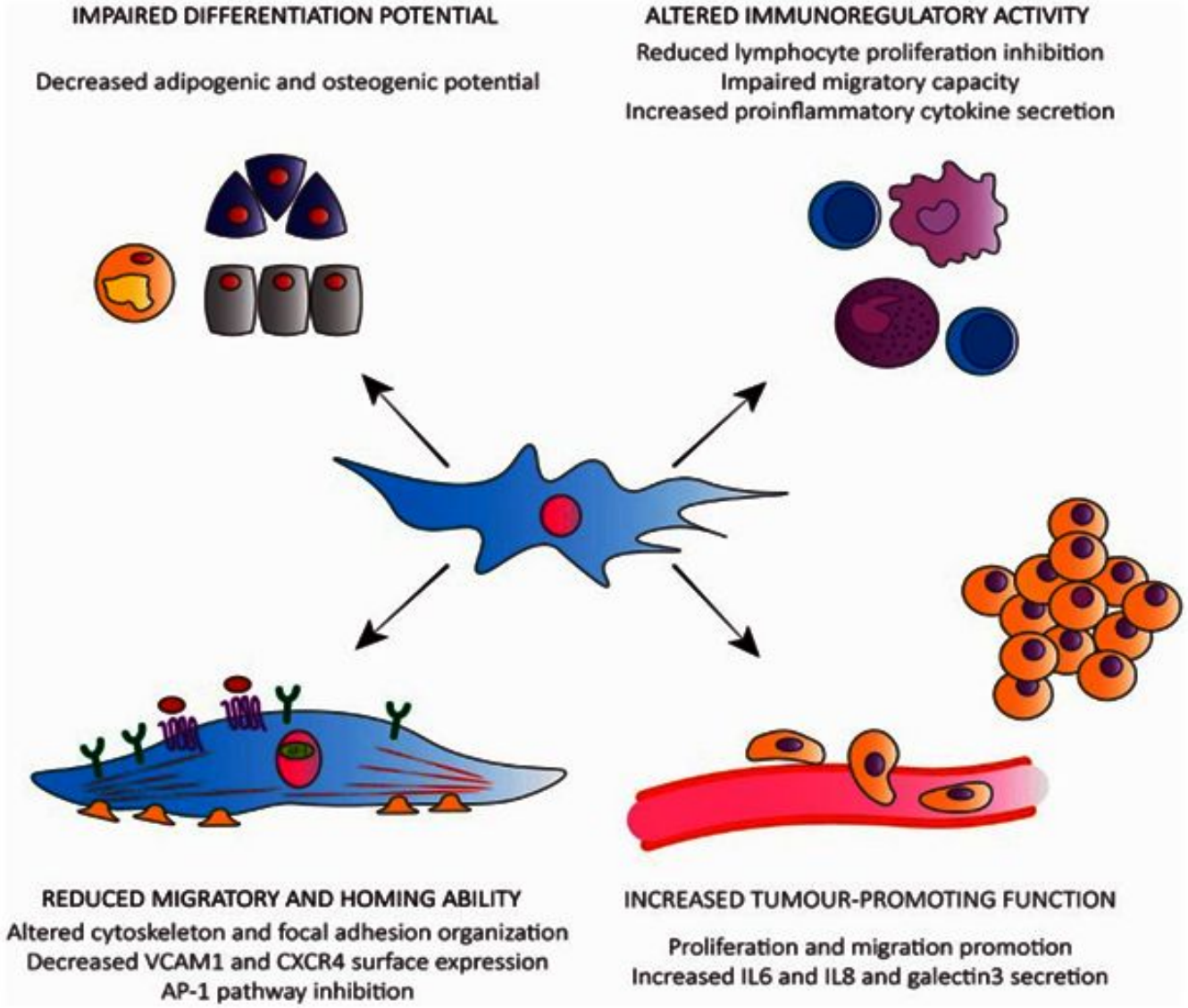
Senescent cells develop a large, flat morphology, display characteristic changes in gene expression, harbour characteristic enlarged and persistent DNA damage nuclear foci (γH2AX and 53BP1) and accumulate a distinct heterochromatin structure, termed senescence-associated heterochromatin foci (SAHFs). Senescent cells secrete factors, including growth factors, proteases and cytokines, with potent autocrine and paracrine activities. senescence-associated secretory phenotype (SASP).

27 proteins and 31microRNA
The cytoskeletal reorganization of hMSC, describing a reduction of myosin-10, redistribution of myosin-9 and secretion of profilin-1

The senescence-associated β-galactosidase (SA-βgal) - is detectable in most senescent cells. However, it is also induced by stresses such as prolonged confluence in culture. The SA-βgal probably derives from the lysosomal β galactosidase and reflects the increased lysosomal biogenesis that commonly occurs in senescent cells.

Immunostaining for proteins such as PCNA and Ki-67, these markers do not distinguish between senescent cells and quiescent or differentiated post-mitotic cells.

Senescent hMSCs secreted higher levels of numerous proteins compared to non-senescent cells: 27 proteins Among the factors with the highest levels of secretions -LEPTIN, Transforming Growth Factor Alpha (TGFA), IL8, EOTAXIN, Interferon Gamma (IFNG), VCAM1, Interferon Beta (IFNB), IL4, and Monocyte Chemotactic Protein-1 (MCP1). 31 miRNAs, differentially expressed in senescent hMSCs compared to control cells.



Changing in the MSC surface markers during prolonged cultivation was reported to be related with decreased homing ability of hMSCs .
A strong decrease in VCAM1 expression, an important mediator of MSC interaction with endothelial cells and subsequent MSC homing .

For the use of MSCs in therapy, methods that allow the generation of large populations of MSCs without affecting their properties of differentiation or immunomodulation need to be established.

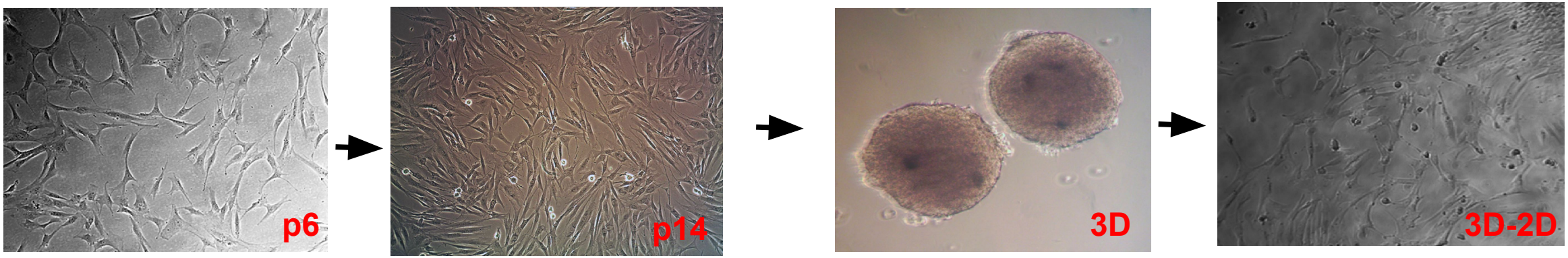
The antioxidant *N*-acetyl-L-cysteine (NAC), a precursor of glutathione and a direct ROS scavenger, has been used as a therapeutic agent to ameliorate the damaging effects of ROS (Lin et al., 2005).
Other **antioxidants** such as ascorbic acid and inhibitors of p38/MAPK or mTOR can markedly improve ROS-mediated injury in MSCs and lead to full recovery (Choi KM et al., 2008).

The introduction of hTERT into MSCs resulted in a substantial multiplication of their replicative lifespan accompanied by the preservation of a normal karyotype, **elongation of telomeres and loss of the senescent phenotype without impact on differentiation ability** (Takeuchi M et al., 2007; Simonsen JL et al., 2002).
Several small molecular compounds, such as **aspirin and vitamin C, as well as FGF-2**, have been developed to activate the endogenous telomerase of MSCs, achieving similar effects of improved proliferative and osteogenic potential in recent research (Wei F et al., 2012). However, **this is ill-advised for clinical applications given the small but possible risk of malignant transformation.**

Knockdown of p16/CDKN2A (Gu Z et al., 2012) or **silencing of RB** (Galderisi U et al, 2006) in MSCs rescues the senescent phenotype and increases the proliferation rate and clonogenicity. But, silencing of these tumor-suppressor-genes disrupts differentiation potential and **increases tumorigenesis risks.**

Knockdown or silencing of **miR-195 significantly increases hTERT**, **phosphorylation of AKT and FOXO3** expression and induces telomere re-lengthening in senescent MSCs (Gharibi B , 2012).
Exogenous **FGF-2, PDGF and EGF** has been reported to **increase proliferation ability and delay MSC senescence, without affecting osteogenesis and adipogenesis for therapeutic use.** Lysophosphatidic acid (**LPA)**

Модель эксперимента



Spheroids with 3000 cells/25ul drop
7000 cells/25ul drop
10000 cells /25ul drop
2 and 3 days culture

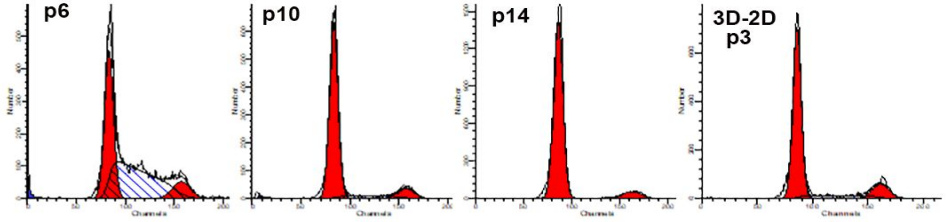
1. **Характеристика MSCs ранних и поздних пассажей и после сфер. Динамика клеточного цикла, маркеров старения –β-Gal – есть.**
2. **WB: p21; p16; p53 (нужно).**
3. **Кариотипирование на раннем пассаже, позднем и после сферы (есть на Fet MSCs, собрано на AD MSCs).**
4. **Экспрессия маркеров MSCs (FLOW) – нужно на AD MSCs. Есть на Fet MSCs. Abs обещали.**
5. **Способность к остеогенной дифференциации – есть на Fet MSCs, RT-PCR for markers на AD MSCs ? –Suppl Inf**

Cell Cycle Characterization of AD MSCs lines 1 & 2

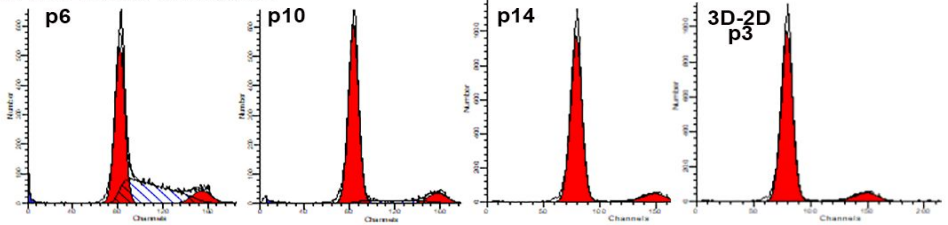
Figure1

Cell Cycle analysis

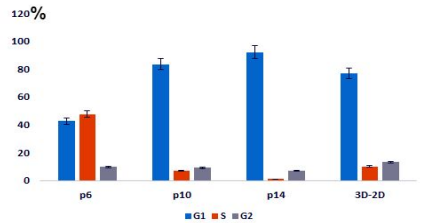
A LINE1 MSC 1519000139



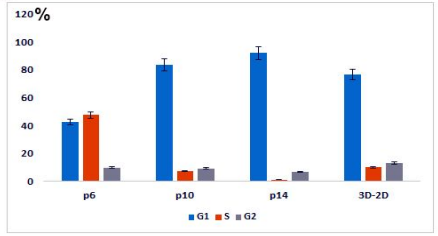
B LINE2 MSC 1515000031



C



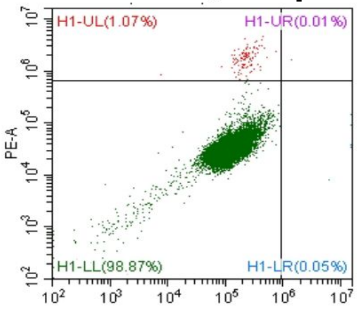
D



E

pH3 (Ser10)

LINE1 p14



LINE2 p14

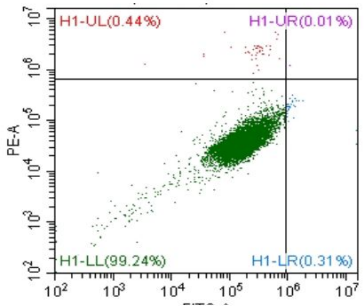
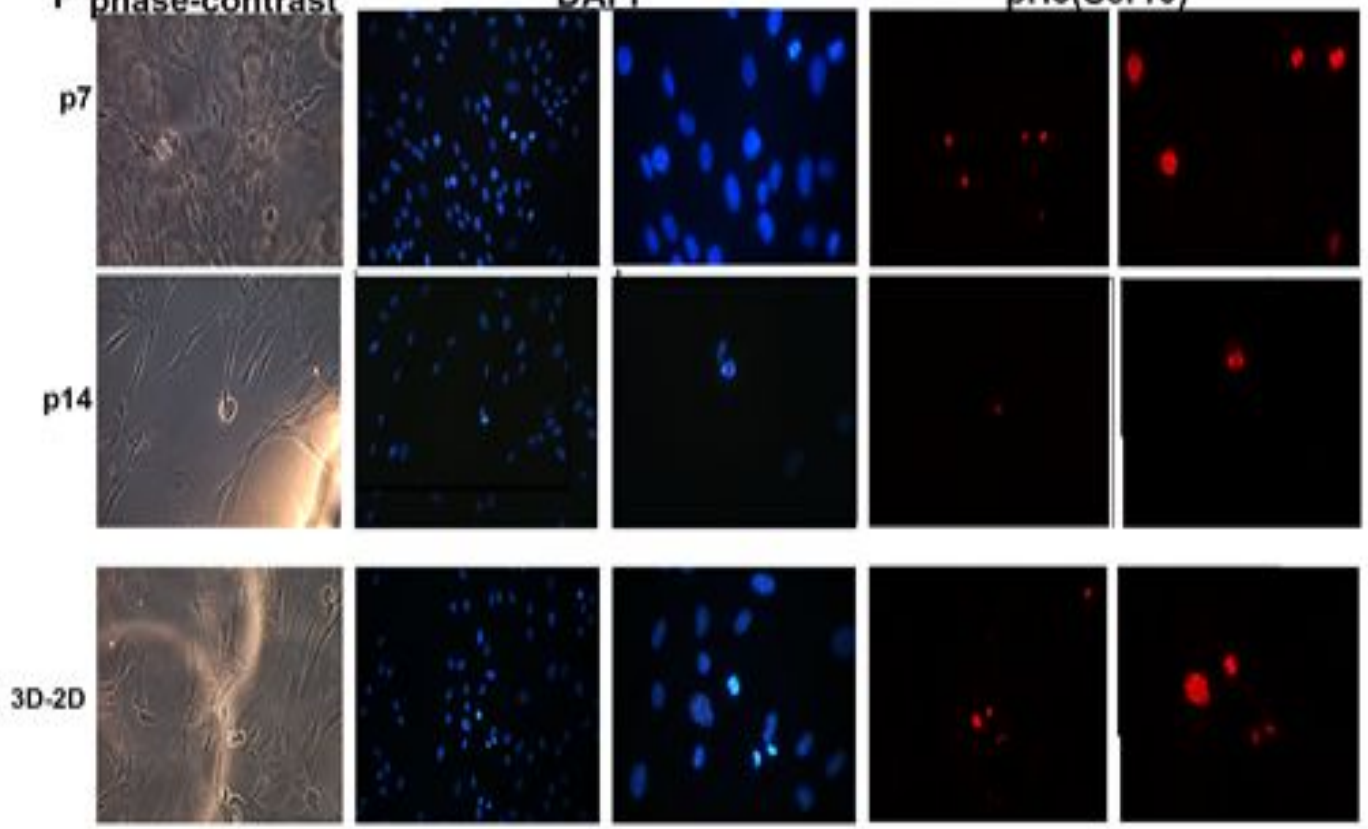


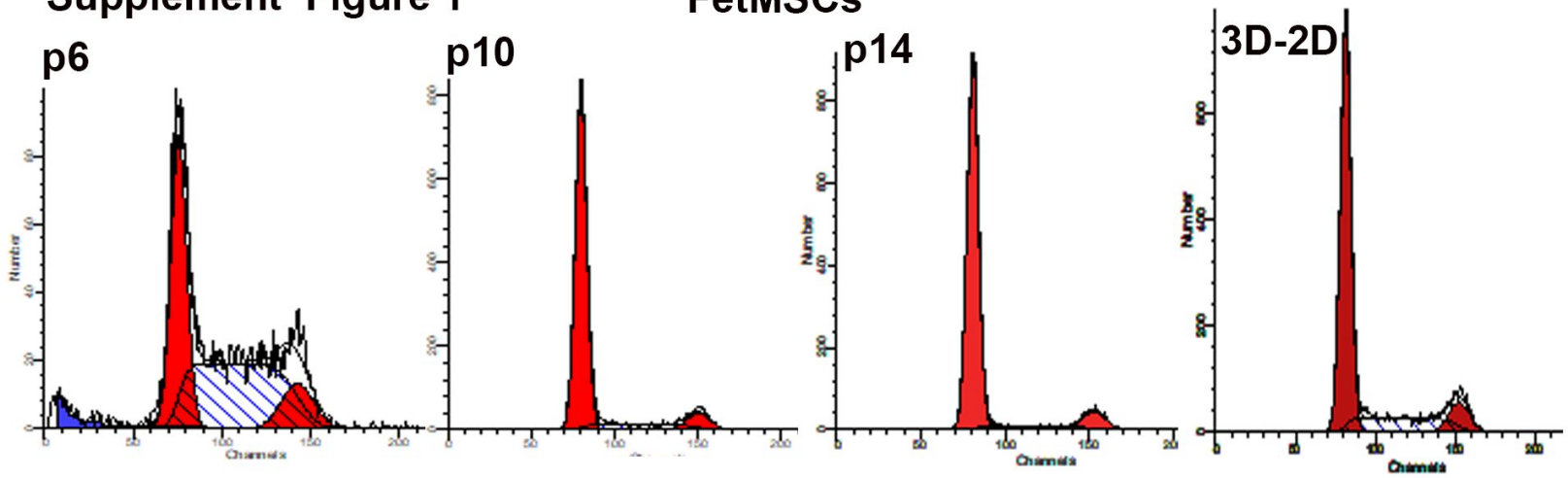
Figure 1 (continued)

F phase-contrast DAPI pH3(Ser10)



Supplement Figure 1

FetMSCs



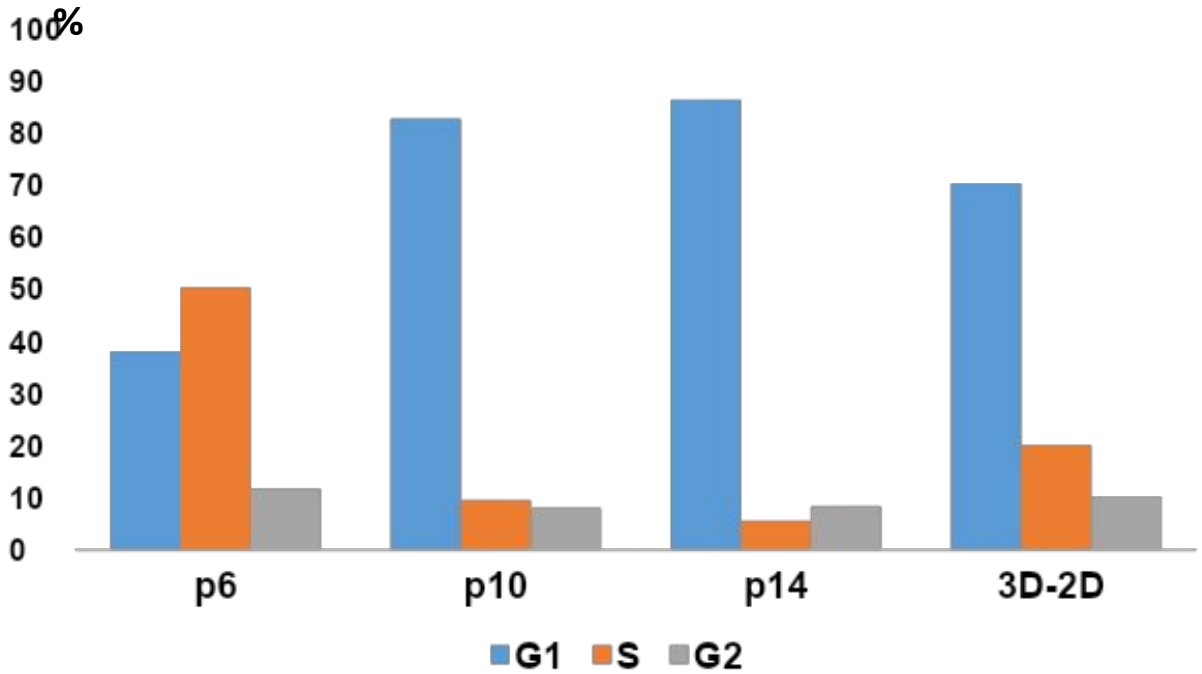
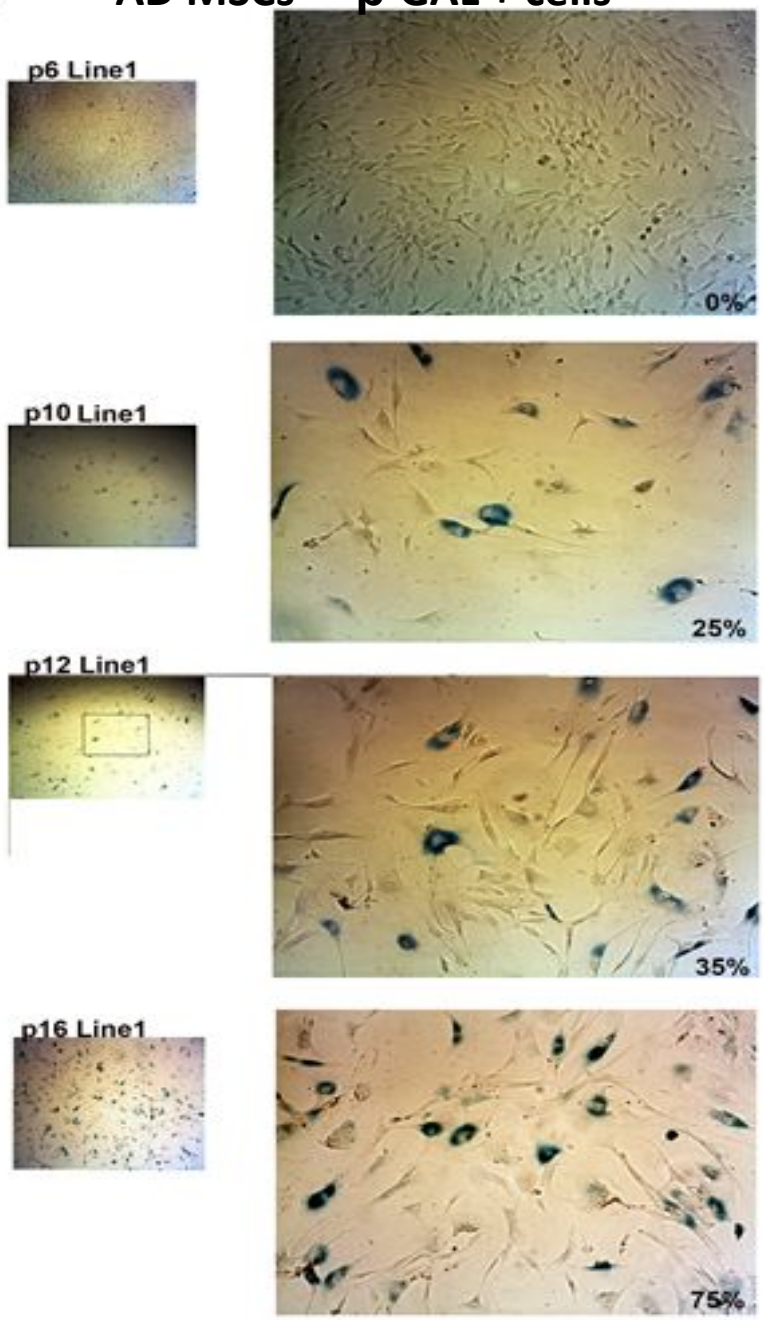


Figure 2
A

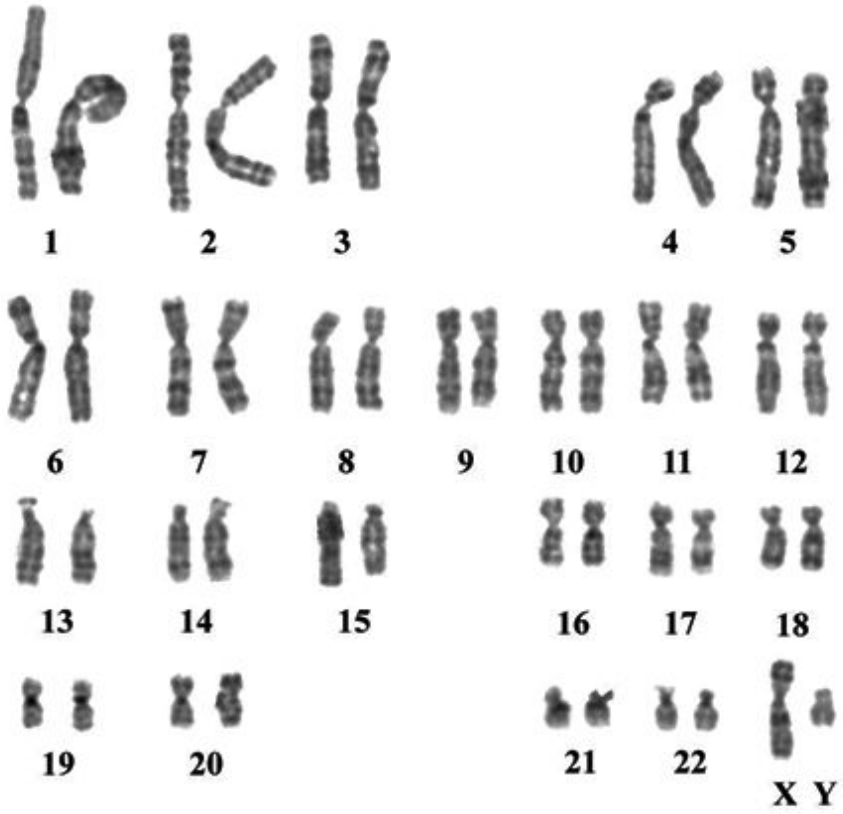


Нужно:

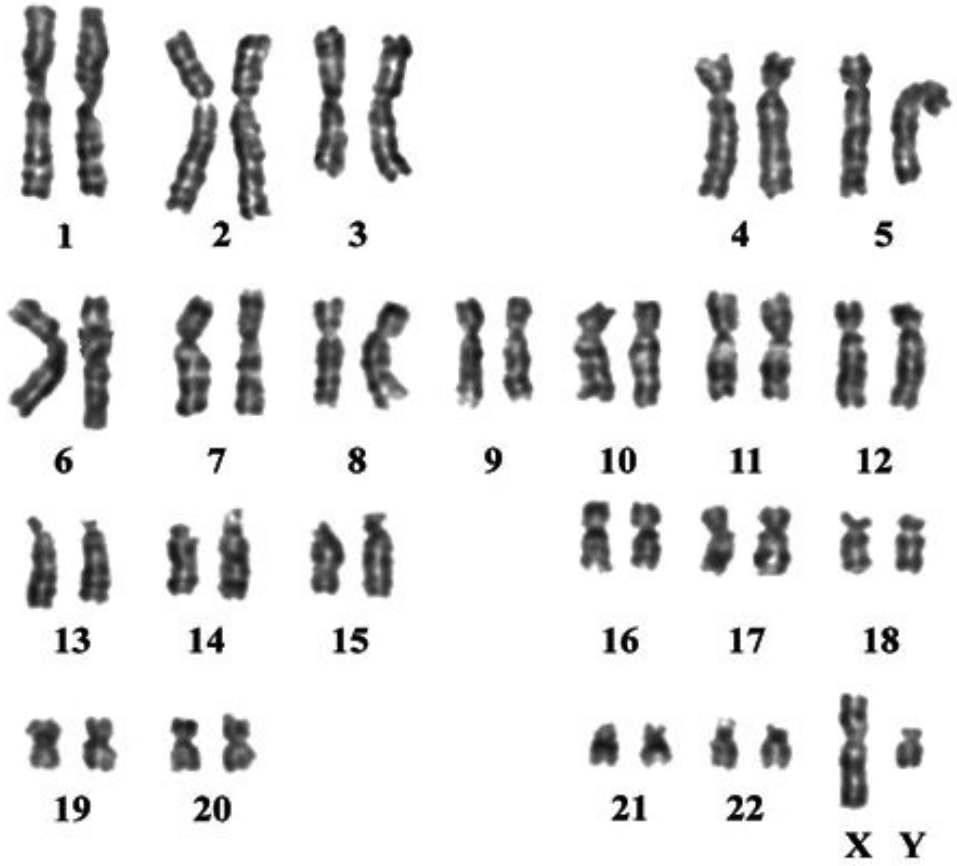
1. **Анализ на длину теломер ?**
2. **Анализ на β-Gal+ в 3D-2D- p3- есть**
3. **WB : p21; p16; p53, Cyclin A, Cyclin B1**

КАРИОТИПИРОВАНИЕ Fet MSCs

p7



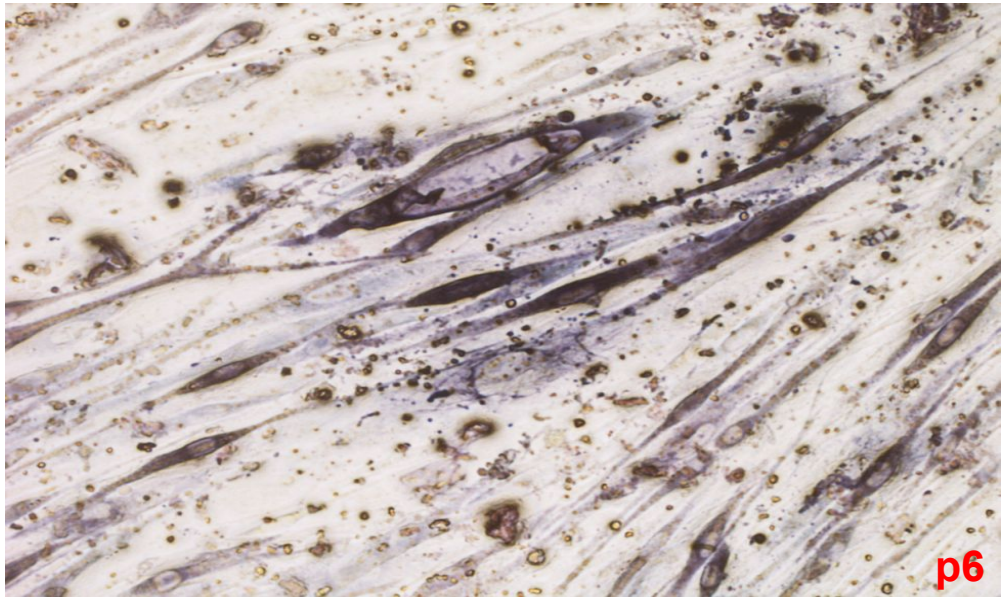
3D-2D /p3

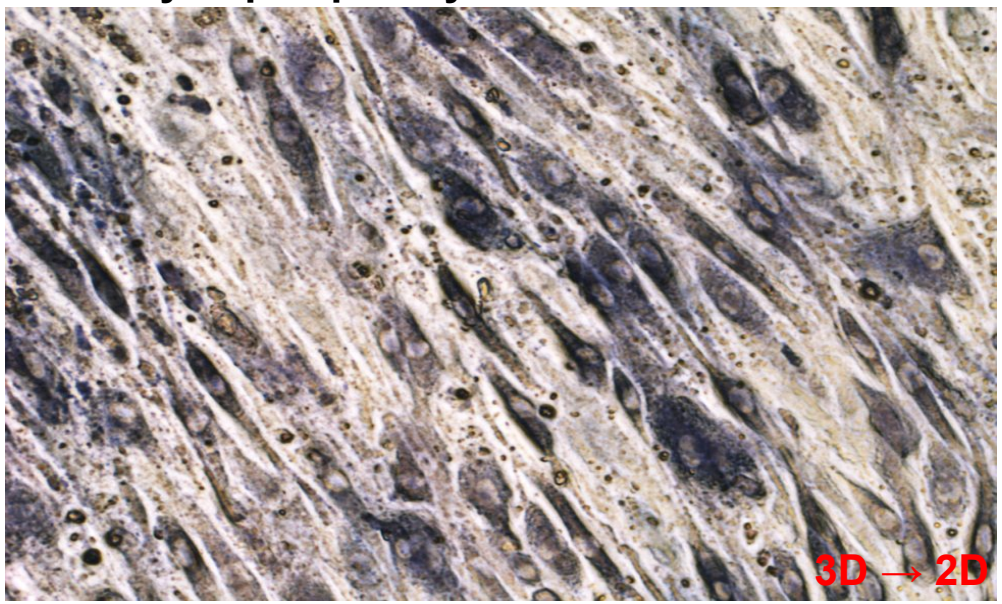


Кариотипирование AD MSCs -+

Остеогенная дифференцировка Fet MSCs

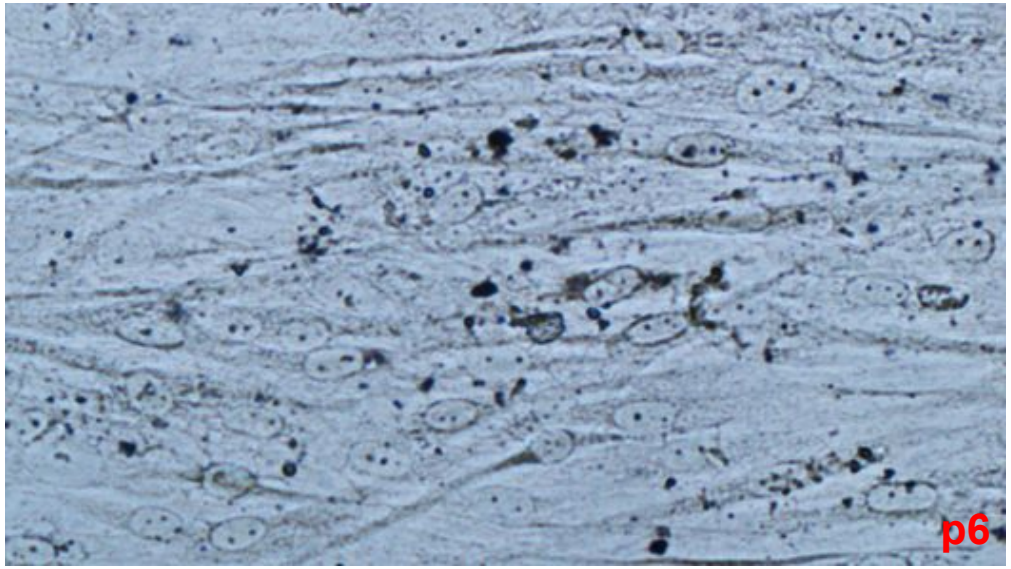
Окрашивание на щелочную фосфатазу

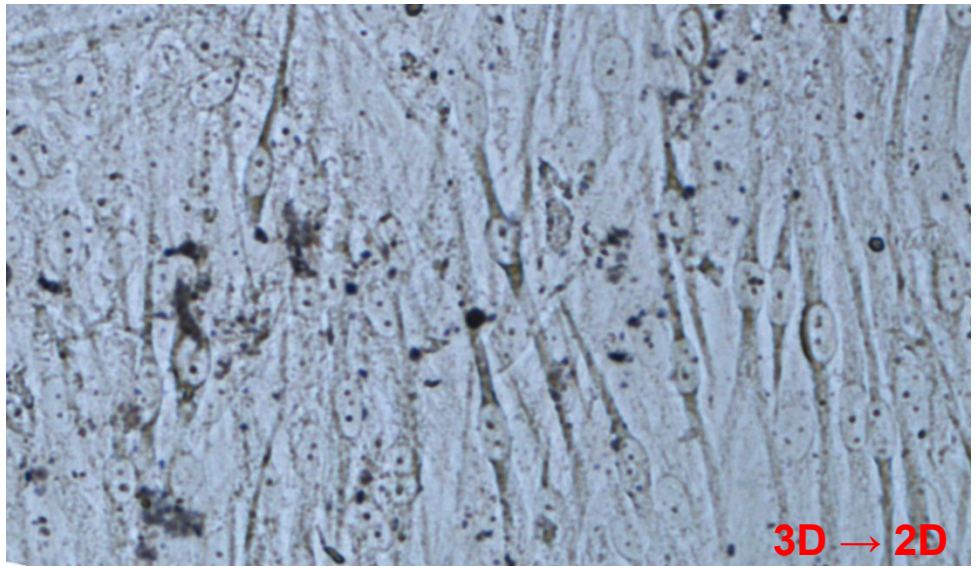




активность щелочной фосфатазы - раннего маркера остеогенеза

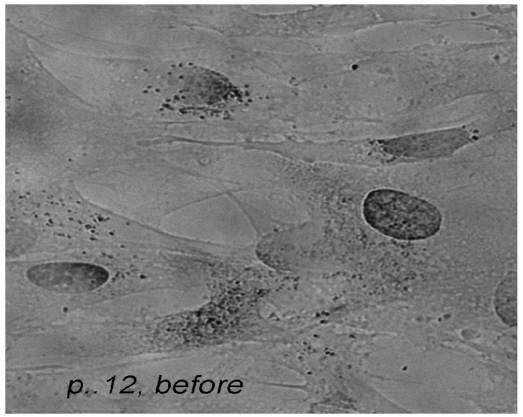
Окрашивание по Van Kossa (нитратом серебра)

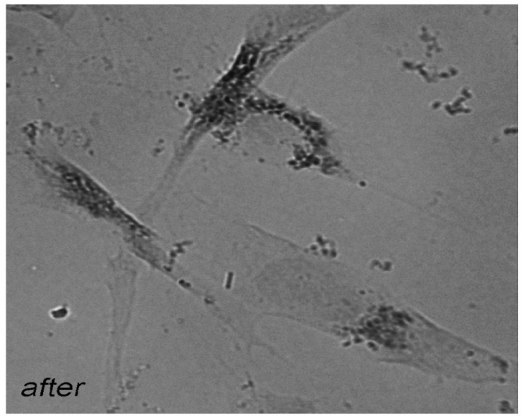


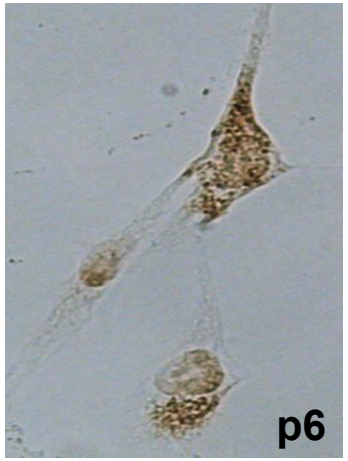


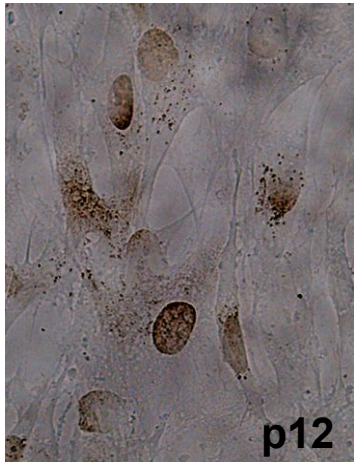
подтверждено образованием оссификатов и фосфатов кальция

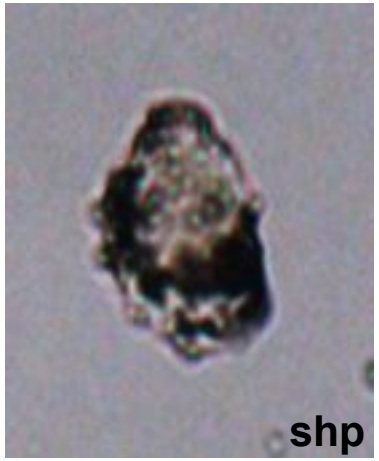
Автофагоцитоз (реакция на кислую фосфатазу по Гомори) Fet MSCs

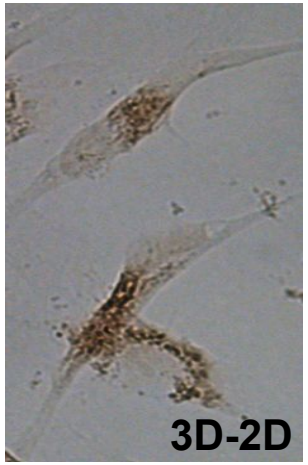


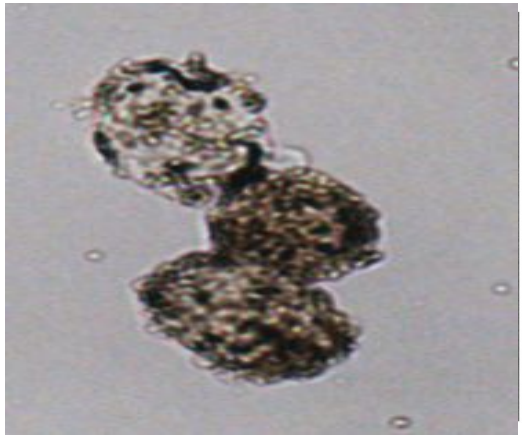


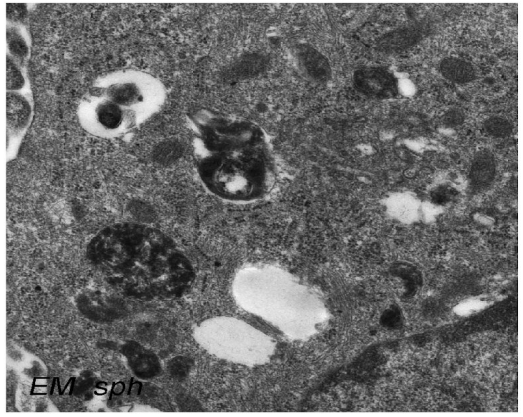




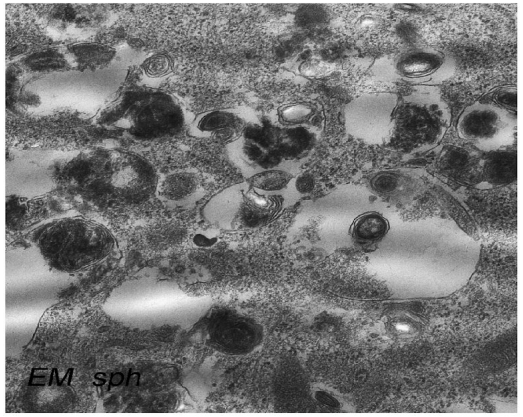


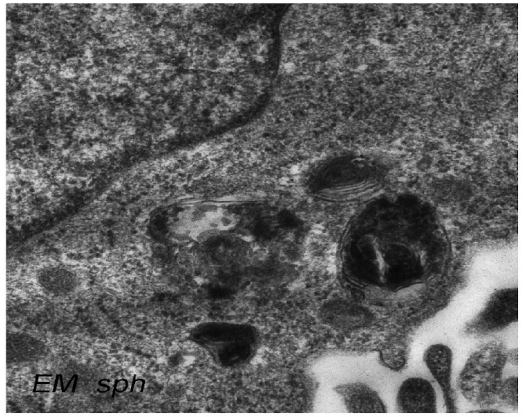


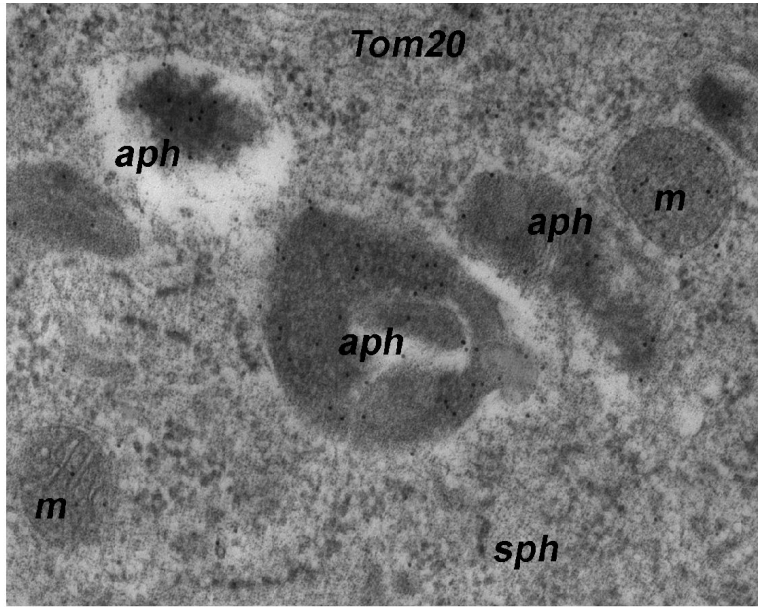


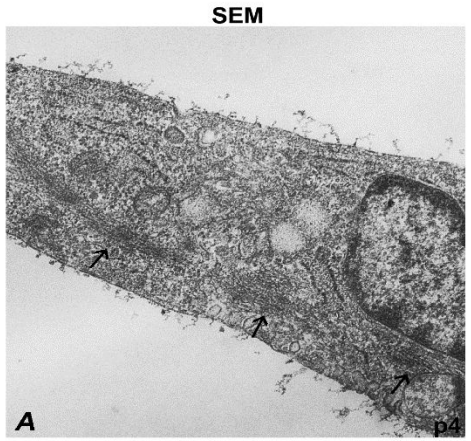


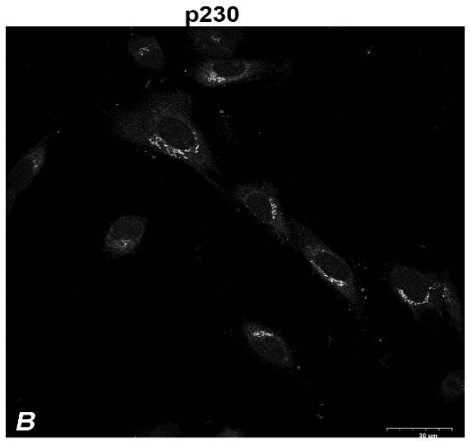
Активность AcPase лизосомах, демонстрирует продвинутую стадию автофагоцитоза (переваривания содержимого)

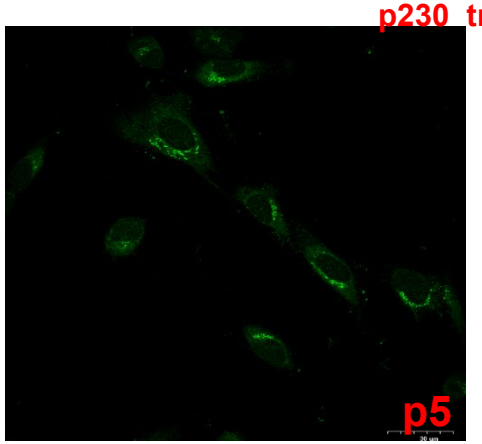


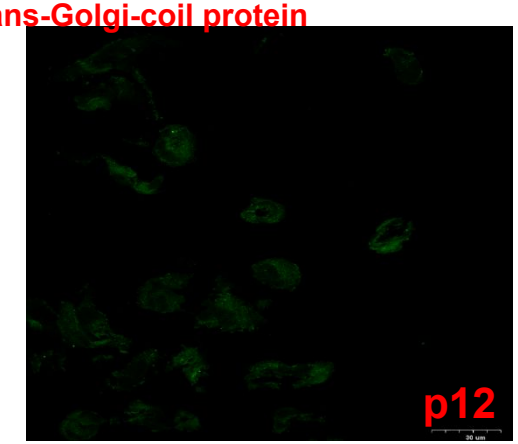


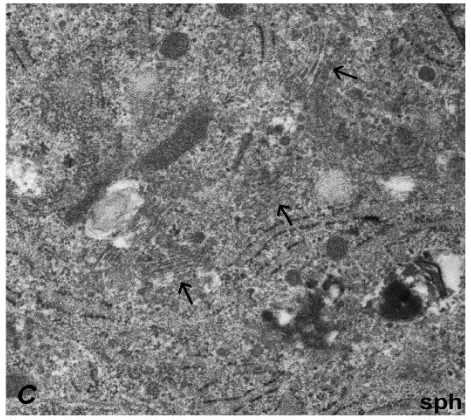


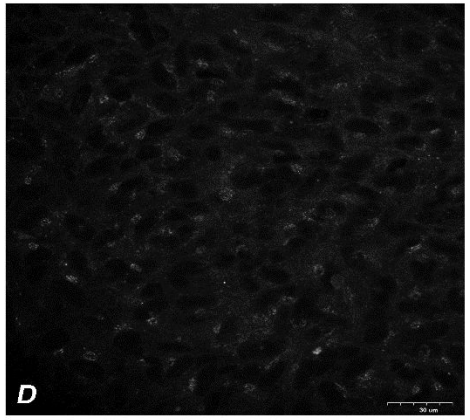


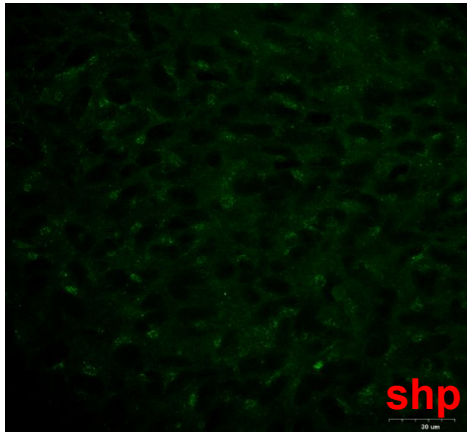


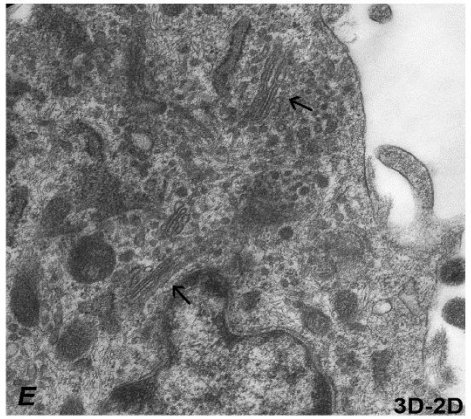


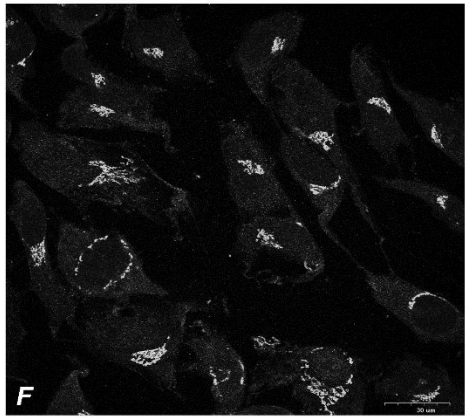


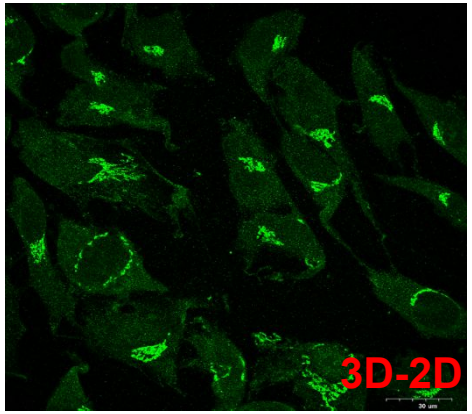










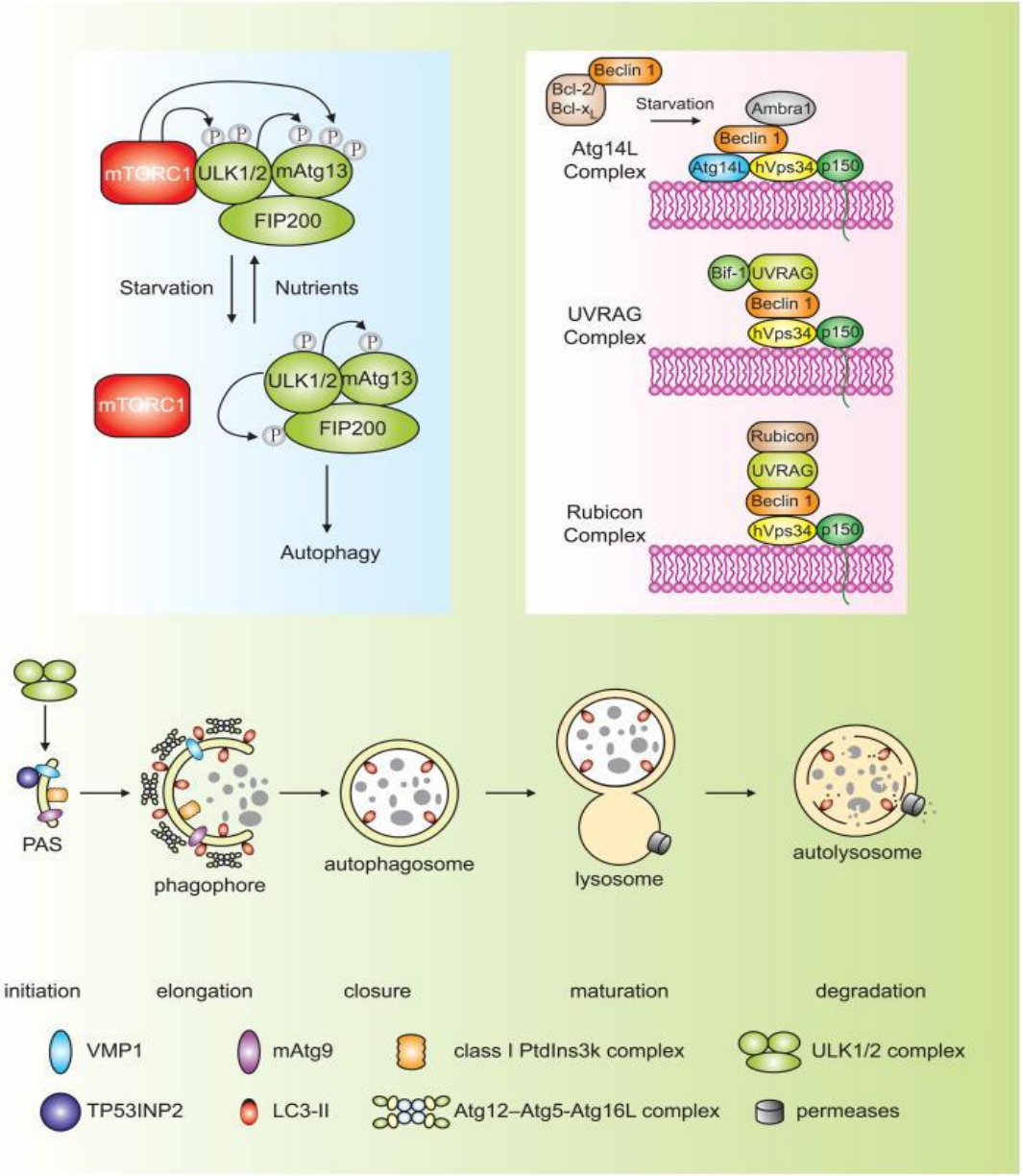


p230/golgin-245 is a *trans*-Golgi coiled-coil protein that is known to participate in regulatory transport from the *trans*-Golgi network to the cell surface.

p230 and MACF1 cooperatively play an important role in the formation of phagophore through starvation-induced transport of mAtg9-containing membranes from the TGN.

p230 detected in autophagosomes/autolysosome with p62 or LC3 during autophagosome biogenesis.

Происходит ли омоложение популяции в сфероидах за счет усиленного аутофагоцитоза ?

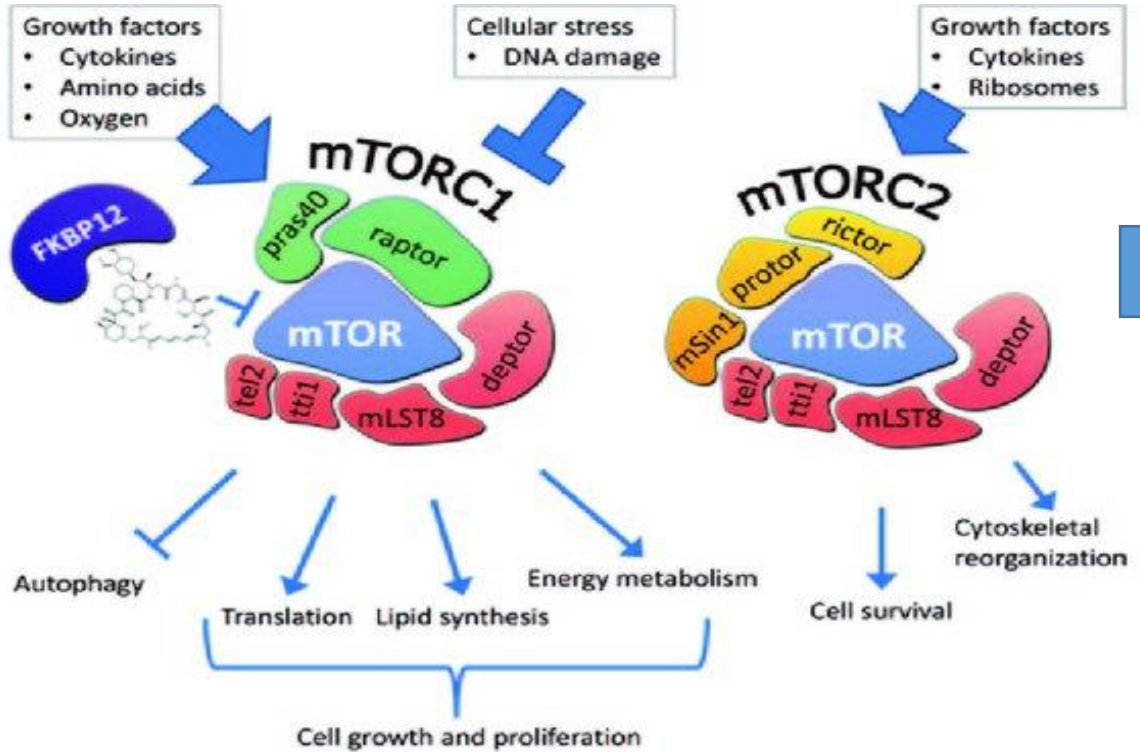


Regulatory components for autophagy induction include the **ULK1** and **ULK**2 complexes that contain various Atg proteins. The association of mTORC1 with this complex and the activity of mTORC1 depend on the nutrient status.
Under nutrient-rich conditions, mTORC1 is associated with the ULK1 and ULK2 complexes, and phosphorylates ULK1, ULK2, and mAtg13; upon inactivation of mTORC1 by nutrient starvation, mTORC1 disassociates, mAtg13, ULK1 and ULK2 are partially dephosphorylated, and activation of ULK1 and ULK2 promotes phosphosphorylation of FIP200. There are at least three class III PtdIns3K complexes (light red box at right), that are involved in autophagosome formation or clearance. The Atg14L (Atg14L-Beclin 1-hVps34-p150) and UVRAG (UVRAG-Beclin 1-hVps34-p150) complexes are required for autophagy, whereas the Rubicon complex (Rubicon-UVRAG-Beclin 1-hVps34-p150) negatively regulates autophagy. Ambra1 and Bif-1 are essential for induction of autophagy, through direct interaction with Beclin 1 and UVRAG, whereas Bcl-2 binds to Beclin 1 and disrupts the Beclin 1-associated hVps34 complex, thereby inhibiting autophagy

The mammalian/mechanistic **target of rapamycin (mTOR)** is a key component of cellular metabolism that integrates nutrient sensing with cellular processes that fuel cell growth and proliferation

rapamycin

Rapamycin interacts with FKBP12 and inhibits mTORC1.



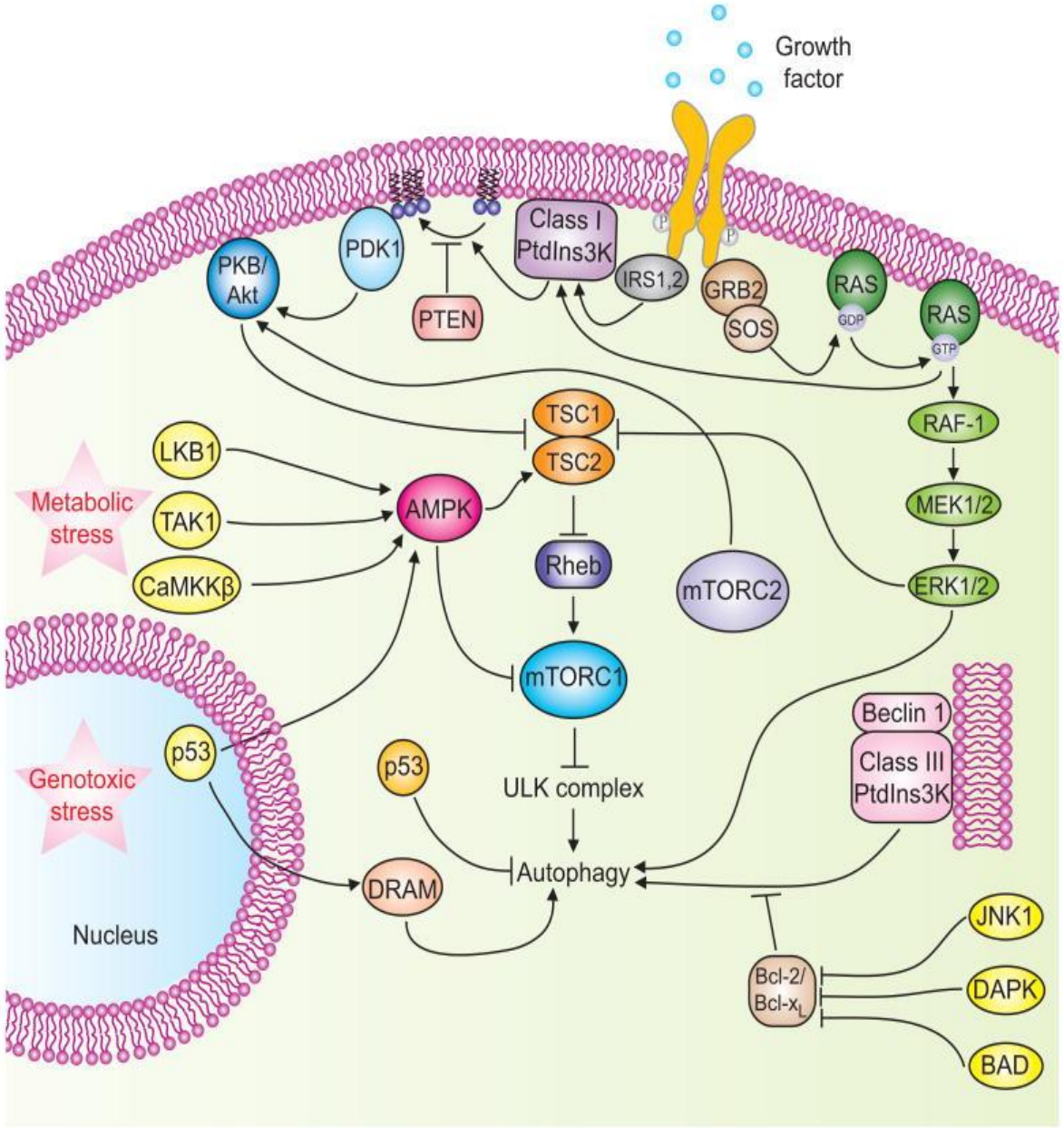
rapamycin

<u>Rapamycin induces autophagy in a wide variety of cell types by inhibiting the activity of TOR</u> as part of the multi-component TOR Complex 1 (TORC1). Control of autophagy by TORC1 signaling is largely responsible for the potent effect of starvation as an autophagy inducer.

Inhibition of TOR signaling thus leads to both activation of autophagy and inactivation of S6K, a positive regulator of autophagy.

Rapamycin: Current and Future Uses

Signaling cascades involved in the regulation of mammalian autophagy



Activation of growth factor receptors stimulates the class I PtdIns3K complex and small GTPase Ras, which leads to activation of the PtdIns3K-PKB-mTORC1 pathway and the Raf-1-MEK1/2-ERK1/2 pathway. PKB and ERK1/2 phosphorylate and inhibit the GTPase-activating protein complex TSC1/TSC2, leading to the stabilization of Rheb-GTPase, which, in turn, activates mTORC1, causing inhibition of autophagy. Activated ERK1/2 also stimulates autophagy. mTORC2 inhibits autophagy through the phosphorylation and activation of PKB. Metabolic stress, such as high AMP/ATP ratios resulting from energy depletion, or an increase in the cytosolic free Ca^{2+} concentration or cytokines, cause the AMP-activated protein kinase (AMPK) to be phosphorylated and activated by LKB1, CaMKKβ and TAK1. AMPK phosphorylates and activates TSC1/TSC2, leading to inactivation of mTORC1 and autophagy induction.
Genotoxic and oncogenic stresses result in nuclear p53 stabilization and activation, which stimulates autophagy through activation of AMPK or upregulation of DRAM. In contrast, cytosolic p53 has an inhibitory effect on autophagy. Anti-apoptotic proteins, Bcl-2 or Bcl-X_L, associate with Beclin 1 and inhibit the Beclin 1-associated class III PtdIns3K complex, causing inhibition of autophagy.

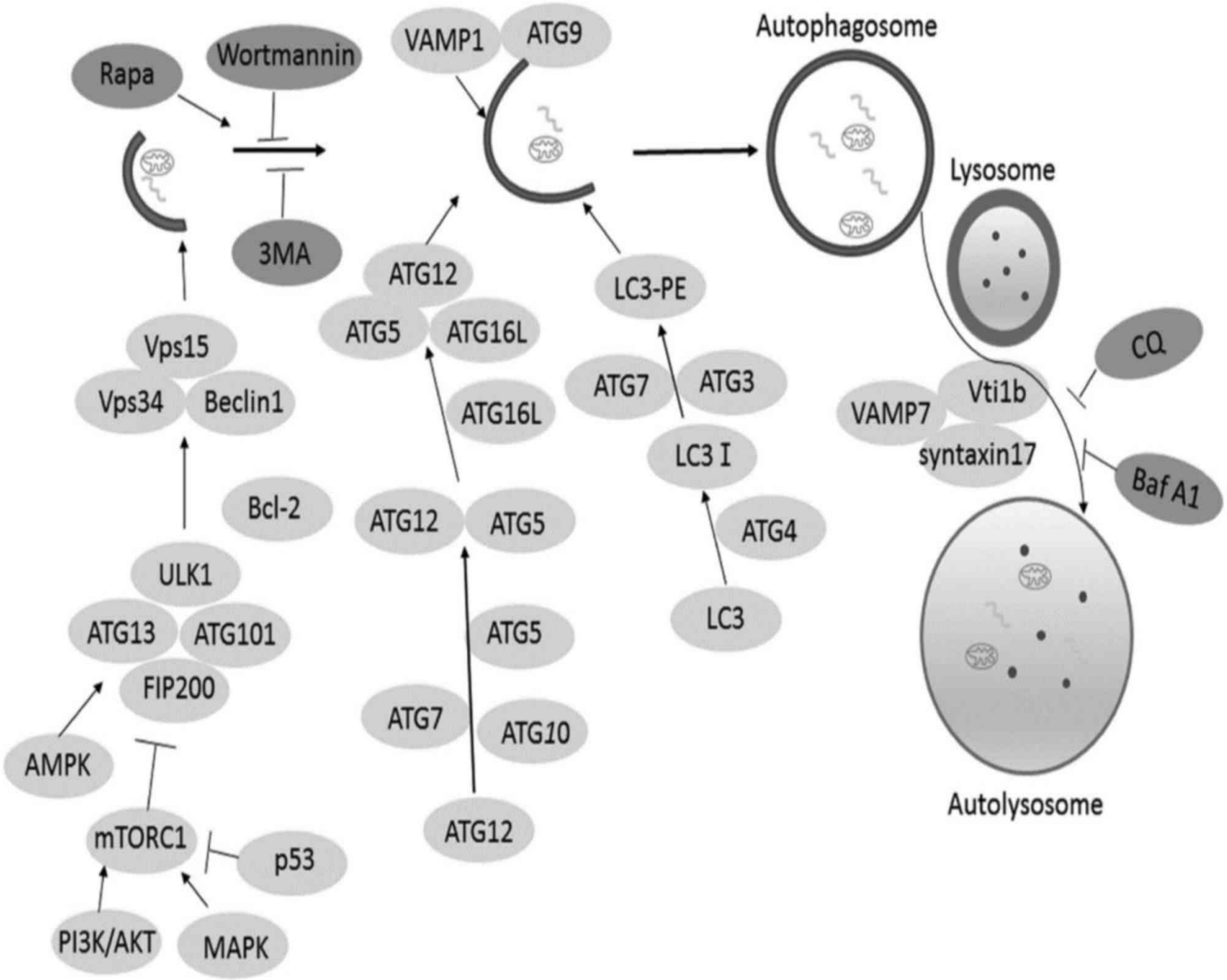
Decreased Production of Reactive Oxygen Species in 3D-mesenhcymal Stem Cell Spheroids Leads to Increased Therapeutic Efficacy via Autophagy Induction Shobha Regmi1, Yeungnam University, Gyeongsan, Korea.

ABSTRACT. In previous studies, 3D-MSC spheroids showed enhanced antiinflammatory effect and higher cell survival. In this study, we aimed to investigate the molecular signaling pathways responsible for the enhancement of cell viability in 3D-MSC, particularly focusing on autophagy and reactive oxygen species (ROS).

Method 3D-MSC spheroids were prepared by using hanging drop technique. Cell viability, ROS production, and autophagy activation in 3D-MSC were compared with that of 2D-cultured MSC

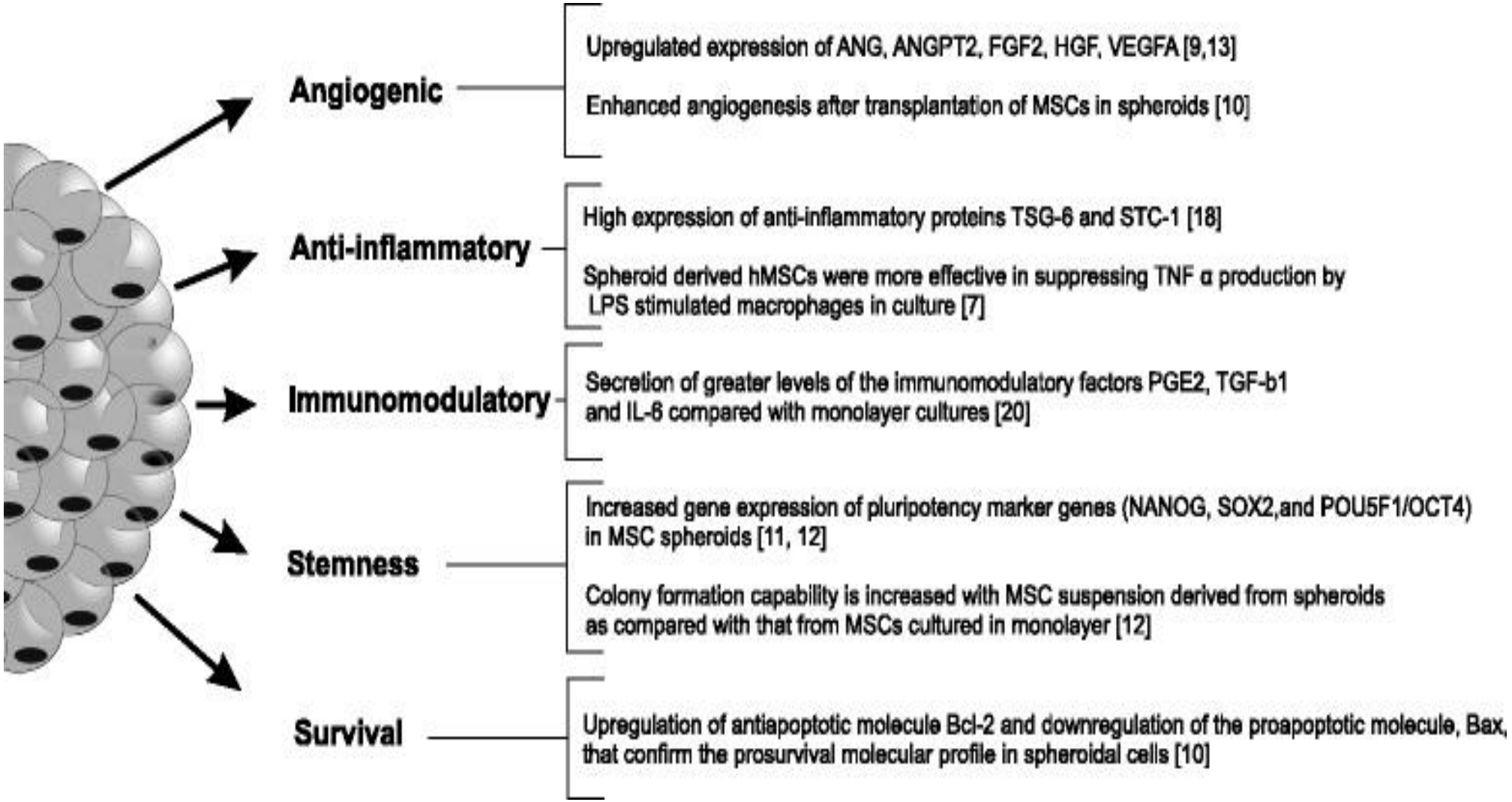
Results 3D-MSC showed higher cell viability, low ROS production, and upregulation in the expression of antioxidant proteins such as catalase, SOD2, and **hemooxygenase-1 (HO-1**). Inhibition of HO-1 by gene silencing in the 3D-MSC led to an increase in ROS production. In addition, HO-1 induction upregulated the catalase expression and attenuated ROS production in the MSC. Interesting, **HO-1 induction further induced autophagy activation**. Furthermore, inhibition HIF-1α resulted in HO-1 downregulation in 3D-MSC. This suggested **HO-1/ HIF-1α axis** may be involved in autophagy activation and cell survival in 3D-MSC. In vivo, silencing of autophagy in 3D-MSC caused decreased effectiveness of the MSC in ameliorating colitis in mice.

Conclusion The attenuation of ROS production in 3D-MSC led to an enhancement in MSC survival via the induction of autophagy. Therefore, the therapeutic effectiveness of 3D-MSC is at least, in part, mediated by autophagy induction.



PI3K/AKT and MAPK inhibit autophagy by regulating mTOR signaling pathway, p53 serves the opposite effect.
AMPK upregulates autophagy by activating ULK1 complex. Bcl-2 inhibits autophagy by interacting with Beclin1.
Rapamycin promotes the nucleation step of autophagosome, but wortmannin and 3MA inhibit this step.
CQ and Baf A1 impair the autophagic flux by inhibiting the fusion autophagosome and lysosome.

AMPK, 5′ AMP-activated protein kinase;
ULK1, Serine/threonine-protein kinase ULK1;
CQ, chloroquine;
BAF, bafilomycin A1;
3MA, 3-methyladenine.



Petrenko et al., 2017. The therapeutic potential of threedimensional multipotent mesenchymal stromal cell spheroids . Stem Cell Research & Therapy (2017) 8:94

HO-1/ HIF-1α axis may be involved in autophagy activation and cell survival in 3D-MSC

To validate this assumption, autophagy need to be assessed by:

(1) Histochemical staining - +

(2) transmission electron microscopy (TEM) -+

(3) Immunofluorescence (LC3 and p62) , p53

(4) WB analysis of LC3B-II (autophagosomal surface protein) and p62 (SQSTM1, an autophagic substrate), mTOR1, mTOR2, ERK1/2

(5) autophagic flux assay with lysosomal inhibitor

Additional WB Antibodies against phospho-ULK (Ser757) (#6888, 1:2000),
phospho-ULK1 (Ser555) (#5869, 1:2000),
ULK1 (#8054, 1:2000),
phospho-Beclin-1 (Ser93) (#14717, 1:2000),
Beclin-1 (#3738, 1:2000),
phospho-AMPKα (Thr172) (#2535, 1:2000),
AMPKα (#2532 S, 1:2000),
p62/SQSTM1 (#5114, 1:2000) from Cell Signaling
ATG4A (ab108322, 1:3000), LC3B (ab51520, 1:3000) from Abcam

WB for autophagy

p6 p10 p14 shp 2D/p3 2D/p6

Spheroids with 3000 cells/25ul drop
7000 cells/25ul drop
10000 cells /25ul drop

WB/FLOW for stemness : OCT4, NANOG, SOX2

? To block autophagocytosis at shp – rescue effect?
MitoSOX Red-stained human MSCs . Tom20

Most accurate way to measure autophagy is with an autophagic flux assay defined as the new formation of autophagosomes and their subsequent fusion with the lysosome.

LC3 is a unique component of the autophagic machinery because it is incorporated into the newly forming autophagosome membrane but is then degraded along with the autophagosome contents after lysosomal fusion

(1) Untreated cells, (2) Cells treated with the stimulus of interest (starvation for 16hrs), (3) Cells treated with a lysosomal inhibitor, i.e. chloroquine at 40μM for 2hrs, and (4) Cells starved for 16hrs with chloroquine (40μM) added for the last 2hrs of treatment.

(2) Whole cell lysate from these samples is then loaded onto a 12% polyacrylamide gel to ensure sufficient separation of the LC3-I and LC3-II band and probed with and LC3 antibody. Densitometric analysis of the LC3-II band can then be used to assess autophagic flux.

(3) It is important to note that the LC3-I band is not indicative of autophagic flux and should not be analyzed, nor should the LC3-II band be normalized to the LC3-I band.

(4) An advantage to monitoring **p62** to measure autophagic flux is that lysosomal inhibitors are not necessary, because unlike LC3-II, p62 does not usually increase when autophagy is induced. However, changes in p62 can often be subtle compared to LC3-II flux, probably because of additional mechanisms of regulation.

Одним из специфических свойств МСК является колониеобразование. При этом установлено, что только около 30% колониеобразующих мезенхимальных клеток являются мультипотентными, т. е. способными к дифференцировке в остеогенном, адипогенном и хондрогенном направлениях

CFE assay

СПАСИБО!