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Antioxidant pretreatments of mesenchymal stem cells against oxidative stress and respiration deficiency. The investigation of cellular and molecular mechanisms.

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Devant le JURY

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General introduction

Introduction

Les cellules souches mésenchymateuses (CSMs) ont été largement étudiées en raison de leur capacité à se différencier en adipocytes, chondrocytes et ostéoblastes, présentant un potentiel thérapeutique important dans le domaine de la médecine régénérative. Il a été montré que la greffe de CSMs est une approche prometteuse pour la réparation et la restauration des lésions tissulaires, cependant, la faible survie des CSMs greffées est une difficulté majeure pour le développement de ces nouvelles thérapies.

Au cours des dernières années, plusieurs approches ont été développées pour améliorer la survie des cellules greffées. Par exemple, il a été démontré que le prétraitement avec des facteurs de croissance (le FGF-2, le BMP-2, le facteur de croissance analogue à l'insuline IGF-1) et l'utilisation de supports tels que des matrices tridimensionnelles améliorent l'efficacité de l'implantation des CSMs. De plus, la transplantation des CSMs pré-conditionnées à l'hypoxie semble améliorer la capacité de ces cellules à réparer le site lésé via une survie accrue des cellules implantées et l'activation de voies de signalisation d'AKT et de l'angiogenèse. Des CSMs génétiquement modifiées surexprimant les signaux « pro-survie» cellulaires tels que Bcl-2 et AKT se sont également révélées plus résistantes à l'apoptose *in vitro* et *in vivo*.

Bien que les stratégies de pro-survie des CSMs se soient avérées efficaces dans plusieurs études, elles ne préservent pas les cellules de la mort cellulaire programmée, l'anoikis, induite par la perte ou l'absence des attachements matriciels. Les espèces oxygénées réactives (ROS) peuvent augmenter les signaux d'anoikis dans les CSMs transplantées dans la zone blessée. De plus, des preuves récentes suggèrent que la perturbation des contacts des intégrines dans les fibroblastes peut conduire à un détachement cellulaire qui est précédé d'une augmentation des niveaux de ROS intracellulaires. Les ROS peuvent également induire une réponse inflammatoire et nuire à l'adhésion cellulaire, entraînant la mort cellulaire. Des travaux antérieurs ont démontré que les ROS, une des causes majeures de la blessure après ischémie / reperfusion, peuvent entraver l'adhésion et la propagation des CSMs , et que la neutralisation des ROS améliore l'adhésivité des CSMs greffées dans le cœur infarci, conduisant à des effets bénéfiques pour la réparation cardiaque. Ainsi, la co-injection de CSMs avec des antioxydants (vitamine E, mito-TEMPO, astaxanthine, vitamine C,…) pourrait améliorer la viabilité et le potentiel d'intégration des CSMs et pourrait offrir de nouvelles opportunités thérapeutiques pour le traitement de l'infarctus du myocarde et de l'insuffisance cardiaque.

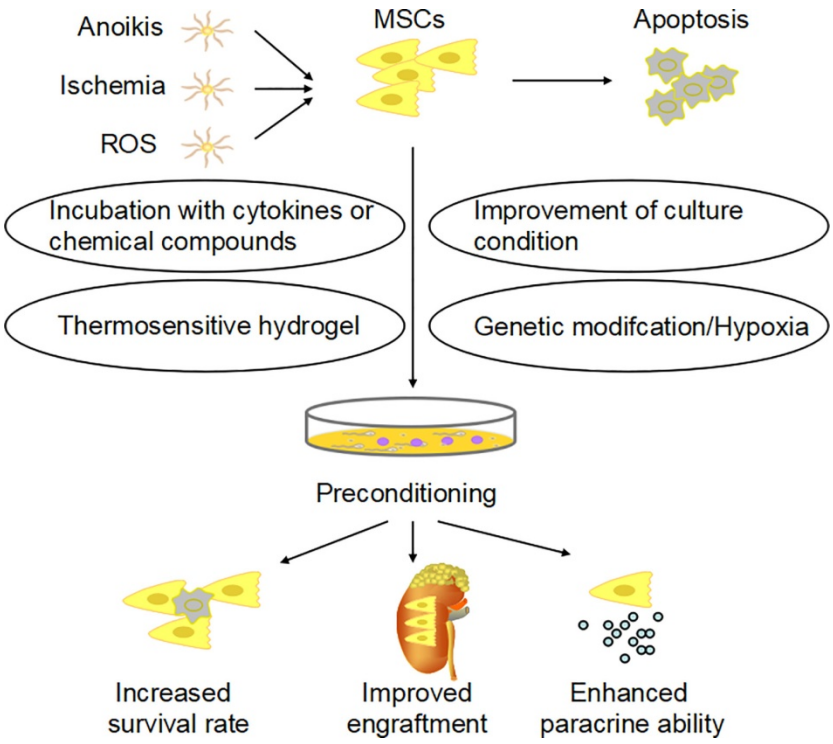
Ainsi, dans cette thèse nous avons étudié la signalisation cellulaire en conditions de stress oxydatif induit par le blocage de la chaine respiratoire cellulaire et l'éventuel effet bénéfique les antioxydants dans la survie des CSMs *in vitro*.

Ce manuscrit de thèse est organisé en 5 chapitres :

Le **Chapitre 1** est consacré à une revue de la littérature sur la biologie redox des cellules souches et des stratégies récentes pour le contrôle du stress oxydatif et du devenir des cellules souches.

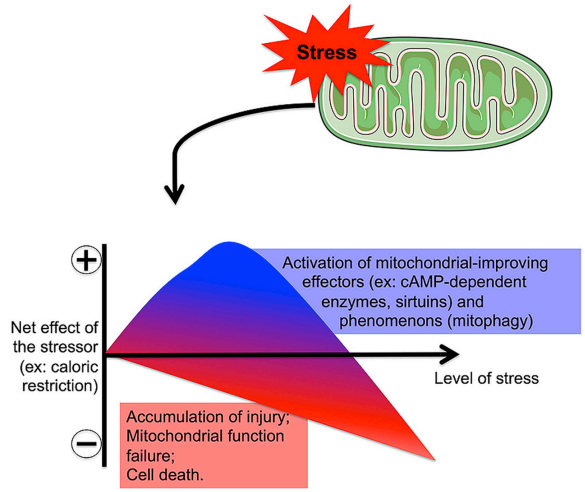
Résumé du Chapitre 1 :

Les capacités à se renouveler et à se différentier permettent aux CSMs transplantées d'exercer des effets régénératifs dans les tissus lésés par des mécanismes comprenant des effets paracrines, tels que les effets immunosuppresseurs et anti-apoptotiques, la vascularisation et la différenciation mais aussi la stimulation des cellules souches progénitrices locales. Cependant, le problème majeur dans cette thérapie est le faible taux de survie des CSMs après la transplantation. La mort cellulaire via l'anoïkis est causée en partie par le stress oxydatif induit par l'hypoxie et la perturbation du métabolisme énergétique. Pour améliorer la survie et l'adhésion cellulaire des CSMs transplantées, diverses stratégies ont été étudiées, y compris le prétraitement avec des facteurs de croissance ou des cytokines et la conception des nano-échafaudages biomimétiques mais également le préconditionnement hypoxique et des modifications génétiques pour induire la surexpression de HIF1α et d'autres acteurs moléculaires en aval.



Journal of Cellular and Molecular Medicine, 2018, DOI: (10.1111/jcmm.14035)

Par ailleurs, plusieurs études effectuées pour évaluer l'effet du préconditionnement des cellules souches avec des antioxydants ont montré un effet bénéfique dans la survie des cellules souches transplantées. Bien que les ROS soient un sous-produit physiologique de la phosphorylation oxydative, les altérations du taux d'oxydation mitochondriale avec une charge excessive de ROS ont été traditionnellement associées aux conditions pathologiques. Cependant, les ROS ont également été suggérées comme promoteurs de la mitohormesis, un processus dans lequel de faibles concentrations non cytotoxiques d'espèces réactives de l'oxygène favorisent l'homéostasie mitochondriale. Il a été montré que des faibles taux d'hypoxie et de ROS ne sont pas nocifs pour les cellules souches et induisent la prolifération et le maintien de leur



Free Radical Biology and Medicine, 2019
https://doi.org/10.1016/j.freeradbiomed.2019.07.017

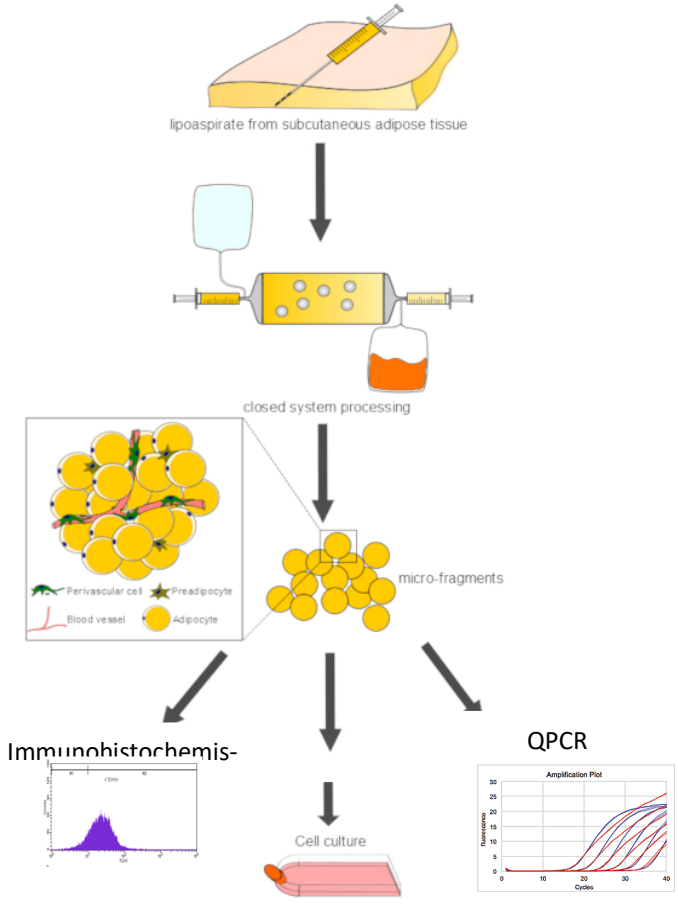
caractéristiques souches. Pour ces raisons, les systèmes de signalisation impliqués dans la régulation de l'homéostasie mitochondriale sont des candidats attrayants pour le développement de nouvelles stratégies thérapeutiques.

L'identification d'une stratégie prometteuse pour prévenir le stress oxydatif et induire la pré-dynamisation des CSMs permettrait d'améliorer l'homing et la survie des cellules souches transplantées. L'une de ces stratégies pourrait être un prétraitement de l'hypoxie et l'ajout des antioxydants aux CSMs avant leur transplantation dans les tissus endommagés. Cependant un équilibre entre l'éventuelle consommation d'antioxydants et la mitohermèse induite par les ROS devra être trouvé pour améliorer la durée de vie et la survie des cellules souches.

Dans le **Chapitre 2** nous avons choisi de présenter les cellules souches que nous avons utilisées dans toutes les études de la thèse.

Résumé du Chapitre 2 :

En comparaison avec d'autres cellules souches et en particulier celles issues de la moelle osseuse, les cellules souches mésenchymateuses dérivées du tissu adipeux (AD-MSC) sont prometteuses pour la thérapie régénérative en raison de leur facilité de récupération et leur potentialité à se différentier. L'isolement de cellules souches du tissu adipeux est de réalisation simple et permet d'obtenir un rendement cellulaire élevé. Cependant, les études ont souligné que les AD-MSC présentaient également un potentiel très élevé de propriétés telles que la prolifération, l'homing dans les tissus hypoxiques lésés et de multi potence et donc d'excellentes potentialités thérapeutiques. Dans les conditions de culture primaire, l'obtention d'un faible nombre de cellules conduit à réaliser plusieurs passages pour obtenir un nombre de cellules suffisant, dans ce cas les cellules souches deviennent sénescentes ; pour cette raison la récolte à haut rendement est également très importante pour les études *in vitro*. Ainsi, dans cette thèse, nous avons choisi de travailler avec des AD-MSC extraites de tissu adipeux humain obtenu après liposuccion (*voir schéma ci-contre*).



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Les cellules obtenues ont été caractérisées selon les recommandations de la Société internationale de thérapie cellulaire en suivant les trois critères minimaux pour la définition des CSM: (i) l'adhérence plastique, (ii) l'expression des marqueurs : CD73, CD90 et CD105, et l'absence d'expression de : CD11b ou CD14, CD19 ou CD79α, CD45 et de HLA-DR. Nous avons analysé l'expression de certains de ces marqueurs par cryométrie en flux,

(iii) leur potentiel de différenciation tri-lineage en adipocytes, chondrocytes et ostéoblastes. Nous avons évalué leur potentiel après culture dans les milieux appropriés et détection des marqueurs spécifiques par QPCR.

Dans les **Chapitres 3A et 3B** sont présentés deux articles de recherche sur l'effet des antioxydants, le mito-TEMPO((2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride) et le NAC (N-acétylcystéine) respectivement sur des cellules souches soumises au stress oxydatif induit par l'antimycine A en tant que bloqueur de la chaîne de transport d'électrons mitochondriale.

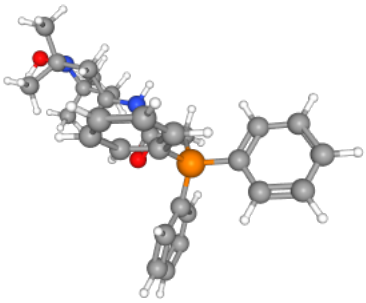
Résumé du Chapitre 3A :

Les travaux réalisés dans ce chapitre ont été acceptés (sous presse) pour publication dans : **Journal of Cellular Physiology**. *Mitochondria-targeted antioxidant mito-TEMPO alleviate oxidative stress induced by antimycin A in human mesenchymal stem cells*/ **DOI:10.1002/jcp.29495** (sous presse)

Comme nous l'avons décrit précédemment, une des raisons de la faible viabilité des CSMs transplantées est un déficit de la chaîne respiratoire dans les tissus endommagés lié au stress oxydatif et au métabolisme énergétique. Cela affecte leur sécrétome, leur survie, l'homing et par conséquent les résultats thérapeutiques. D'autre part, les fonctions essentielles des CSMs sont régulées par leur statut redox et leur métabolisme énergétique. Il est donc nécessaire de mettre en place des stratégies innovantes pour soutenir à la fois la chaîne respiratoire des CSMs et en parallèle, neutraliser le stress oxydatif.

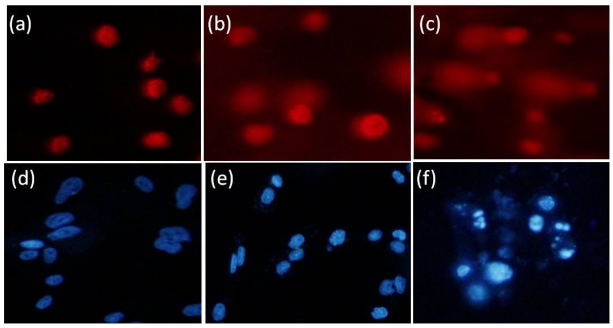
Ainsi, la revigoration des cellules souches par un preconditionnement avec des antioxydants contre le stress oxydatif pourrait être une stratégie envisageable pour améliorer les résultats de la thérapie cellulaire.

De divers antioxydants, le mito-Tempo (mito-T) (voir formule chimique dans la figure; https://pubchem.ncbi.nlm.nih.gov/compound/Mito-TEMPO) est l'un des antioxydants puissants qui pourraient cibler et neutraliser le stress oxydatif mitochondrial. Le mito-T est une substance hybride (une combinaison de l'antioxydant nitroxyde de pipéridine TEMPO avec le cation lipophile triphénylphosphonium), qui peut facilement traverser les bicouches lipidiques et s'accumuler dans les mitochondries jusqu'à plusieurs centaines de fois. Pour identifier les mécanismes sous-jacents liés à une protection des CSMs au stress oxydatif, les cellules ont été prétraitées avec le mito-T.



Dans cette étude, pour l'induction de l'hypoxie et du OS dans les CSMs isolées du tissu adipeux humain, l'antimycine A (AMA) a été utilisée. L'AMA induit le stress oxydatif via le blocage du cytochrome C et conduit à la

libération de quantités élevées de ROS et à la dépolarisation de la membrane mitochondriale. Les réponses cellulaires ont été analysées, y compris la viabilité cellulaire et l'arrêt du cycle cellulaire de CSMs exposées à l'AMA, au mito-T, au potentiel antioxydant, à l'homéostasie redox et aux voies de signalisation dans les MSC sous stress oxydatif.



Analysis of nucleus integrity. Panels a, b and c respectively show the Comet assay of the untreated cells, the cells co-treated with mito-T and AMA, and the cells treated with AMA. Panels d, e and f respectively represent the DAPI staining of the untreated cells, the cells co-treated with AMA and mito-T, and the cells treated with 40 μM of AMA after 48 h. AMA: antimycin A, mito-T: mito-TEMPO [Color figure can be viewed at wileyonlinelibrary.com]

Sur la base de nos résultats, nous avons montré une protection de la viabilité cellulaire et une gènoprotection des CSMs sous stress lors qu'elles étaient prétraitées par le Mito-T. Nous avons étudié les voies de signalisation cellulaires impliqués dans la réponse au stress, en particulier la voie du Nrf2, un facteur de transcription clé car il contrôle l'expression des gènes antioxydants et cytoprotecteurs via la séquence régulatrice appelée élément de réponse antioxydant (ARE).

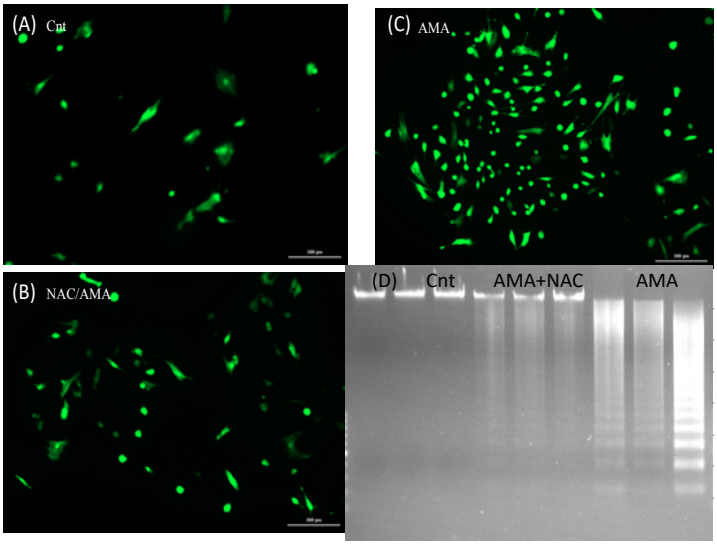
Lors de l'exposition des CSMs à des conditions de stress oxydatif élevé en utilisant l'AMA, les cellules n'ont pas réussi à récupérer. L'utilisation de mito-T a atténuée les dommages induits par le stress oxydatif à la fois par les fonctions directes du piégeage des radicaux libres et l'interaction en termes de voies de signalisation cellulaire, y compris la régulation à la hausse de la voie Nrf2. Le traitement a apporté aussi aux CSMs une résistance plus élevée à l'apoptose induite par le stress oxydative, ce qui est en corrélation avec la voie Nrf2 requise pour gérer le stress oxydative. Ces résultats pourraient ouvrir la voie à la thérapie par cellules souches pour les lésions tissulaires induites par l'hypoxie.

Résumé du Chapitre 3B : *Les résultats obtenus dans cette partie feront l'objet d'un article scientifique*

Dans cette partie, nous avons évalué l'activité du N-acétyl systéine (NAC), précurseur du glutathion, un antioxydant endogène humain. Le NAC a montré son efficacité chez l'homme *in vivo* dans le traitement de surdosage de paracétamol, des preuves solides existent également pour soutenir le NAC en tant que protecteur rénal et en tant qu'agent mucolytique. Dans la plupart des essais cliniques les effets secondaires du NAC ne diffèrent pas significativement du placebo. Le NAC a montré son efficacité lorsqu'il existe des preuves claires du stress oxydatif en tant que physiopathologie majeure ; la recherche sur les mécanismes d'action sous-jacents et la sécurité à long terme du NAC sont nécessaires pour envisager son utilisation dans de nouvelles indications.Nous avons émis l'hypothèse que le NAC pourrait cibler directement et indirectement les radicaux libres impliqués dans l'homéostasie énergétique des CSMs sous hypoxie et de ce fait présenter une efficacité accrue en comparaison des résultats obtenus avec le Mito-T. Dans cette étude, les CSMs dérivées du tissu adipeux humain ont été exposées à l'AMA en tant qu'inhibiteur de la chaine respiratoire cellulaire.

Sur la base de nos résultats, lors de l'exposition des MSCs à l'état de déficience de la chaîne respiratoire induit par l'AMA, les cellules n'ont pas réussi à piéger les radicaux libres et le métabolisme énergétique.

Le prétraitement des MSCs avec le NAC a montré atténuer les dommages causés à l'ADN, l'apoptose cellulaire, le stress oxydatif via la voie Nrf2 / Sirt3 en conditions de stress. Selon nos hypothèses nous attendions que le Nrf2 soit légèrement régulé à la baisse par le co-traitement du NAC, ce qui pourrait avoir un impact de récupération sur les ROS intracellulaires induits par l'AMA, mais il a été observé de manière surprenante que le NAC affectait directement la signalisation cellulaire et avait un effet synergique sur l'expression du Nrf2 en conditions basales. Les antioxydants en aval, y compris la catalase, NQO1 et SOD2, ont été régulés à la hausse dans des conditions de stress induit par l'AMA, mais atténués via un traitement NAC. Malgré ces résultats encourageants, nos résultants montrent que le NAC n'a pas d'effet ni sur le potentiel de la membrane mitochondriale ni sur la production d'ATP. Cette étude nous a permis de clarifier l'activité protectrice du NAC dans les MSCs sous stress mitochondrial causé par l'AMA.



Fluorescent microscopy and flow cytometry determination of oxidative stress within the stem cells. Panels A, B, and C represent the fluorescence intensity in the untreated control cells, the cells treated with AMA and NAC, and the cells treated with AMA, respectively Panel D represents the DNA ladder assay in triplicate.

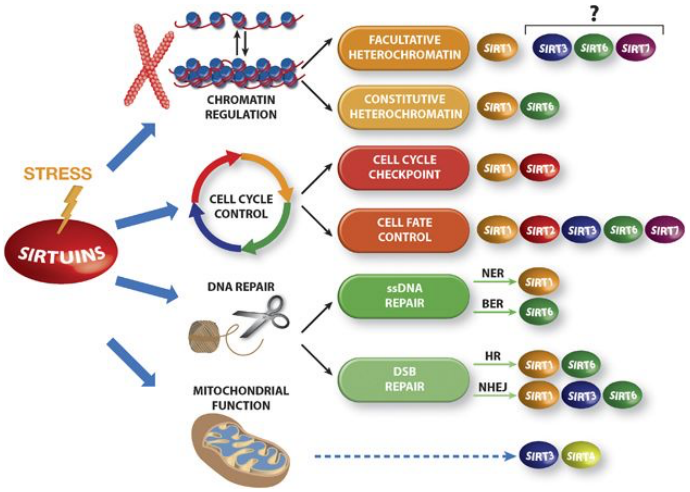
En conclusion de cette partie, nous avons mis en évidence qu'avec ce type de traitement antioxydant, nous pouvions préserver les CSMs du stress oxydatif induit par l'AMA, mais que nous n'apportons pas de solution au blocage du métabolisme énergétique et à l'arrêt de la production de l'ATP dans les conditions d'hypoxie.

Pour cette raison, et afin de trouver des nouvelles cibles thérapeutiques, nous avons analysé les données bibliographiques publiés sur d'autres paramètres que conditionnent également le devenir des cellules souches après l'implantation.

Dans le **Chapitre 4** nous présentons une revue de littérature (qui fera l'objet d'un article) avec une discussion critique concernant la nidification des cellules souches dans les lésions ischémiques hypoxiques et la reprogrammation des cellules souches dans des nouvelle niches dysfonctionnelles avec des acteurs macromoléculaires complexes.

La niche de cellules souches fait référence au microenvironnement local dans un tissu dans lequel les cellules

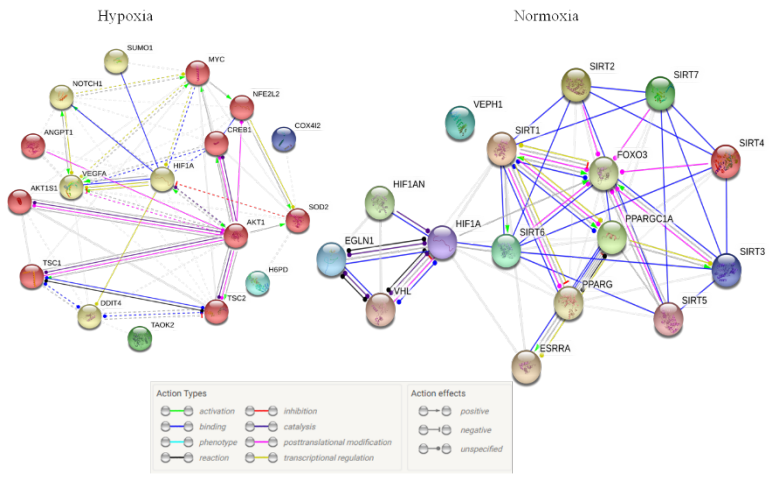
souches sont maintenues dans un état indifférencié et auto-renouvelable et reçoivent des stimuli qui déterminent leur sort. La niche est une structure complexe et dynamique qui transmet et reçoit des signaux par le biais de médiateurs cellulaires et acellulaires. L'oxygène est l'un des composants critiques de la niche. De faibles tensions en oxygène (hypoxie) maintiennent des états indifférenciés des phénotypes des cellules souches embryonnaires, hématopoïétiques, mésenchymateuses et neurales et influencent également la prolifération et l'engagement du destin cellulaire. Les mécanismes de nidification et de devenir des cellules souches dans la niche dysfonctionnelle sont des questions cruciales auxquelles il faut répondre pour concevoir une thérapie cellulaire réussie. Le site de lésion est capable d'induire une sénescence ou une apoptose en fonction des conditions environnantes hostiles, de l'hypoxie, de l'insuffisance respiratoire et du stress oxydatif. Il a été prouvé que l'hypoxie physiologique est vitale pour l'homing, l'auto-renouvellement et la différenciation des cellules souches dans la niche.



Oncogene **33,** 3764–3775 (2014)
doi:10.1038/onc.2013.344

Les principaux acteurs macromoléculaires impliqués dans le soutien à la survie et à la réadaptation des cellules souches dans une nouvelle niche dysfonctionnelle nécessitent d'être analysés et leurs rôles compris. Le facteur 1-alpha inductible par l'hypoxie (Hif1-α) est le principal régulateur transcriptionnel de la réponse des cellules à l'hypoxie et de l'adaptation des cellules souches dans une nouvelle niche. De plus, cette protéine est régulée par interaction avec les Sirtuins. Les Sirtuins (figure ci-contre) sont des enzymes dépendantes de NAD+ hautement conservées qui regulent le statut énergétique cellulaire et modulent la transcription des gènes, la stabilité du génome et le métabolisme énergétique en réponse aux signaux environnementaux pour adapter l'homing et le destin des cellules souches.

Dans ce chapitre une discussion critique est présentée sur la nidification des cellules souches dans les lésions ischémiques hypoxiques et la reprogrammation des cellules souches dans une nouvelle niche dysfonctionnelle. Les interactions Sirtuins / HIF1-α dans le devenir des cellules souches implantées sont discutées. Pour explorer les réseaux d'interaction des protéines codés par tous les gènes significatifs, une analyse des processus biologiques été réalisée par analyse bioinformatiques par le logiciel Search Tool for the Retrieval of Interacting Genes (STRING).

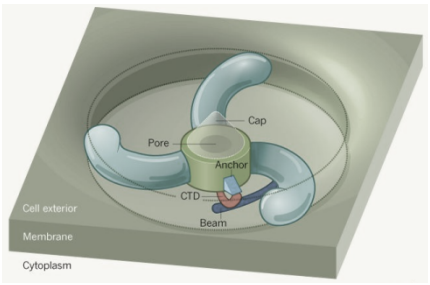


Nous avons extrait les réseaux de la base de données STRING (http://STRING- db.org/) qui permet de consolider *in silico* les données d'association protéine-protéine connues et prévues pour un grand nombre d'organismes.

Dans le **Chapitre 5A**, nous présentons un article d'analyse bibliographique actuellement en cours de révision dans le journal Cell and Tissue Research ; Manuscript No: CTRE-D-19-00265/Title: *The role of Piezo proteins and cellular mechano-sensing in tuning the fate of transplanted stem cells.*

Dans cette revue nous analysons le rôle des canaux piézoélectriques ou protéines Piézo, dans le devenir des cellules souches après l'implantation. Les protéines Piézo ont récemment été reconnues comme des canaux ioniques spéciaux assurant la médiation de la détection mécanique et de la mécanotransduction. La différenciation des cellules souches peut être modulée par une combinaison de signaux internes et externes, y compris des signaux mécaniques provenant du micro-environnement. Bien que de nombreux agents chimiques et biologiques aient été reconnus dans la régulation du destin des cellules souches, la question de savoir si les cellules souches peuvent directement détecter les signaux mécaniques pour choisir la différenciation en une lignée spécifique est encore mal connue. Le succès de tout effort de transplantation de cellules souches, cependant, repose sur une compréhension approfondie du sort de ces cellules sous différents signaux, y compris des signaux mécaniques.

Les protéines Piézo constituent une famille de canaux ioniques excitateurs directement déclenchés par



des forces mécaniques. Ces canaux ioniques participent à la mécanotransduction cellulaire et donc à la conversion des forces mécaniques en signaux biologiques. Les signaux mécaniques jouent un rôle important dans la traduction des informations des forces mécaniques telles que les contraintes de cisaillement et les charges de traction, ainsi que la rigidité et la topographie de la matrice

extracellulaire vers les voies de signalisation intracellulaires, liés à l'homing et à la différenciation des cellules souches.

Cette revue met en évidence l'importance des canaux ioniques à déclenchement mécanique exprimés par les cellules souches humaines, ainsi que de la mécanotransduction et de la mécano-mémoire passée dans le destin des cellules souches transplantées. Il est possible d'envisager que le contrôle des forces mécaniques spécifiques des tissus dans les matrices tridimensionnelles utilisées comme support pour l'implantation des cellules souches permettrait une amélioration ou encore la régulation de l'homing, de la différenciation et du sort des cellules souches transplantées.

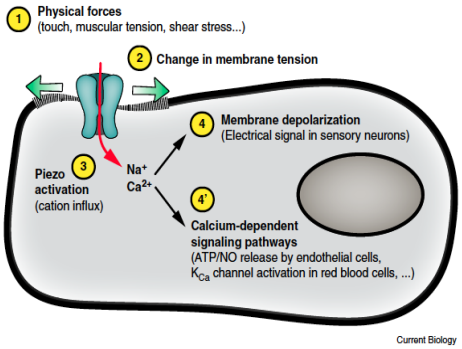


Figure 1. Piezo-dependent mechanotransduction.
Various mechanical stimuli exerted on cells induce changes in plasma membrane tension, eliciting Piezo channel opening. The resulting cation influx can trigger sensory neuron firing or activation of intracellular calcium signaling pathways.

Current Biology 27, R243–R258, April 3, 2017 © 2017 Elsevier Ltd

Les contraintes de cisaillement et les charges de traction, ainsi que la rigidité et la topographie de la matrice extracellulaire jouent un rôle très important dans l'activation des voies de signalisation intracellulaires, liés à l'homing et à la différenciation des cellules souches. Cette revue met en évidence le rôle des canaux ioniques à déclenchement mécanique exprimés par les cellules souches humaines, ainsi que le rôle de la mécanotransduction et de la mécano-mémoire passée dans le destin des cellules souches transplantées

Le **Chapitre 5B** est consacré au travail publié dans l'article :

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REVIEW ARTICLE

Journal of Cellular Physiology WILEY

The role of Hippo signaling pathway and mechanotransduction in tuning embryoid body formation and differentiation

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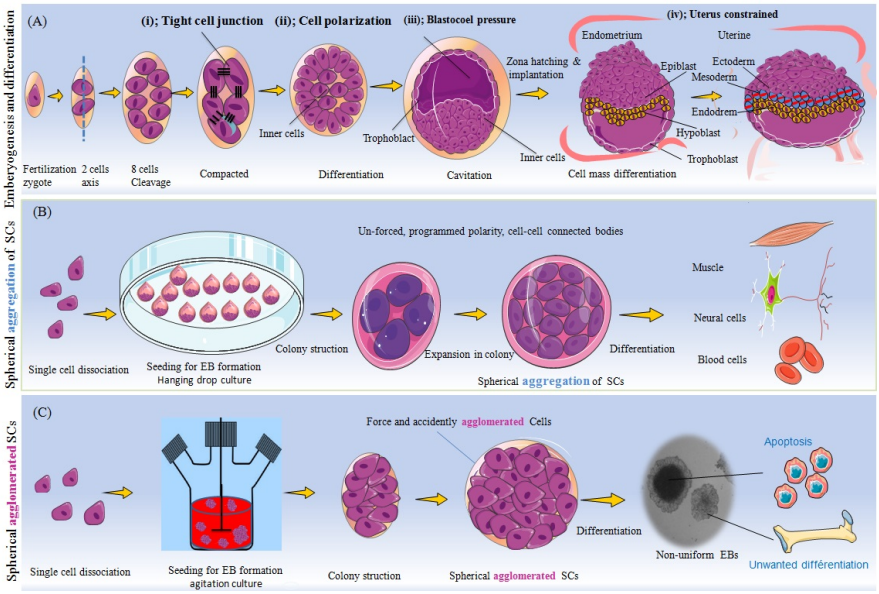
Nous présentons dans cette partie une revue des données de la littérature ainsi qu'une analyse *in silico* des facteurs intervenant dans le devenir des cellules souches embryonnaires (CSE) cultivées *in vitro.* Les agrégats de cellules souches pluripotentes appelés corps embryoïdes (EB) sont des modèles tridimensionnels souvent utilisés pour évaluer la différenciation des CSE. *In vivo*, la différentiation de ces cellules dépend des signaux mécaniques et biochimiques environnants liés à la voie de signalisation Hippo et à la mécanotransduction. La question reste posée concernant la signalisation cellulaire *in vitro* dans les EB. Des données récentes montrent que la formation et la différenciation d'EB ne sont pas aléatoires, qu'elles imitent plutôt le processus naturel de développement de l'embryogenèse. L'expression des marqueurs phénotypiques de l'endoderme et de l'ectoderme a révélé l'aptitude des EB à créer des cellules des trois couches germinales. Il existe plusieurs méthodes pour induire la formation d'EB à partir de CSE. D'après les données publiées, le système idéal devrait éviter la construction d'EB de grandes tailles, les agglomérations de cellules, la non-polarité des cellules et les contraintes mécaniques élevées.

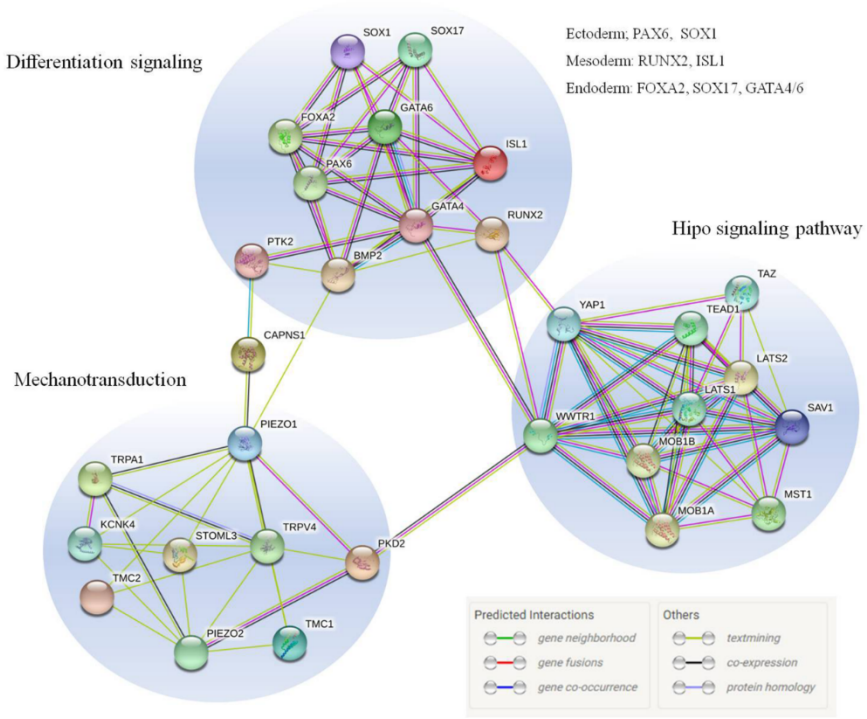


The effect of mechanical forces, cell polarity and cell arrangement, as well as cell-cell junctions in the formation and differentiation of embryoid bodies

Par ailleurs, l'analyse d'interactions des réseaux-protéine-protéine montre qu'il existe des interactions entre la mécanotransduction et la signalisation Hippo avec des marqueurs de différenciation phénotypiques. Cependant, la différenciation hétérogène typique des EB est un défi important pour la génération de types cellulaires définis et peut donc être influencée par la méthode de culture et la formation des EB. Par la cascade de mécanotransduction, les cellules traduisent des signaux mécaniques en signaux biochimiques contrôlant plusieurs aspects des mœurs cellulaires, y compris la prolifération et la différenciation.

Les protéines piezo, en tant que canaux ioniques activés par la tension membranaire et les signaux mécaniques, ont un rôle essentiel dans la traduction des contraintes mécaniques aux cellules souches. Au cours de la dernière décennie, des études approfondies ont clarifié l'importance de la voie Hippo dans la régulation de la taille des organes et plusieurs mécanismes sont proposés à cet égard. De toute

évidence, la polarité cellulaire et les complexes d'adhésion jouent un rôle clé dans la modulation de la voie Hippo.



Dans la figure est schématisée la signalisation Hipo en se basant sur des associations protéiques directes et indirectes dans la détermination de la prolifération et de la différenciation des cellules souches. Le réseau d'interaction a été réalisé à l'aide de la base de données STRING v. 10.5 (http://www.string-db.org). L'interaction entre la mécanotransduction et la signalisation Hippo est prédite, car la polycystine-2 (PKD2) qui fonctionne comme un canal cationique est impliquée dans la mécanosensation.

Dans la dernière partie du manuscrit nous présentons une discussion suivie d'une conclusion ainsi que des perspectives à venir concernant les possibles voies de soutien des cellules souches en conditions hypoxiques.

Nos résultats permettent de conclure que la seule l'utilisation d'antioxydants dans le soutien des cellules souches en conditions hypoxiques ne suffit pas, car le métabolisme énergétique reste dysfonctionnel. De plus il apparaît clairement qu'une étude linéaire de l'effet des prétraitements sur des cellules souches, y compris les prétraitements antioxydants et également le préconditionnement à l'hypoxie ne sont pas appropriés pour l'évaluation du devenir cellulaire. En effet, les signalisations cellule-cellule, l'adaptation des cellules souches par HIF1α / Sirtuins, les composants de la niche native et des systèmes de mécanotransduction pourraient chacun d'entre eux modifier les résultats linéaires obtenus avec des antioxydants dans le soutien des cellules souches en conditions d'hypoxie. De ce fait, il est nécessaire de comprendre de l'ensemble des facteurs intervenant et de les intégrer dans l'étude.

Introduction

Mesenchymal stem cells (MSCs) have been widely studied because of their potential to differentiate into adipocytes, chondrocytes and osteoblasts, and with significant therapeutic potential in the field of regenerative medicine. It has been shown that grafting MSCs is a promising approach for repairing and restoring tissue damage, however, the poor survival of grafted MSCs is a major abstacle for the development of these new therapies.

In recent years, several approaches have been developed to improve the survival of transplanted stem cells. For example, it has been shown that pretreatment with growth factors (FGF-2, BMP-2, insulin-like growth factor IGF-1) and the use of carriers such as three-dimensional matrices improve the efficiency of MSCs after implantation. In addition, transplanting of hypoxia pre-conditioned MSCs appears to improve the ability of these cells to repair the injured tissues via increased survival of cells and activation of AKT signaling pathways and induction of angiogenesis. Genetically engineered MSCs for overexpressing of cellular "pro-survival" signals such as Bcl-2 and AKT have also been shown to be more resistant of cells to apoptosis in vitro and in vivo.

Although MSCs pre-treatment strategies have been shown to be effective in several studies, they do not completely preserve cells from programmed cell death, due to the other issues in injured site including, anoikis, induced by the loss or absence of matrix adherent.

Reactive oxygen species (ROS) can increase the signals of anoikis in MSCs transplanted into the injured area. In addition, recent evidence suggests that disruption of integrin contacts in fibroblasts can lead to cell detachment which is preceded by increased levels of intracellular ROS. ROS can also induce an inflammatory response and impair cell adhesion, resulting in cell death. Previous works have demonstrated that ROS, one of the major causes of ischemia/reperfusion injury, can block the nesting and migration of MSCs, and that the neutralization of ROS improves the adaptation of grafted MSCs in the infracted heart, leading to beneficial effects for heart repair. Thus, the co-injection of MSCs with antioxidants (vitamin E, mito-TEMPO, astaxanthin, vitamin C,…) could improve the viability and the integration potential of MSCs and could offer new therapeutic opportunities for the treatment of myocardial infarction, heart failure and other hypoxic injured tissues.

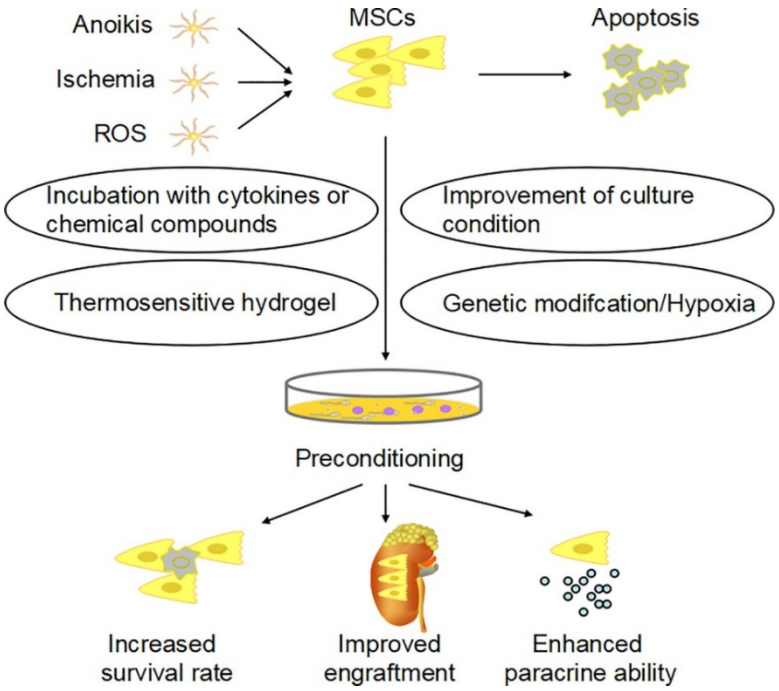
Thus, in this thesis we studied the cell signaling under conditions of oxidative stress induced by blockage of the cellular respiratory chain and the possible beneficial effect of antioxidants in the in vitro survival of MSCs.

This thesis manuscript is organized into 5 chapters:

Chapter 1 is devoted to the literature review on stem cell redox biology and recent strategies for controlling of oxidative stress and the fate of stem cells.

Summary of Chapter 1:

The capacities to selfrenewl and differentiation allows to the MSCs to use of them in regeneration of damaged tissues via mechanisms, including paracrine effects, such as immunosuppressive and anti-apoptotic effects, vascularization and differentiation but also stimulation of the local progenitor stem cells. However, the major problem in stem cell therapy is the low survival rate of MSCs after transplantation. Cell death via anoikis is caused in part by oxidative stress induced by hypoxia and disruption of energy metabolism. To improve the survival and homing of transplanted MSCs, various strategies have been reported, including pretreatment with growth factors or cytokines and the design of biomimetic nano-scaffolds but also hypoxic preconditioning and genetic modifications for over-expression of hypoxia transcription factor (HIF1α) and other it's downstream molecular players.

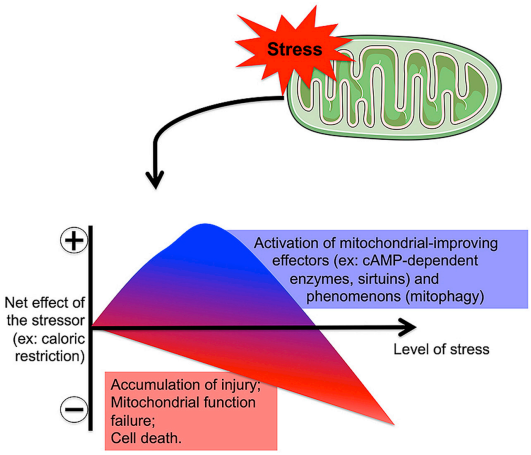


Journal of Cellular and Molecular Medicine,2018,DOI: (10.1111/jcmm.14035)

In addition, several studies carried out to assay the effect of preconditioning stem cells with antioxidants have shown a beneficial effect in the survival of trans-planted stem cells.

Although ROS are a physiological by-product of oxidative phosphorylation, alterations in the rate of mitochondrial oxidation with an excessive ROS release have traditionally been associated with pathological conditions. However, ROS have also been suggested as promoters of mitohormesis, a process in which low non-cytotoxic concentrations of ROS promote mitochondrial homeostasis and cell's selfrenewl. Low levels of hypoxia and ROS have been shown not to be harmful

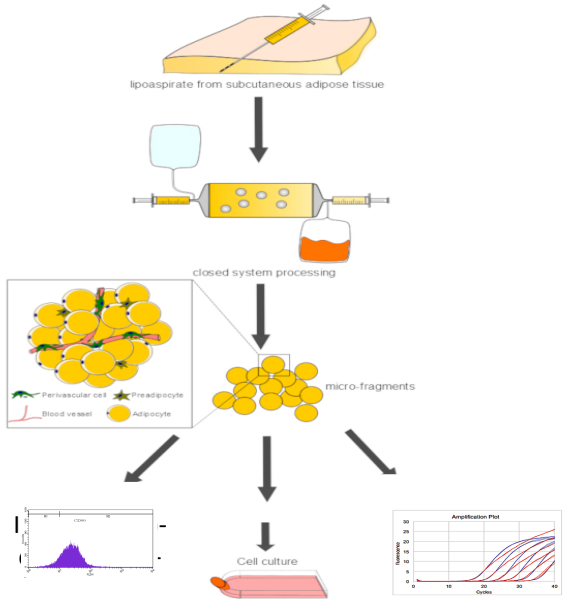
to stem cells and induce the proliferation and maintenance of their stemness. For these reasons, the signaling mechanisms involved in the regulation of mitochondrial homeostasis are attractive candidates for the development of new therapeutic strategies. The identification of a promising strategy to prevent oxidative stress and induce the pre-adaptation of MSCs would improve the homing and survival of transplanted stem cells. One of these strategies could be hypoxia and antioxidants pretreatment of MSCs before transplanted into damaged tissue. However, a balance between the possible treatment of antioxidants and mitohermesis induced by ROS must be found to improve the lifespan, survival and programming to the fate.



Free Radical Biology and Medicine, 2019
https://doi.org/10.1016/j.freeradbiomed.2019.07.017

In Chapter 2 we have present the adipose drived stem cells isolation, characterization and screening that we used in all of the thesis studies.

Summary of Chapter 2:



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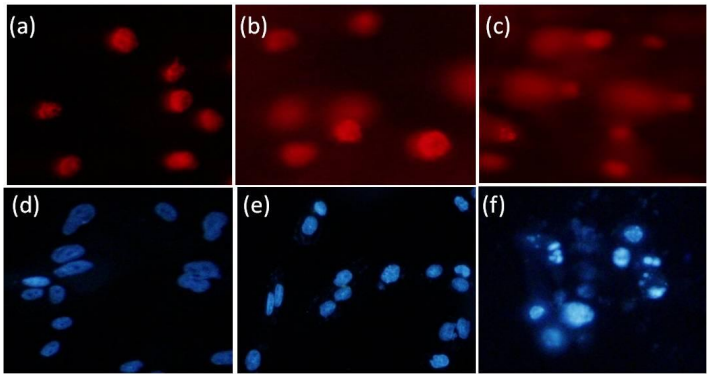
In comparison with other stem cells and in particular those isolated from the bone marrow, the MSCs derived from adipose tissue (AD-MSC) are promising for regenerative therapy because of their ease of accessible, recovery and their potential to homing. The isolation of stem cells from adipose tissue is simple and with high yield of stem cells to be obtained.

However, studies have reported that AD-MSCs also have a very high potential for properties such as proliferation, homing in injured hypoxic and injured tissue and therefore excellent therapeutic



Analysis of nucleus integrity. Panels a, b and c respectively show the Comet assay of the untreated cells, the cells co-treated with mito-T and AMA, and the cells treated with AMA. Panels d, e and f respectively represent the DAPI staining of the untreated cells, the cells co-treated with AMA and mito-T, and the cells treated with 40 μM of AMA after 48 h. AMA: antimycin A, mito-T: mito-TEMPO [Color figure can be viewed at wileyonlinelibrary.com]

potential. In primary culture conditions, obtaining a low number of cells leads to making several passages to obtain a sufficient number of cells for injection, in this case the stem cells become senescent; for this reason, high yield harvesting is also very important for in vitro studies. Thus, in this thesis, we have chosen to work with AD-MSC extracted from human adipose tissue obtained after liposuction (see diagram opposite). The isolated cells were characterized according to the recommendations of the International Society for Cell Therapy by following the three minimum criteria for the definition of MSCs:

(i) plastic adhesion, (ii) expression of markers: CD34, CD90 and CD105, and the absence of expression of: CD14, CD19 or CD79α, and CD45 . We analyzed the expression of some of these markers by flow cryometry,

(iii) Their potential for tri-lineage differentiation in to adipocytes, chondrocytes and osteoblasts. We evaluated their homing and paracrin secretion potential after culture in hypoxia condition.

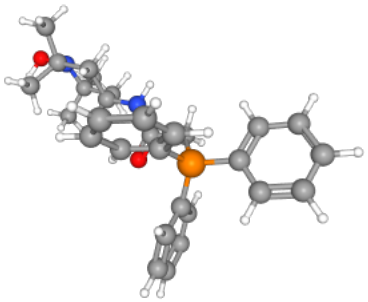
In Chapters 3A and 3B are presented two research articles on the effect of antioxidants, mito-TEMPO ((2- (2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino) -2-oxoethyl) triphenylphosphonium chloride) and NAC (N-acetylcysteine) respectively on stem cells subjected to oxidative stress induced by antimycin A as a blocker of the mitochondrial electron transport chain (ETC).

Summary of Chapter 3A:

The work presented in this chapter has been accepted in the journal of cellular phidiology. Mitochondria-targeted antioxidant mito-TEMPO alleviate oxidative stress induced by antimycin A in human mesenchymal stem cells / DOI: 10.1002 / jcp.29495 .As we have previously described, one of

the reasons for the low viability of transplanted MSCs is a deficit in the respiratory chain in damaged tissue linked to oxidative stress and energy metabolism. This affects their secretome, survival, homing and therefore therapeutic outcomes. On the other hand, the essential functions of MSCs are regulated by their redox status and their energy metabolism. It is therefore necessary to improve the strategies to support both the respiratory chain of MSCs and at the same time neutralize oxidative stress.

Thus, reinvigoration of stem cells by preconditioning with antioxidants against oxidative stress could be a possible strategy to improve the cell therapy success. Among diferent antioxidants, mito-Tempo



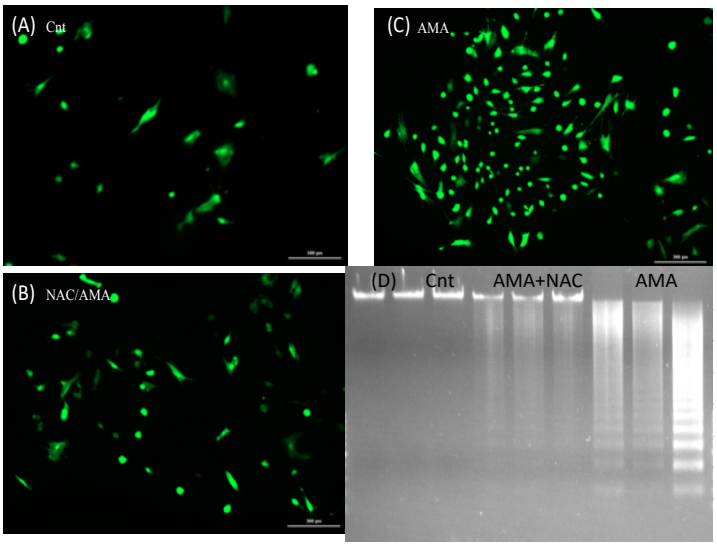
(mito-T) (see chemical formula in the figure; https://pubchem.ncbi.nlm.nih.gov/compound/Mito-TEMPO) is one of the powerful antioxidants that could target and neutralize mitochondrial oxidative stress. Mito-T is a hybrid substance (a combination of the antioxidant piperidine nitroxide TEMPO with the lipophilic cation triphenylphosphonium), which can easily cross lipid bilayers and accumulate in the mitochondria up to several hundred times. To identify the underlying mechanisms linked to the protection of MSCs from oxidative stress, the cells were pretreated with mito-T. In this study, for the induction of hypoxia in the MSCs isolated from human adipose tissue, antimycin A (AMA) was used. AMA induces oxidative stress via the blocking of cytochrome C and leads to the release of high amounts of ROS and depolarization of the mitochondrial membrane. Then, the responses were analyzed, including cell viability and cell cycle arrest of MSCs exposed to AMA, antioxidant potential of mito-T, redox homeostasis and signaling pathways in MSCs under oxidative stress.

Based on our results, we have shown protection of cell viability and genoprotection of MSCs under oxidative stress when they were pretreated with Mito-T. We have studied the cellular signaling pathways involved in the stress response, in particular the Nrf2 pathway, a key transcription factor because it controls the expression of antioxidant and cytoprotective genes via the regulatory elements called antioxidant response element (ARE) .

When exposing the MSCs to high oxidative stress conditions using AMA, the cells failed to recover.

The use of mito-T attenuated the damage induced by oxidative stress both by the direct functions of free radical scavenging and the interaction in terms of cell signaling pathways, including upregulation of some cell signaling pathway especially Nrf2 pathway. The treatment also couses that MSCs be resistance to oxidative stress-induced apoptosis, which is correlated with the Nrf2 pathway required to manage oxidative stress. These findings could pave the way for stem cell therapy for hypoxia-induced tissue damage.

Summary of Chapter 3B: The results obtained in this part will be the subject of a scientific article



Fluorescent microscopy and flow cytometry determination of oxidative stress within the stem cells. Panels A, B, and C represent the fluorescence intensity in the untreated control cells, the cells treated with AMA and NAC, and the cells treated with AMA, respectively Panel D represents the DNA ladder assay in triplicate.

In this section, we have assessed the activity of N-acetyl cystein (NAC), a precursor to glutathione, a human endogenous antioxidant. NAC has been shown to be effective in humans in the treatment of paracetamol overdose, there is also strong evidence that, NAC act as a mucolytic agent. In most clinical trials the side effects of NAC did not differ significantly from placebo. NAC has been shown to be effective when there is clear evidence of oxidative stress as a major pathophysiology; research on the underlying mechanisms of action and long-term safety of NAC is necessary to consider its use in new treatments. We hypothesized that the NAC could directly and indirectly target the free radicals involved in the energy homeostasis of MSCs under hypoxia and therefore present an increased efficiency compared to the results obtained with Mito-T treatment. In this study, MSCs derived from human adipose tissue were exposed to AMA as an inhibitor of the respiratory chain reaction.

Based on our results, when exposing MSCs to AMA-induced respiratory chain deficiency, cells failed to scavenge free radicals and energy metabolism. Pretreatment of MSCs with NAC has been shown

to reduce damage to DNA, cellular apoptosis, oxidative stress via the Nrf2 / Sirt3 pathway under stress conditions. According to our hypotheses, we expected that Nrf2 would be slightly downregulated by the co-treatment of NAC, which could have a recovery impact on intracellular ROS induced by AMA, but it was surprisingly observed that NAC directly affected cell signaling and had a synergistic effect on the expression of Nrf2 in basal conditions. Downstream antioxidants, including catalase, NQO1 and SOD2, were upregulated under AMA-induced stress conditions, but attenuated via NAC treatment. Despite these encouraging results, our results show that NAC has no effect either on the potential of the mitochondrial membrane or on the production of ATP. This study allowed us to clarify the protective activity of NAC in MSCs under mitochondrial stress caused by AMA.

In conclusion of this part, we highlighted that with this type of antioxidant treatment, we could preserve the MSCs from oxidative stress induced by AMA, but that we couldnot recover the energy metabolism and the ATP production under hypoxic conditions.

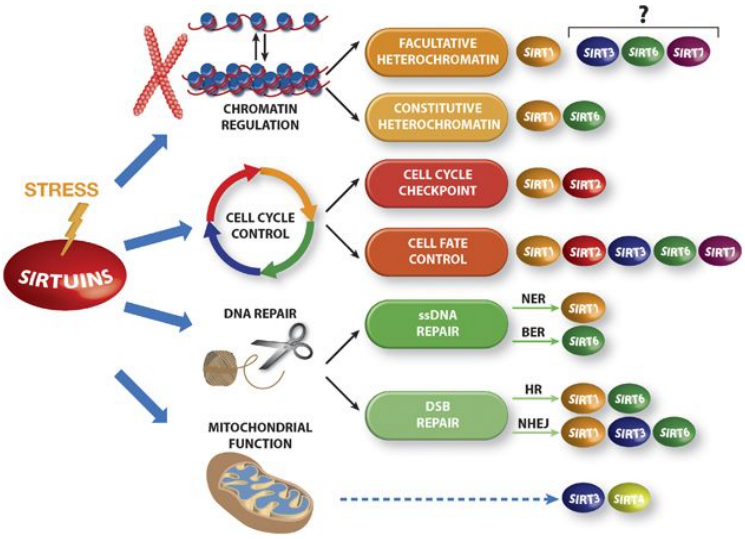
For this reason, and in order to find new therapeutic targets, we analyzed the published bibliographic data on other parameters that also determine the fate of stem cells after implantation.

In Chapter 4 we present a literature review (which will be the subject of an article) with a critical discussion concerning the nesting of stem cells in hypoxic ischemic lesions and the reprogramming of stem cells in new dysfunctional niches with a complex macromoleculars players.

The stem cell niche refers to the local microenvironment in a tissue in which the native stem cells are kept in an undifferentiated and self-renewing state and receive internal and external stimuli that determine their fate. The niche is a complex and dynamic structure that transmits and receives signals through cellular and acellular mediators.

Oxygen is one of the critical components of the niche. Low oxygen tensions (hypoxia) maintain undifferentiated states of the phenotypes of embryonic, hematopoietic stem cell, mesenchymal and neural stem cells and also influence the proliferation and commitment of cell fate. The mechanisms of nesting and becoming stem cells in the dysfunctional niche are crucial questions that must be answered in order to design a successful cell therapy. The lesion site is capable of inducing senescence or apoptosis depending on harsh surrounding conditions, hypoxia, respiratory failure and oxidative stress.

Physiological hypoxia has been reported to be vital for homing, self-renewal and differentiation of stem cells in the niche. The main macromolecular actors involved in supporting the survival and rehabilitation of stem cells in a new dysfunctional niche need to be analyzed and their roles understood. The factor 1-alpha inducible by hypoxia (Hif1-α) is the main transcriptional regulator of the response of cells to hypoxia and of the adaptation of stem cells in a new niche. In addition, this protein is regulated by interaction with the Sirtuins.

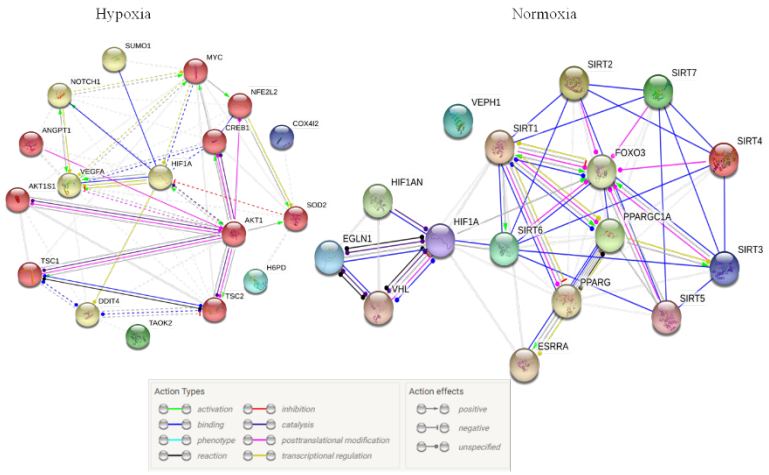


Oncogene **33,** 3764–3775 (2014)
doi:10.1038/onc.2013.344

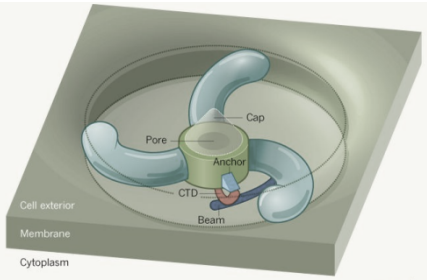
The Sirtuins (figure opposite) are highly conserved NAD + dependent enzymes that regulate cellular energy status and modulate gene transcription, genome stability and energy metabolism in response to environmental signals to adapt homing and fate stem cells.

In this chapter a critical discussion is presented on the nesting of stem cells in hypoxic ischemic lesions and the reprogramming of stem cells in a new dysfunctional niche. The Sirtuins / HIF1-α interactions in the fate of implanted stem cells are discussed.

To explore the interaction networks of proteins encoded by all significant genes, an analysis of biological processes was carried out by bioinformatics analysis using Search Tool for the Retrieval of Interacting Genes (STRING) software. We have extracted the data from the STRING database (http: // STRING- db.org/) which makes it possible to consolidate in silico the protein-protein interactions data known and expected for a large number of organisms.



In **Chapter 5A**, we present a bibliographic review article currently under review in the journal of Cell and Tissue Research; Manuscript No: CTRE-D-19-00265 / Title: The role of Piezo proteins and cellular mechano-sensing in tuning the fate of transplanted stem cells.



In this review we analyze the role of piezo channels or Piezo proteins in the fate of stem cells after implantation. Piezo proteins have recently been recognized as special ion channels mediating mechanical detection and mechanotransduction. Differentiation of stem cells can be modulated by a combination of internal and external signals, including mechanical signals from the microenvironment. Although many chemical and biological agents have been recognized in regulating the fate of stem cells, the question of whether stem cells can directly detect mechanical signals to choose differentiation into a specific line is still poorly understood. The success of any stem cell transplant effort, however, depends on a thorough understanding of the fate of these cells under different signals, including mechanical signals.

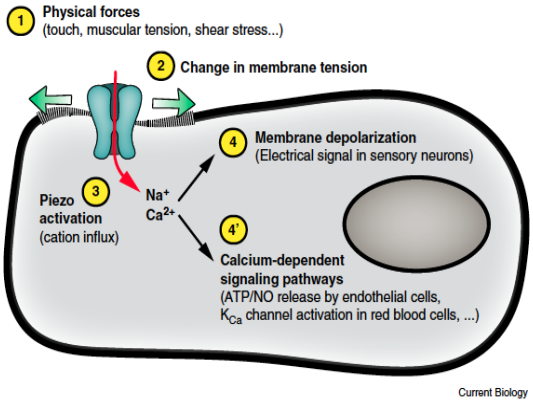


Figure 1. Piezo-dependent mechanotransduction.
Various mechanical stimuli exerted on cells induce changes in plasma membrane tension, eliciting Piezo channel opening. The resulting cation influx can trigger sensory neuron firing or activation of intracellular calcium signaling pathways.

Current Biology 27, R243–R258, April 3, 2017 © 2017 Elsevier Ltd

Piezo proteins constitute a family of excitatory ion channels directly triggered by mechanical forces. These ion channels participate in cellular mechanotransduction and therefore in the conversion of mechanical forces into biological signals. Mechanical signals play an important role in the translation of information from mechanical forces such as shear stresses and tensile loads, as well as the rigidity and topography of the extracellular matrix towards the intracellular signaling pathways, linked to homing. and differentiation of stem cells.

This review highlights the importance of mechanically triggered ion channels expressed by human stem cells, as well as mechanotransduction and past mechanical memory in the fate of transplanted stem cells. It is possible to envisage that the control of the specific mechanical forces of the tissues in the three-dimensional matrices used as support for the implantation of the stem cells would allow an improvement or the regulation of the homing, the differentiation and the fate of the transplanted stem cells.

Shear stresses and tensile loads, as well as the rigidity and topography of the extracellular matrix play a very important role in the activation of intracellular signaling pathways, linked to the homing and differentiation of stem cells. This review highlights the role of mechanically triggered ion channels expressed by human stem cells, as well as the role of mechanotransduction and past mechanical memory in the fate of transplanted stem cells

Chapter 5B is devoted to the work published in the article:

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REVIEW ARTICLE

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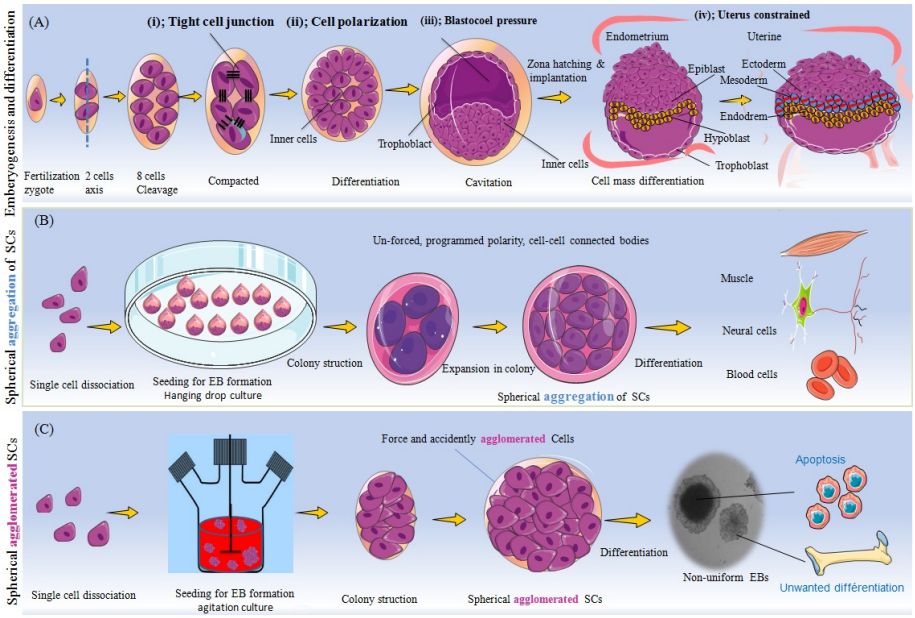
The role of Hippo signaling pathway and mechanotransduction in tuning embryoid body formation and differentiation

Abolfazl Barzegari[1] | Virginie Gueguen[2] | Yadollah Omidi[3,6] | Alireza Ostadrahimi[4,7] | Mohammad Nouri[1,5] | Graciela Pavon-Djavid[2]

In this section, we present a review of the data from the literature as well as an in silico analysis of the factors involved in the pluripotent stem cell aggregates called embryoid bodies (EBs). EBs are three-dimensional models often used to assess the differentiation of stem cells invitro. In vivo, the differentiation of these cells depends on the surrounding mechanical and biochemical signals linked to the Hippo signaling pathway and to mechanotransduction. The question remains regarding cell signaling in vitro in EB. Recent data show that the formation and differentiation of EB is not random, rather it mimics the natural process of development of embryogenesis. Expression of phenotypic markers of the endoderm and ectoderm revealed the ability of EBs to create cells from the three germ layers. There are several methods to induce EB formation from CSE. According to the published data, the ideal system should avoid the construction of large EBs, cell agglomerations, non-polarity of cells and high mechanical stresses.

Furthermore, the analysis of protein-protein network interactions shows that there are interactions between mechanotransduction and Hippo signaling with phenotypic differentiation markers. However, the heterogeneous differentiation typical of EBs is an important challenge for the generation of defined cell types and can therefore be influenced by the culture method and the formation of EBs. Through the mechanotransduction cascade, cells translate mechanical signals into biochemical signals controlling several aspects of cell mores, including proliferation and differentiation.

The piezo proteins, as ion channels activated by membrane tension and mechanical signals, have an essential role in the translation of mechanical stresses to stem cells. Over the past decade, in-depth studies have clarified the importance of the Hippo pathway in regulating organ size and several mechanisms are proposed in this regard. Clearly, cell polarity and adhesion complexes play a key role in modulating the Hippo pathway.



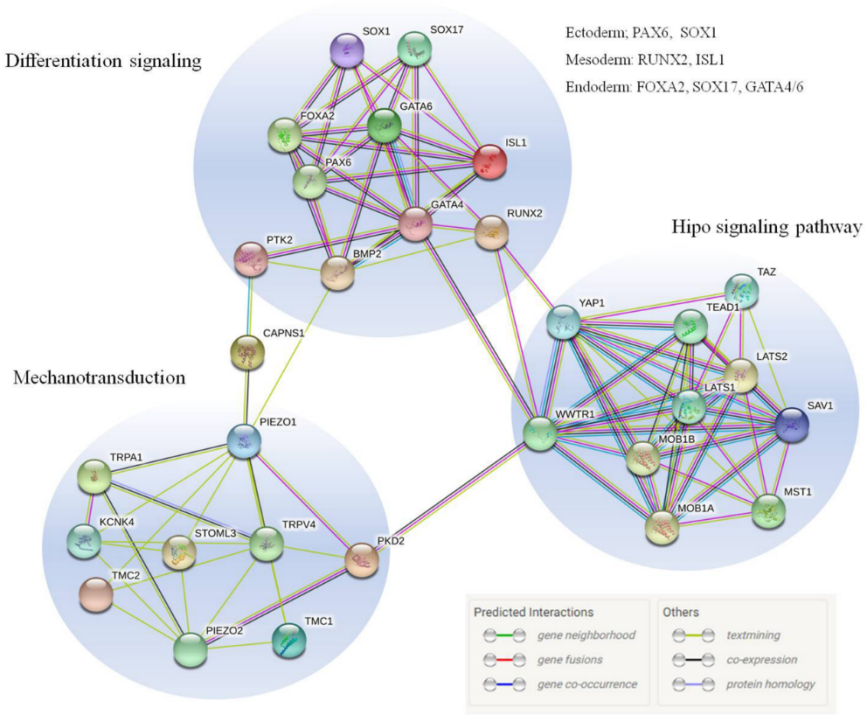
The effect of mechanical forces, cell polarity and cell arrangement, as well as cell-cell junctions in the formation and differentiation of embryoid bodies

In the figure is diagrammed the Hipo signaling based on direct and indirect protein associations in determining the proliferation and differentiation of stem cells. The interaction network was created using the STRING v 10.5 database. (http://www.string-db.org). The interaction between mechanotransduction and Hippo signaling is predicted because polycystin-2 (PKD2) which functions as a cation channel is involved in mechanosensation.

In the last part of the manuscript we present a discussion followed by a conclusion as well as future perspectives concerning the possible ways of supporting stem cells in hypoxic conditions.

Results allow us to conclude that the mere use of antioxidants to support stem cells in hypoxic conditions is not enough, since energy metabolism remains dysfunctional. In addition, it is clear that a linear study of the effect of pretreatments on stem cells, including antioxidant pretreatments and also preconditioning for hypoxia are not suitable for the evaluation of cell fate. Indeed, cell-cell signaling, adaptation of stem cells by HIF1α / Sirtuins, components of the native niche and mechanotransduction

systems could all neutralize the linear results of antioxidants in supporting stem cells in hypoxic conditions. Therefore, it is necessary to understand all the factors and to integrate a maximum of the components



Key words: Antioxidants, Cell signaling, Mesenchymal Stem Cells, Oxidative stress; Reactive oxygen species; Stem cell niche

1 Chapter

Hypoxia and oxidative stress in Biology of Mesenchymal Stem Cell: an Overview

In this chapter we discussed about the paradox between antioxidants and oxidants in the biology of cells especially in view of stem cells. A major problem in stem cell therapy is the low survival rate after transplantation due to the cell death via oxidative stress inducing by hypoxia. On the other side, to improve the survival and adhesion of the transplanted stem cells, the hypoxic preconditioning need to induce the HIF1α protein. Also, short-term oxidative stress may also be important in prevention of apoptosis by induction of a process called mitohormesis. This chapter highlights the impact of physiological ROS in adaptation to the hypoxia condition and programming to the fate. Then, the role of antioxidants players in hypoxic condition and the crosstalk with ROS homeostasis will discussed.

Abstract

The potential of MSCs in self-renew and differentiate into a variety of cell lineage have made these cells a attractive candidate for stem cell therapy. The main mechanisms by which MSCs exert regenerative activity in injured tissues include paracrine effects, such as immune-suppressive and antiapoptotic effects, vascularization and differentiation or local progenitor stem cells stimulation.

However, a major problem in MSCs therapy is the poor surviving after transplantation due to cell death via anoikis and oxidative stress induced by hypoxia and energy metabolism disruption. To enhancing of the survival and adhesion of cells of the transplanted MSCs, different strategieshave been investigated, including pretreatment with growth factors or cytokines and designing the biomimetic nano-scafolds but also the hypoxic preconditioning, and genetic modifications to induce the overexpression of HIF1α and other downstream molecular players. On the other way, several studies showed that hypoxia and ROS are not harmful to cells and induce proliferation and maintaining their stemness. The paradigm is between consumption of antioxidants and ROS induced mitohermesis in lifespan and surviving of stem cells. Several studies were done to evaluate the effect of preconditioning of stem cells with antioxidants and showed beneficial effects of this strategy in the surviving of transplanted stem cells. Then the studies for evaluation of pre-treatment of antioxidants same as pre-conditioning in hypoxia conditions are necessary and could clarify the molecular

players in this way.

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Keywords: Oxidative stress; Reactive oxygen species; Antioxidants, Mesenchymal stromal/stemcells (MSCs , Mitohormesis)

1. Introduction

Oxidative stress" as a concept in redox biology and medicine has been formulated in 1985; at the beginning of 2020, approx, 214557 Pub Med entries show for this term. Oxidative stress is a phenomenon induce by an imbalance between oxygen reactive species (ROS) production and accumulation, in tissues and the ability of our tissues to detoxify of it[1]. ROS, usually generated as by-products the metabolism of oxigen; despite this, environmental stressors (i.e., radiations, heavy metals and pollutants) and xenobiotics contribute to greatly increase ROS production, therefore causing the imbalance that leads to cell and tissue damage[2]. In fact, when the concentration of reactive species is not controlled by internal defense mechanisms such as antioxidants (tocopherols, ascorbic acid, glutathione, and others) or cellular enzymes involved in oxygen radical scavenging (SOD, catalase, peroxidase, and superoxide dismutase), oxidative damage occurs to proteins, lipids, and DNA, which could lead to cytotoxicity, genotoxicity, and even tissue damages[3]. In addition, nucleotide mutation in genomic DNA is mostly indirect and caused by reactive oxygen species (ROS) generated, e.g. superoxide radical, OH and H_2O_2.

Then, Oxidative stress is thought to be involved in the development of many disease of human including cancers[2] Parkinson's disease,[3] Alzheimer's disease,[5] atherosclerosis,[6] heart failure,[7] myocardial infarction,[8][9] vitiligo,[13] autism,[14] and depression[16]. However, ROS can be beneficial, as they are used by the immune system as a way to remove of pathogens[18] Short-term oxidative stress may also be important in prevention of aging by induction of a process called mitohormesis[19]. Further, ROS act as cellular secondary messengers in redox signaling. Thus, oxidative stress can cause disruptions in normal mechanisms of cellular signaling and the fate of cells[4].

In the stem cellular physiology, ROS homeostasis is very important in maintain of stemness and induction of fate that in the next section are explained.

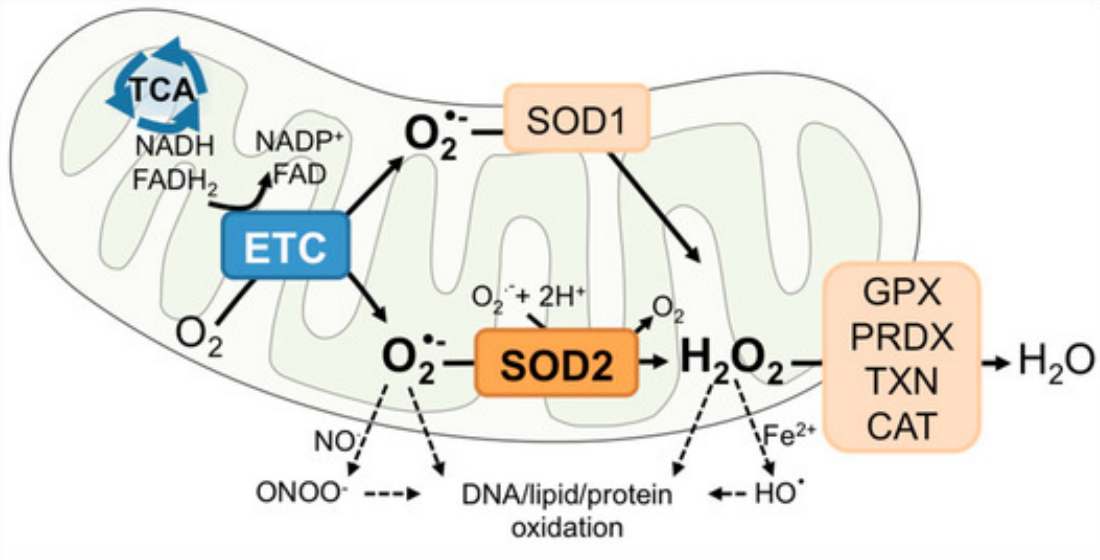

Figure 1. Free radical toxicity induced by xenobiotics or hypoxia and the subsequent detoxification by cellular enzymes. Oxidative stress is a phenomenon induces by an imbalance in the accumulation of ROS tissues and the ability of a biological system to detoxify these reactive products. enzymes involved in oxygen radical scavenging are catalase, peroxidase, and superoxide dismutase, SOD.

2. Free radicals and cell signaling

Although ROS historically viewed as purely harmful, recent evidence suggests that they function as important physiological regulators of intracellular signaling pathways. The specific effects of ROS are modulated in large part through the covalent modification of specific cysteine residues found within redox-sensitive target proteins. Oxidation of these specific and reactive cysteine residues in turn can lead to the reversible modification of enzymatic activity[4]. ROS were originally shown to have signaling properties when they were found to act as secondary messengers [5]. However, not all ROS can be act in signaling mechanisms. Only ROS with a substrate specificity that generates reversible oxidation, such as H_2O_2, are likely to trigger signaling cascade in in vivo physiological settings [6].

ROS could act as messenger signal directly to proteins via amino acid oxidation, the most common reaction being oxidation of cysteine residues[7]. ROS could cause change in the functional of different proteins and thus these types of modifications have recognized ROS as vital regulators of cellular signaling[8]. Such enzyme and proteins are known as redox sensors, meaning that they are directly adapted by ROS, undergoing a structural change as a result of the oxidative modification ; this change influences their bio-function, stability, and cell localization[9]. Although ROS could modify protein functions, the conflicting is also correct: a vast number of proteins have been reported to modulate ROS levels[10]. Interestingly, many of these redox sensor enzymes and proteins that are directly modulated by ROS in response to oxidative stress are also found to be crucial regulators of stem cell fate. Among these proteins are transcription factors that have been linked

to the regulation of cellular antioxidant system. These include members of the forkhead box O (FOXO) family, PR domain containing 16 (PRDM16), nuclear factor erythroid 2 (NRF2), and the p53 (TRP53) tumor suppressor [11-13] .

Other transcription factors, such as nuclear factor κB (NFκB), mediate the transactivation by ROS of hypoxia inducible factor 1α (HIF1α)[14]. Furthermore, other protein types, such as ATM (ataxia telangiectasia mutated kinase), apurinic/apyrimidinic (AP) endonuclease1/redox factor 1 (APE/REF1) protein, protein kinase B (AKT) protein kinases, PTEN (phosphate and tensin homolog), p38 mitogen-activated protein kinase (MAPK), mammalian target of rapamycin (mTOR) and and sirtuins (specifically SIRT1 and SIRT3) are also considered to be redox sensors. All of mentioned cell signaling interplay in the proliferation and differentiation of stem cells and the investigation and study of them is necessary to control stem cells behavioral in native or dysfunction niches.

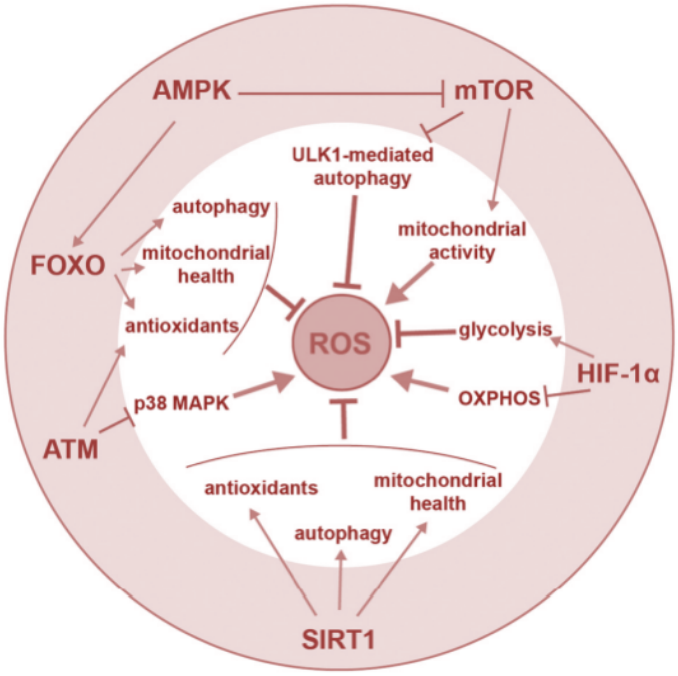

Fig. 2 A crosstalk of signaling players in th determines the final ROS concentration and impacts cell fate. Various pathways influence ROS levels by regulating mitochondrial activity, glycolysis, autophagy, expression of antioxidative enzymes or stress-responsive signaling cascades. AMPK: AMPactivated protein kinase; mTOR: mammalian target of rapamycin; FOXO: forkhead box class O family; ATM: ataxia telangiectasia mutated; hypoxia-inducible factor 1α (HIF-1α); SIRT1: sirtuin 1; p38 MAPK: p38 mitogen activated protein kinase.

3. **Free radical in cell metabolism**

Cellular metabolism is the sum of catabolic and anabolic mechanisms that involve the chemical conversion of carbon substrates to generate energy in the form of ATP , or to produce macromolecular in the form of nucleotides, lipids and amino acids in the process of anabolic . The balance between the catabolic and anabolic processes can shift depending on the cellular process. The cell's growth process and proliferation require mostly anabolic processes to generate building blocks for DNA, protein and membranes.

One of the major ways in which metabolism can affect signaling pathways is through alterations of ROS levels. In turn, ROS can directly react with various proteins, such as kinases, phosphatases or transcription factors, to alter processes that regulate cell cycle progression, apoptosis, quiescence or differentiation [15]. Furthermore, ROS can also directly modify metabolic enzymes or proteins that participate in nutrient-sensing pathways to direct the metabolic flux[16-17] . In these contexts, ROS can be considered as signaling molecules that take part in the crosstalk between metabolism and stem cell fate decisions. Manipulating metabolic pathways used by stem cells with either genetic approaches or drugs can directly affect whether stem cells remain quiescent, self-renew or differentiate [18-19]. Importantly though, metabolism can affect cell fate through multiple ROS-independent mechanisms or via mechanisms where the influence of ROS on metabolism is less obvious. Such mechanisms include changes in the epigenetic landscape brought about by metabolite abundances, as well as the 'moonlighting' functions of metabolic enzymes beyond their role in catalyzing metabolic reactions [20-21]. In the next section the changing in embolism of stem cells by ROS and the effect of it in the fate of stem cells are explained.

4. ROS as a **mediator** of stem cell fate and programming

An appropriate balance between self-renewal and differentiation is vital for stem cell function during both early development and tissue homeostasis throughout life[22]. Recent evidence suggests that this balance is partly regulated by ROS, which, in synchrony with metabolism, mediate the cellular redox state[23].

One of the eventual applications of stem cell biology is the generation of healthy differentiated cells to repair damaged or deteriorated tissues and organs. Given that ROS may influence a vast array of biological processes, and that we are limited in our knowledge of which species of ROS are implicated in any given physiological setting, it seems an immense challenge to explore how ROS metabolism can be manipulated to generate stem cells and influence stem cell fate. However, the study of metabolism and ROS mediated mechanisms of stem cell fate regulation has led to improved differentiation and reprogramming protocols. Differentiation of ESCs towards the cardiac lineage has been shown to rely on H_2O_2 signaling induced by NOX4 upregulation[24-25] . In the case of the cardiac lineage, not only are ROS important for differentiation, but the exclusive use of oxidative phosphorylation in cardiomyocytes compared with pluripotent stem cells (PSCs) can be taken advantage of to improve purification and differentiation efficiency[26-27] . Interestingly, the degree of activation of mitochondrial metabolism is related to mouse ESC fate determination[28] .

Finally, a recent study in human HSCs demonstrated that glutamine metabolism and pentose phosphate pathway-mediated generation of nucleotides is required for erythroid lineage commitment[29]. Chemical inhibition of these metabolic pathways led to commitment towards myeloid and granulocytic fates. Notably, as in ESCs, differentiation of MSCs towards adipocytes or neuron-like cells has also been shown to employ NOX4-mediated H_2O_2 signaling, as well as mitochondrial ROS[30-31] . Further studies are required to reveal whether manipulation of ROS through metabolic pathways or directly can direct differentiation of other types of stem cells to various lineages. The several studies showed that pre-conditioning of stem cell in invitro with hypoxia and or with pretreatment of antioxidant could manipulate the ROS homeostasis in the stem cells lead to the induction of molecular players involving in the programming to the fate.

5. Hypoxia and **oxidative** stress pre-conditioning of stem cells

The physiological niches for MSCs in the bone marrow and other sites have much lower oxygen tension. When used as a therapeutic tool to repair tissue injuries, MSCs cultured in standard conditions must adapt from 21% oxygen in culture to less than 1% oxygen in the ischemic tissue. Therefore it is examined the effects of pre-culturing MSCs in hypoxic conditions (1%–3% oxygen) to elucidate the best conditions that enhance their tissue regenerative potential[32].

It demonstrated that, MSC cultured in hypoxia activate the Akt signaling pathway while maintaining their viability and cell cycle rates. Hypoxia-induced apoptosis can be circumvented by preconditioning cells in less severe hypoxic conditions (1%–3% O2) for a period of time before exposing them to the severe ischemia at the site of injury in other cell types [32].

Other reports have observed that in addition to maintaining stem cells viability when cultured in 2%–5% O2, they also increase their proliferation rate after an initial lag phase [33-34]. There is a discrepancy in the field, however, about how hypoxia affects the self-renewal and differentiation potential of MSCs. A number of studies have found MSCs cultured in hypoxic conditions to be able to differentiate more and to have better self-renewal[33,35].

6. **Antioxidant** pre-treatments

Several studies were done to evaluating of the effect of preconditioning of stem cells with antioxidants showed that this strategy in the surviving of transplanted stem cells was suitable. For example the studies demonstrated that Vitamin E pretreatment enabled MSCs to counteract H_2O_2-induced oxidative stress in vitro. In that study, the proliferative markers, proliferating cell nuclear antigen (PCNA) and Ki67 were up-regulated, along with the increase in the viability of MSCs[36]. It was shown that N-acetyl-Lcysteine (NAC) affects cellular differentiation and function in hypoxia condition[36]. NAC accelerate the differentiation of osteoblastic cells, and local implantation of the collagen sponge containing NAC enhanced bone regeneration in a large bone defect in the rat femur [37]. The biological mechanism underlying bone formation by local transplantation of BMSCs pretreated with NAC in association with the interaction with the

immune system is poorly understood. So, in our studies we use antioxidants NAC and mito-TEMPO to possible supporting the mesenchymal stem cells from oxidative stresses induced by respiration deficiency.

7. **Conclusion**

The most prominent capabilities of MCSs which make them promising for therapeutic applications are their capacity to endure and implant in the target tissue. However, the therapeutic applications of these cells are limited due to their early death within the first few days following transplantation. Therefore, to improve cell therapy efficacy, it is necessary to manipulate MSCs to resist severe stresses imposed by microenvironment. The cell therapy of damaged tissue which is linked to hypoxia condition, failed with oxidative stress (OS) and mitochondrial dysfunctions. Also, MSC biology, as their longevity and functions are affected by oxidative stress (Figure 3).

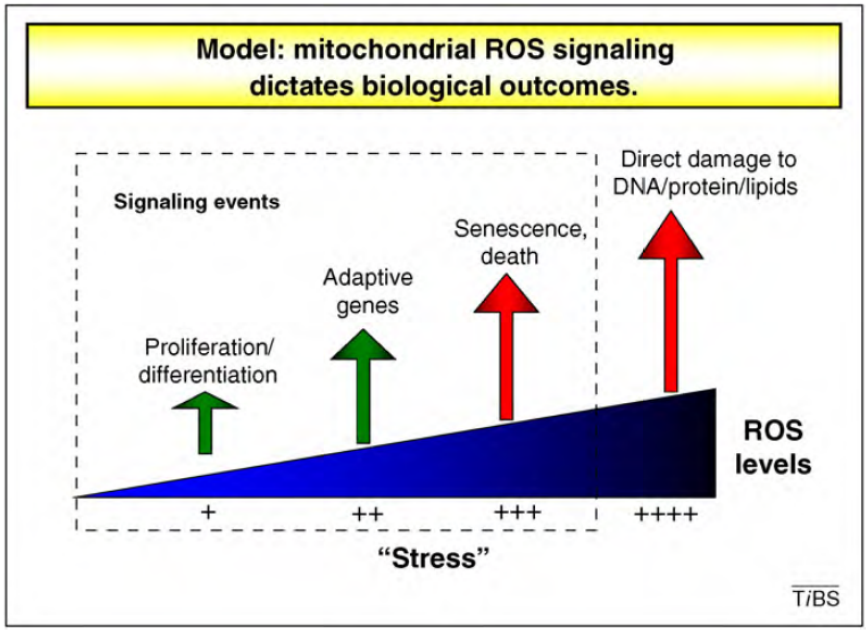

Figure 3. Mitochondrial ROS levels are crucial for biological outcomes. Low levels of mitochondrial ROS production are required for cellular processes such as proliferation and differentiation. An induction in ROS production will lead to adaptive programs including the transcriptional upregulation of antioxidant genes. Even higher levels of ROS will signal the initiation of senescence and apoptosis. Non-signaling, irreversible damage to cellular components is only observed under the highest levels of cellular ROS(Hamanaka & Chandel, 2010)

In general, increased reactive oxygen species (ROS) inhibit MSC proliferation, increase senescence, enhance differentiation, and inhibit MSC immunomodulation. Therefore, there is a great need to identify a promising

strategy to prevent oxidative stress or pre-invigoration of MSCs. One of these strategies is the hypoxia and antioxidants pre-treatment of stem cells before their transplantation in damaged tissue and hypoxia condition. It could affect the homing and surviving of transplanted stem cells. In this chapter we review the redox biology of stem cells and preconditioning of them to the supporting in hypoxic condition. Another strategy is the pre-treatments of stem cells with robust antioxidants but there are some paradigms about the oxidative stress and antioxidants. We don't know till that antioxidants or free radicals are useful for stem cells surviving and fate. In this way the conundrum of dietary antioxidants and mitohormesis must be resolved.

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2 Chapter

Mesenchymal Stem Cells: Isolation, *In Vitro* Expansion and Characterization

Adipose tissue as a stem cell source has several advantages compared to other sources. It is easily accessible in large quantities with minimal invasive harvesting procedure, and yields a high amount of stem cells, which is essential for autologous and heterologous stem-cell-based therapies with direct injection and without more expansion. Adipose drived stem cells (AD-MSCs) have the highest potentiality for therapy with proliferation potential, possess the highest homing in the hypoxia/ischemic tissues. In this chapter we explained the protocol of isolation and characterization of stem cells from adipose tissues. These cells have used in all of our next studies.

Abstract

In comparison with other stem cells and source especially bone marrow, Adipose derived- mesenchymal stem cells (AD-MSCs) hold promise for regenerative therapy due to some robust potentiality. The harvesting of stem cells from adipose tissue might be easier and with high yield. However, the studies highlighted that, AD-MSCs have showed very high potential of therapeutic properties as well. AD-MSCs have the highest potentiality for proliferation, homing in injury hypoxic tissues and multipotency stemness properties. Due to the fact that primary culture of stem cells after several passages will be aged, so, the high yield harvesting of stem cells is very important in vitro studies as well. So, in this thesis we have choice AD-MSCs and after characterization we have used in next our studies. The protocol of isolation and characterization were explained in this chapter.

Keywords: Isolation of stem cells, Cell surface CD-marker, Stem cells, Differentiation

1. Introduction

The mesenchymal stem cell (MSC) is one of the most interesting of the adult stem cell types. These cells are simply isolated, cultured, and manipulated ex vivo. MSCs exhibit great flexibility and harbor the potential for therapeutic applications, but these cells often poorly identified[1].

MSCs show vast potential for the replacement of injured tissues such as bone, cartilage, tendon, and ligament and in the other damaged tissues have therapeutic potential due to the immune suppressor effecting and paracrin secretome[2].

MSCs are present in the many adult tissues specially bone marrow, adipose tissues, umbilical cord blood (UCB) and these cells are presently recognized through an arrangement of poorly defined physical, phenotypic, and functional properties. Due to their low frequency and the lack of knowledge on cell surface markers and their location of origin, most our data concerning MSCs are derived from in vitro studies[3].

Adipose tissue, has several advantages for isolation of MSCs compared to other stem cell's sources. (i); This tissue ubiquitously available and is easily accessible in large quantities with minimum invasive isolation procedure. (ii); The isolation of adipose-derived mesenchymal stem cells (AD-MSCs) contains a large number of multipotent cells, which is necessary for stem-cell-based therapies and tissue engineering. It has been reported that stem cells in the stroma-vascular fraction (SVF) from adipose tissue usually amount to up to 3% of the whole cells, and this is about 2,500-fold more than the rate of stem cells in bone marrow[4]. A bone marrow transplant contains about 6×10^6 nucleated cells per mL [5], of which only 0.001–0.01% are stem cells [6]. In contrast, the number of SVF cells that can be isolated from subcutaneous liposuction aspirates is approximately $0.5–2.0 \times 10^6$ cells per gram of adipose tissue [5,7-8].As therapeutic properties of MSCs, the studies reported that, AD-MSCs possess several advantages over BMSCs[9]. In vivo study showed that AD-MSCs facilitated the homing of hematopoietic stem cells to the BM better than BMSCs and results suggest that AD-MSCs can be a promising therapeutic alternative to BMSCs[9]. Other study but showed that, after cardiac transplantation, both adipose and bone marrow drives stem cells, the transplanted stem cells death within 4−5 weeks[10].

Anyway, isolation of stem cells are done by their capacity to adhere to plastic and haven't a confident standard protocol. The International Society for Cellular Therapy proposed three minimal criteria for the definition of MSCs: (i) plastic adherence, (ii) expression of CD73, CD90, and CD105, and lack of CD11b or CD14, CD19 or CD79α, CD45, and HLA-DR expression, and (iii) their trilineage differentiation potential into adipocytes, chondrocytes, and osteoblasts[11]. But in the therapeutic potentiality of stem cells are differ. The evidences show that, AD-MSCs are a heterogeneous mixture of cells containing subpopulations of stem and more committed progenitor cells. This is very important that perior of all, stem cells for target therapy must be screened. With considering of mentioned prosperities of AD-MSCs, in the current research we choice AD-MSCs for our studies that summarized the isolation and characterization of them according to the mentioned protocol.

2. Materials and methods

2.1. Sample collection and processing

All samples were obtained with written, informed consent in accordance with the local hospital ethics committee requirements. After informing and filling the consent form, the adipose tissue was collected by the liposuction aspiration from the visceral fat of overweight 7 volunteer (4 men and 3 women) at the Tabriz International Valiasr Hospital (Tabriz, Iran). In the next studies we used MSCs isolated from women with 47 years old due to the some therapeutic potentiality.

2.2. Isolation procedure

The AD-MSCs were isolated from adipose tissue through the following steps: Washing the raw lipoaspirates extensively with phosphate-buffered saline (PBS) containing 5% penicillin/streptomycin (P/S),Upon removal of debris, the samples were placed in a sterile tissue culture plate with 0.075% collagenase type I (1 mg/mL) prepared in PBS containing 2% Penicillin/Streptomycin.

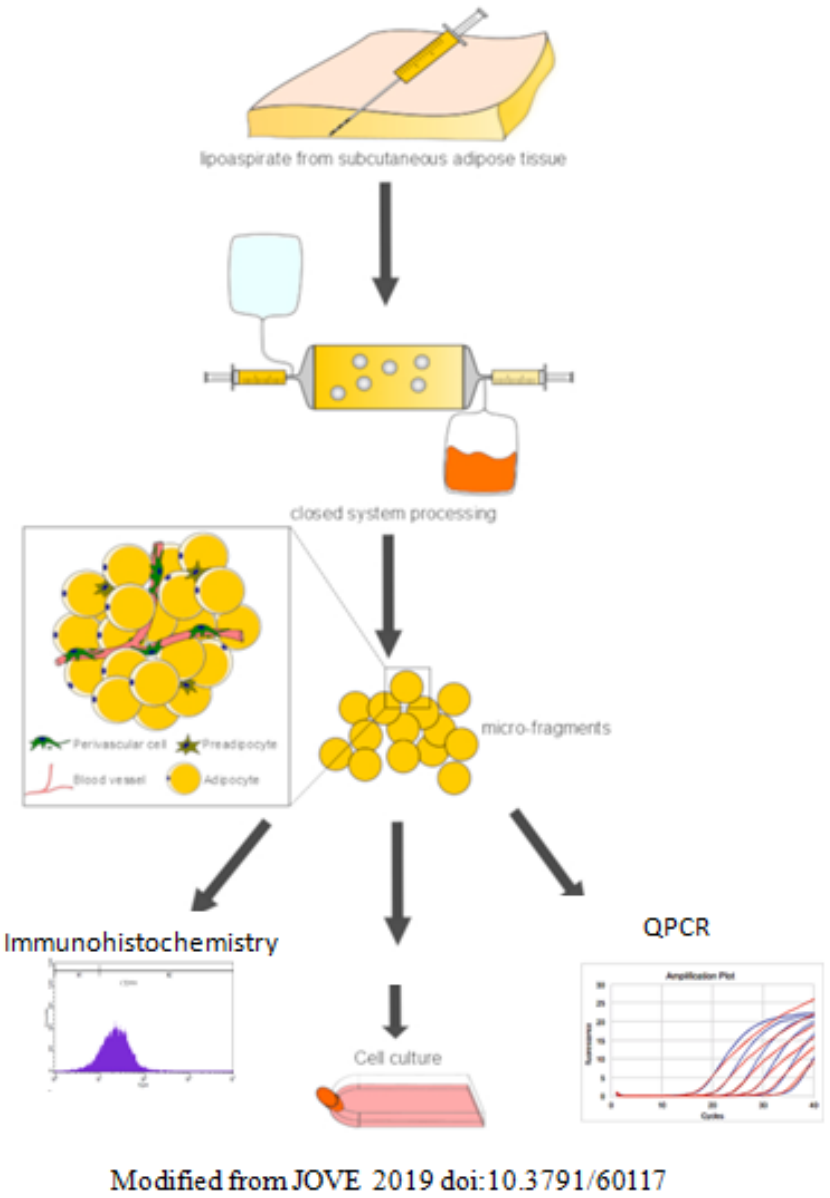


Figure 1. Figure 1: isolation and characterization methodology

After fragmentation of samples in small size, for complete digestion, the samples were incubated for 1 hour at 37°C in a water bath with gentle agitation at 125 rpm. The collagenase digest was then inactivated by adding an equal volume of standard cell culture growth medium (DMEM plus GlutaMAX and 10% FBS). The stromal vascular fraction (SVF) was pelleted by centrifugation at 1300 ×g for 5 minutes. The super-natant was then discarded, and the cell pellet was resuspended in DMEM medium and filtered through a 100 µm cell strainer to remove undigested tissue fragments. The cells were pelleted and resuspended in DMEM-LG medium and incubated at 37°C in 5% CO2. Finally, the cells were used only up to passage 2 for all in vitro assays.

2.3. Characterization of isolated cells

2.3.1. Immunophenotyping

At the second passage of all samples, the morphologically homogeneous population of MSCs were trypsinized and the immunophenotype examined by dual labelling with fluoroscein isothiocyanate (FITC)-conjugated anti-CD105 (Serotec, Oxford, UK), CD90 (BD Biosciences, Oxford, UK) and CD45, CD29,CD34 (Dako, High Wycombe, UK).

2.3.2. Differentiation of MSCs

All isolated AD-MSCs were differentiated into osteogenic and adipogenic lineages according to the protocols[12] and the best one selected for the study. For the osteogenesis, briefly, DMEM low glucose containing 15% FBS was supplemented with 10−7 M dexamethasone, 50 μM ascorbate-2-phosphate and 10 mM β-glycerol phosphate. The culture medium was changed every 3 d for up to 3 weeks. The cells were fixed with methanol for 10 min at room temperature and stained with alizarin red to detect calcified extracellular matrix deposits.

For the adipogenesis, the AD-MSCs were incubated in DMEM low glucose containing 15% FBS supplemented with 1 μM dexamethasone, 10 ng/mL insulin, and 0.5 mM 1-methyl-3-isobutyl-xanthine. The culture medium was changed every 3 d for up to 3 weeks. The cells then were fixed in methanol for 45 min and stained with Oil Red O.

2.3.3 Real time PCR for expression of osteogenic and adipogenic genes

Freshly isolated cells from all volunteer and passage 2 cells were examined for surface molecule expression (Including CD markers and SDF-1-CXCR4/CXCR7 pathway) using the real-time PCR. RNA from 2 passage cells was extracted using the Trizol method according to the manufacturer's protocol. Reverse transcription was performed for the cDNA synthesis. Then, the osteogenic and adipogenic gene expression was examined by the Q-PCR. The normalization of data was done using an external GAPDH gene.

3. Results

3.1. The results of MSCs culture and screening

Culture of AD derived samples, produced a monomorphic confluent adherent layer of elongated fibroblast-like cells that survived multiple passages in DMEM-F12 (Fig 1). Primary culture took between 7 d. (Figure 2).

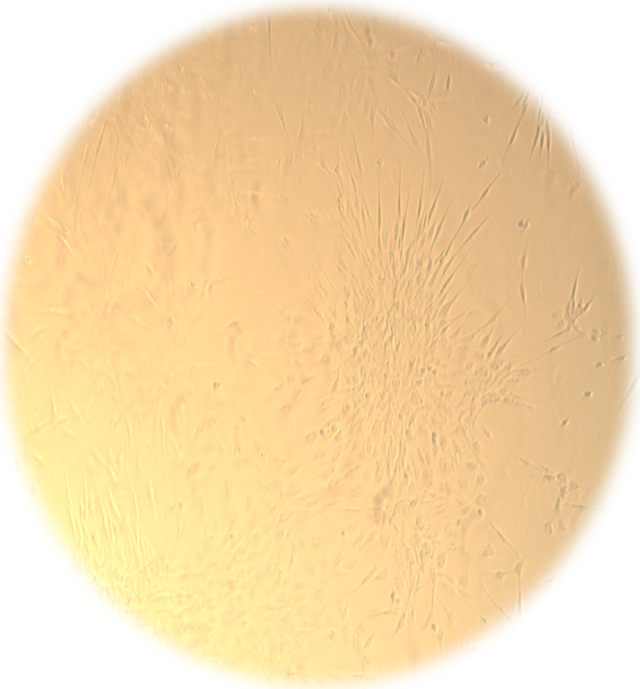


Figure 2 The AD- Mesenchymal Stem Cells in DMEM-F12, the colony of MSCs could be optioned after 7 days.

3.2. Cell Surface phenotype

The immunophenotype characteristics of all isolated AD-MSCs were analyzed by flow cytometry in freshly culturing and after passage 2. After 2 passage, the pure population MSCs positively expressed the CD29 (β1-integrin), CD90, CD105 (endoglin), while they were negative for the CD45 (hematopoietic marker), CD34 (stem/progenitor hematopoietic cells). Figure 1 shows the CD marker characteristics (Figure 3).

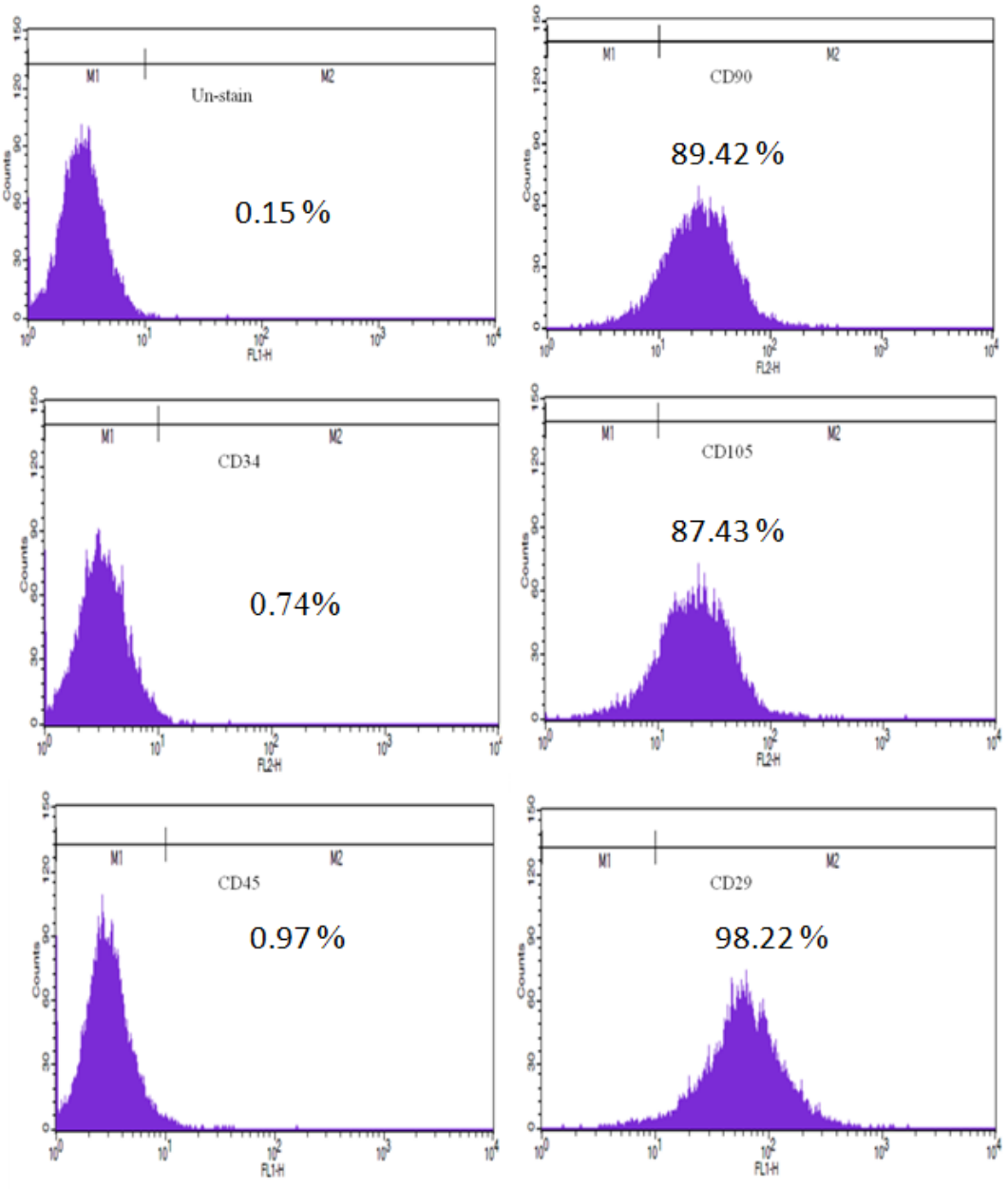


Figure 3 Flow cytometric plots after 2 pasage of isolated MSCs from 47 years old women shows the positive and negative cell surface CD markers. The MSCs were stained using monoclonal antibodies conjugated to FITC. The purity percentage of isolated cells for each CD markers were showed in plots.

3.3. Differentiation studies

At the end of the second passage, BM-derived MSC were successfully differentiated along adipogenic (Fig 3a,c,e) and osteogenic lineages (Fig 4 b,d,f), using methods described earlier. Differentiation of cultured CB and PBSC adherent cells was not attempted because there was no phenotypic or morphological evidence that these were mesenchymal in origin.

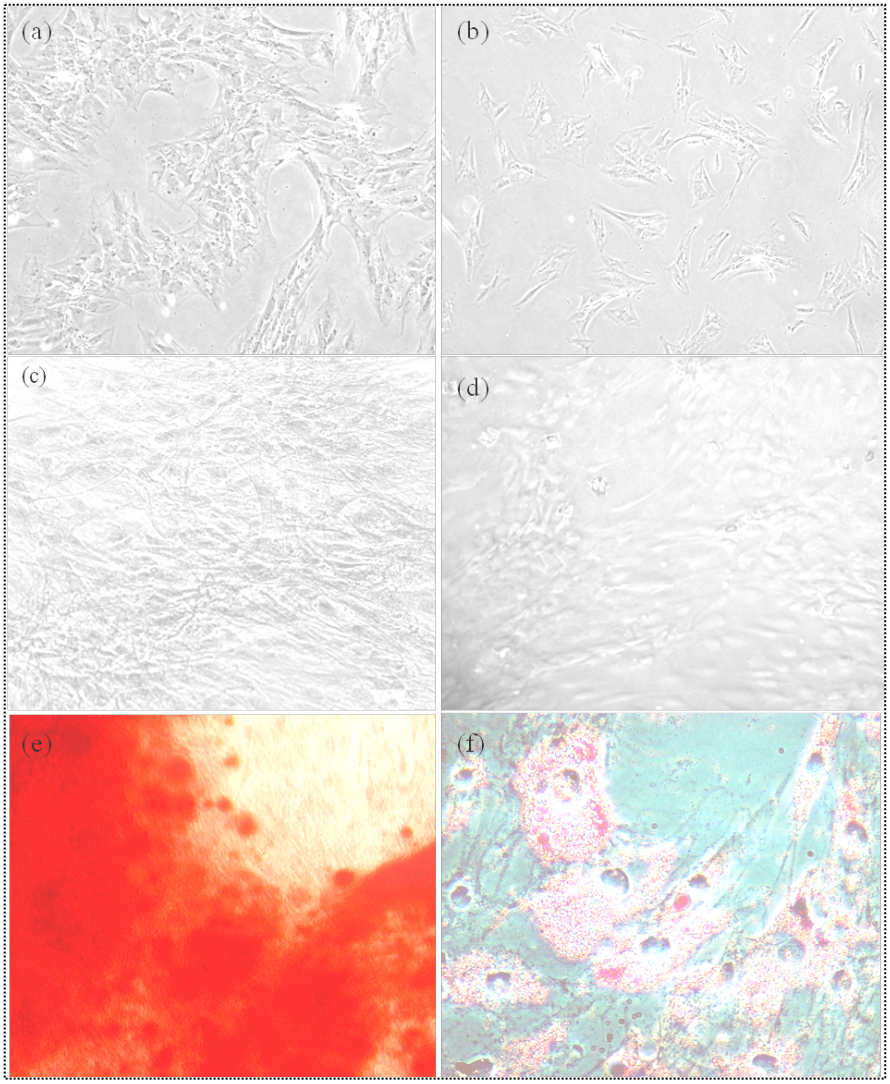
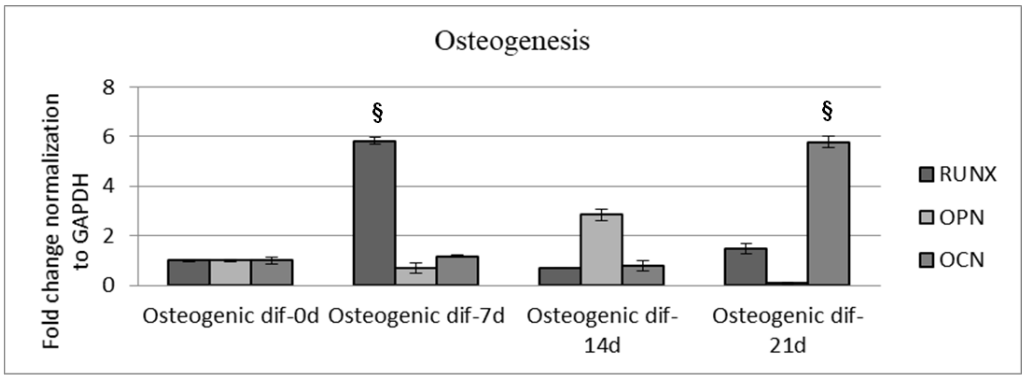


Figure 4 AD-MSCs were differentiated into osteogenic and adipogenic lineages. Panels (a) and (b) show the control cells cultured in DMEM-LG shows no differentiation. (c) The cells cultured in osteogenic media and the same cells. (e) The cells stained with alizarin red after 21 days. (d) The cells seeding in differentiation media after 21 days. (f) The cells stained with Oil red, showing adipogenic lineages.

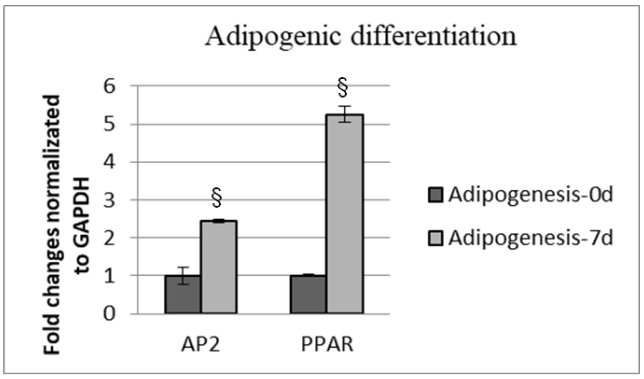
3.4. Real time PCR for expression of osteogenic and adipogenic genes

To study the differentiation towards the osteogenic lineage, several genes expression were investigated, including (i) runt-related transcription factor 2 (RUNX2), (ii) osteopontin (OPN), and (iii) osteocalcin(OCN). And the genes for evaluation of adipogenic differentiation, we looked at (i) adipocyte protein 2 (AP2) and (ii) peroxisome proliferator-activated receptor (PPAR).

(a)



(b)



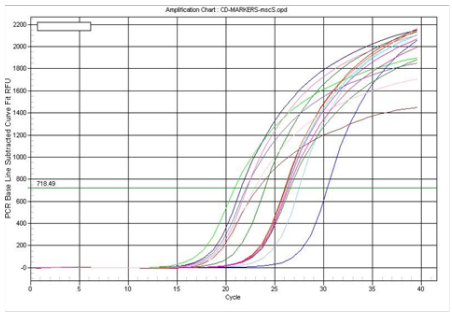


Figure 5. The real-time PCR for the expression of osteogenic and adipogenic genes. The runt-related transcription factor 2 (RUNX2), osteopontin (OPN), and osteocalcin (OCN) were studied for the osteogenic. To evaluate the adipogenic differentiation, adipocyte protein 2 (AP2) and peroxisome proliferator-activated receptor (PPAR) were studied. All differentiation genes were up-regulated step-by-step depending on the cultivation period

Also, the evaluation of therapoitic potentiality of selected MSCs, we investigated under hypoxia the homing involved genes and hypoxia responding genes (Figure 6).

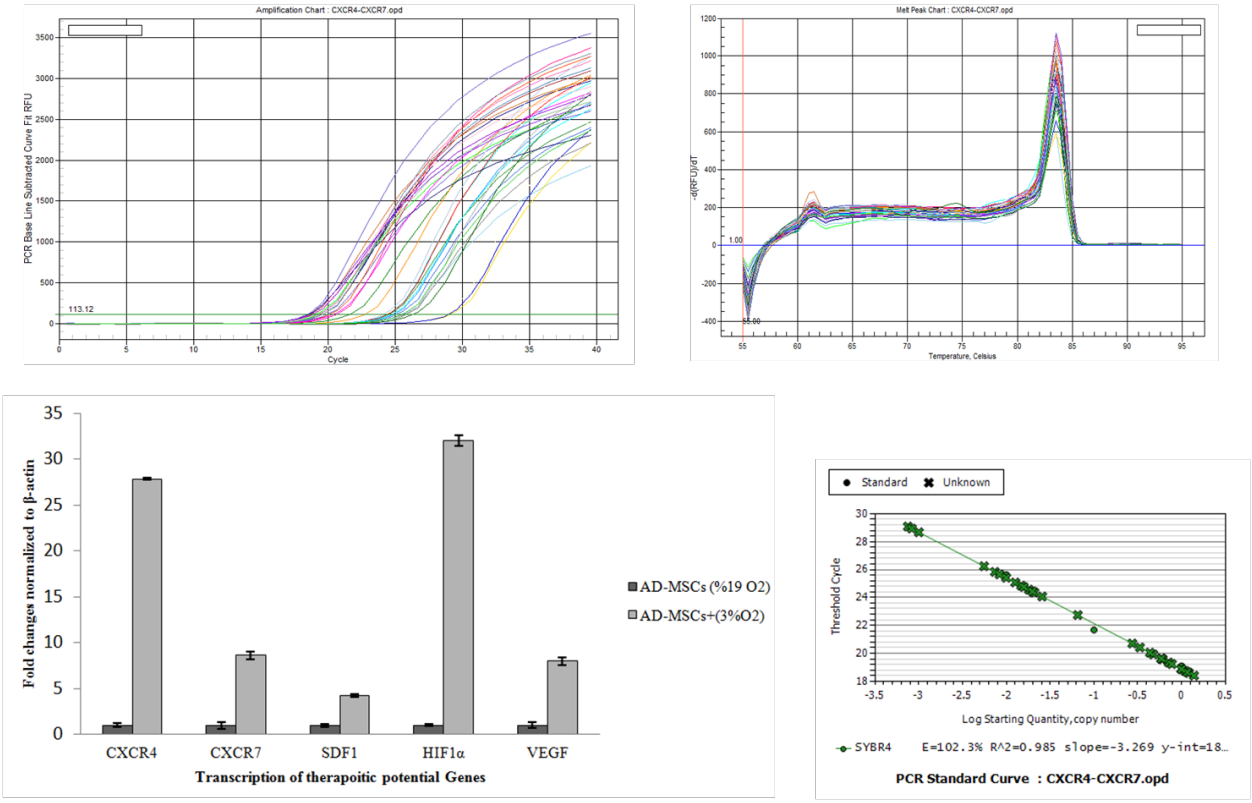


Figure 6. The real-time PCR for the expression of the genes involved in homing in injured hypoxic injured tissues (CXCR4, CXCR7, SDF1) and hypoxia induced factor as a master transcription factor in hypoxia, supporting the MSCs to adaptation in hypoxic condition and VEGF as angiogenesis factor which could secreted by MSCs.

4. Discussion

Various source-derived MSCs have been considered for cell therapeutics. To characterize MSCs from different sources, including human bone marrow (BM-MSCs), adipose tissue (AT-MSCs), and umbilical cord blood-derived MSCs (UCB-MSCs) for surface antigen expression, differentiation ability, proliferation capacity, and paracrine activity were reported by many studies[13-14]. Although MSCs from different tissues have similar levels of surface antigen expression, immunosuppressive activity, and differentiation ability, AD-MSCs had the highest potentiality for therapy[15]. This tissue ubiquitously available and is easily accessible in large quantities with minimum invasive isolation procedure and these cells could homing in hypoxia and injury site better than BM-MSCs. Although, a bone marrow transplant contains only 0.001–0.01% of stem cells however, in contrast, the number of SVF cells that can be isolated from liposuction

aspirates is approximately $0.5–2.0 \times 10^6$ cells per gram of adipose tissue. Also, MSCs derived from adipose tissue (AT-MSCs) possess the highest proliferation potential, followed by MSCs derived from BM and cartilage[16]. In our study, we isolated MSCs from adipose tissues of several volunteer. The prior studies showed that MSCs population even isolated from one sample could show different therapeutic properties. In our study, after characterization of stem cells via cell surface CD markers (CD29, CD90, CD105, CD45, CD34) And differentiation potentiality((RUNX2,OPN, and OCN were studied for the osteogenic and to evaluate the adipogenic differentiation, AP2 and PPAR). We screened the isolated MSCs by expression of several genes involving in hypoxia chemotaxis and homing [17] (CTXR4, CTXR7 and SDF1) and also the cells screened for master transcription factor, HIF1α and for angiogenesis factor, VEGF genes . In the next studies we used this pure primary stem cells.

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3A Chapter

N-acetylcysteine Save Human Mesenchymal Stem Cells from Mitochondrial Oxidative Stress via Nrf2/Sirt3 Signaling Pathways, though Respiration Chain Deficiency and Energy Metabolism yet remains unresolved

Abolfazl Barzegari, Virginie Gueguen, Anne Meddahi-Pellé, Mohammad Nouri, Mohammad Reza Rashidi and Graciela Pavon-Djavid

This chapter describes the first research paper in this thesis. Antimycin a has been used as a stressor with inducer of respiratory deficiency, and mimicking artificially hypoxia. N-acetyl systein (NAC) was chosen. It is precursor to the human indigenous antioxidant, glutathione that could target the free radicals directly and indirectly, involve in the energy homeostasis. NAC is as a could act in both of the cytoplasm and mitochondria to neutralize the free radicals. this antioxidant bioactive substances is a precursor in the synthesis of the native antioxidant, glutathione (GSH), a thiol-containing tripeptide that plays a critical role in maintaining cellular redox homeostasis and other pathways.

Abstract

Mesenchymal Stem Cells (MSCs) have immense therapeutic potential along with paracrine properties; yet this potential has not been reached for a number of reasons. Perhaps one of these reasons is due respiration chain deficiency in damaged tissues linked to the oxidative stress and energy metabolism. This affects the secretome, the survival, the homing and consequently the therapeutic results. On other hand, the core functions of stem cells are critically regulated by their cellular redox status and energy metabolism. Therefore, is necessary to setup the innovative strategies to support the cells respiration chain and in parallel, the neutralize of the oxidative stress. In this study, of various antioxidants, N-acetyl systein (NAC) was chosen. It is precursor to the human indigenous antioxidant, glutathione that could target the free radicals directly and indirectly, involve in the energy homeostasis. In this study, adipose derived MSCs were exposure to antimycin A (AMA) as an inhibitor of cellular respiration via blocking of cytochrome C complex and mitochondrial inducing oxidative stressor. Then several parameters were analyzed, including cell viability/apoptosis, mitochondrial membrane potential, and redox molecular homeostasis. Based on our findings, upon the exposure of the MSCs to the condition of respiration chain deficiency, the cells failed to scavenge the free radicals, and energy metabolism. The use of NAC was found to alleviate the DNA damage, cell apoptosis, oxidative stresses via Nrf2/Sirt3 pathway but, without effect to the mitochondrial membrane potential and ATP production. With antioxidants treatment we could only save the cells from oxidative stresses but the problem of ATP metabolism are going on and energy metabolism yet remains unresolved in the hypoxia condition.

Keywords: Mesenchymal stem cell; N-acetylcystein; Nrf2 signaling pathway; Oxidative Stress, Sirtuin 3

Résumé

Les cellules souches mésenchymateuses (MSCs) ont un immense potentiel thérapeutique ainsi que des propriétés paracrines; pourtant, ce potentiel n'a pas été atteint pour un certain nombre de raisons. Peut-être que l'une de ces raisons est due à un déficit de la chaîne respiratoire dans les tissus endommagés lié au stress oxydatif et au métabolisme énergétique. Cela affecte le sécrétome, la survie, le homing et par conséquent les résultats thérapeutiques. D'autre part, les fonctions essentielles des cellules souches sont réglementées de manière critique par leur statut redox cellulaire et leur

métabolisme énergétique. Par conséquent, il est nécessaire de mettre en place des stratégies innovantes pour soutenir la chaîne respiratoire des cellules et en parallèle, neutraliser le stress oxydatif. Dans cette étude, parmi divers antioxydants, la N-acétyl systéine (NAC) a été choisie. Il est le précurseur de l'antioxydant indigène humain, le glutathion, qui pourrait cibler directement et indirectement les radicaux libres impliqués dans l'homéostasie énergétique. Dans cette étude, les MSCs dérivées de l'adipose ont été exposées à l'antimycine A (AMA) en tant qu'inhibiteur de la respiration cellulaire via le blocage du complexe du cytochrome C et le stress oxydant induisant les mitochondries. Ensuite, plusieurs paramètres ont été analysés, y compris la viabilité / apoptose cellulaire, le potentiel de la membrane mitochondriale et l'homéostasie moléculaire redox. Sur la base de nos résultats, lors de l'exposition des MSCs à l'état de déficience de la chaîne respiratoire, les cellules n'ont pas réussi à piéger les radicaux libres et le métabolisme énergétique. L'utilisation de NAC s'est avérée atténuer les dommages à l'ADN, l'apoptose cellulaire, les stress oxydatifs via la voie Nrf2 / Sirt3 mais, sans effet sur le potentiel de la membrane mitochondriale et la production d'ATP. Avec un traitement antioxydant, nous ne pouvions sauver les cellules que du stress oxydatif, mais le problème du métabolisme de l'ATP se poursuit et le métabolisme énergétique n'est toujours pas résolu dans l'hypoxie.

Mots Clés: Espèces réactives d’oxygène; Sirtuin 3; N-acetylcystein

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1. Introduction

Mesenchymal stem cells (MSCs) are multipotent cells that show the ability to differentiate into multiple lineages including adipocytes, neural cells, vascular endothelial cells, cardiomyocytes, pancreatic β-cells, and hepatocytes [1]. MSCs play a prominent role in tissue regeneration, however, the advances in stem cell therapy are hampered by poor survival and homing of implanted cells. More than 600 clinical trials involving MSCs have been documented in the National Institute of Health (NIH) clinical trial registry (www.clinicaltrials.gov). MSCs have been used for their ability to promote tissue repair [2] and wound healing, for immunomodulation, and as a vehicle for targeted cancer treatments for their tumor-tracking properties [3]. Nevertheless, the limited success of a majority of completed protocols underscores the need to limit the cell death after implantation into the target tissues in order to improve the efficiency of MSC therapies. The deficiency of the respiratory chain and subsequently the energy restriction and redox imbalance in the damaged tissues are of crucial importance for cell survey. In addition, the essential functions of MSCs are regulated by the cellular redox status and the energy metabolism [4]. Reactive oxygen species (ROS) plays a vital role in regulating a range of MSC signaling pathways involved in self-renewal, pluripotency, viability, and genomic stability[5]. Therefore, strategies to protect MSCs respiratory chain and support the programmed functions within the in vivo transplantation are a challenge for the success of new cellular therapies. Recently, several methods have been developed to protect MSCs including genetic modification, retaining viability in vitro, suitable transplantation, supporter scaffolds, and pre-conditioning with antioxidants [6]. N-acetylcysteine (NAC) is an endogenous antioxidant that plays several important roles in cellular function and metabolism [7]. Since the 1960s, NAC has been used as a drug and hence is listed on the World Health Organization (WHO) Model List of Essential Medicines [8]. The administration of NAC-pretreated human MSCs to a bleomycin-induced model of lung injury in nude mice decreased the pathological grade of lung inflammation and fibrosis while enhancing the retention and proliferation of MSCs and improving the survival rate [9]. The capacity of NAC to protect cells and tissues in vivo could be related to its direct and indirect antioxidant properties[10] . The indirect antioxidant effect is related to the role as a precursor in the synthesis of the native antioxidant, glutathione (GSH), a thiol-containing tripeptide that plays a critical role in maintaining cellular redox homeostasis due to its high concentration [7]. GSH is synthesized in the cytosol and subsequently transported to cellular compartments including the mitochondria, nucleus, and ER, where redox buffering is demanded for organelle-specific functions.

Here, the capacity and mechanisms of NAC to protect human MSCs under oxidative stress and

respiratory chain deficiency was studied. Human adipose-derived MSCs (ADMSCs) were exposed to antimycin A (AMA), an inhibitor of cellular respiration, that inhibits specifically oxidative phosphorylation inducing ROS release from the mitochondria[11] . The activity of NAC in blocking oxidative stress, apoptosis, DNA damage, and maintenance of mitochondrial membrane potential were also evaluated. Then, the redox signaling regulation by NAC /AMA interplay was studied by evaluation of, Nrf2, RAD54L, HIF1-A, and mitochondrial Sirtuin3 pathways.

2. Materials and methods

2.1. Chemicals and reagents

N-Acetyl-L-cysteine (NAC; A9165), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), Antimycin A (AMA), dimethyl sulfoxide (DMSO), and Isopropanol (563935) were purchased from Sigma-Aldrich Co. LLC (Saint-Louis, MO, USA). 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA), and Phalloidin BODIPY(B-607) SYBER green from ABI biosystem (4472908) were purchased from Thermo Fisher Scientific (Eugene, OR, USA). Cell culture reagents were all purchased from GIBCO (Life technologies, Carlsbad, CA, USA). Antibodies Nrf2 (sc-365949), SOD2 (sc-137254), VEGF (sc-7269), Casp3 (sc-7272), and B-actin (sc-47778) were purchased from Santa Cruz Biotechnology, Inc.

2.2. Adipose-derived MSCs

According to the published protocols [12], ADMSCs were isolated from the adipose tissue, which was aspirated during liposuction from the visceral fat of an overweight 47-year old woman who had referred to the Tabriz International Valiasr Hospital (Tabriz, Iran) for liposuction. All related ethical issues were considered in performing the liposuction and the informed consent was obtained from the patient for using her adipose tissue. After characterization with cell surface CD marker and detecting the potential of differentiation to adipocyte and lipocyte, the cells were cultured in DMEM-F12 low glucose medium supplemented with 10% (v/v) fetal calf serum and 1% penicillin-streptomycin at 37°C and 5% CO2.

2.3. Cell viability assays

Cell viability was assessed using MTT assay [13]. Cells were seeded (1x104 cells/well) in a 96-well plate and treated with AMA in the concentrations of 5, 10, 20, 40, and 80 µM during 24 and 48 h. After incubation, 200 µL MTT solution (0.5 mg/mL) was added to each well and incubated at 37°C for 4 h. after discarding the supernatant, the formazan crystals were dissolved using 200 µL Sorenson's buffer (0.1 M Glycine, 0.1 M sodium chloride, PH=10.5). The absorbance of the samples was recorded at 570 nm on a microplate reader (Bio TEK microplate readers, Winooski, USA) and the IC50 was determined.

2.4. Determination of NAC neutralizing potential on AMA-induced oxidative stress

The cells were seeded at the density of 104 cells/well in 96-well cell culture plates during 24 h; then the cells were treated with different concentrations of NAC including 1, 2, 4, 8, 14 mM for 24 h. After 24 h, the NAC-treated cells were treated with IC50 concentration of AMA at 48 h.

2.5. Genotoxicity of AMA and protective effect of NAC

The MSCs were pretreated with NAC (8 mM) for 24 h, and then, the stress conditions were induced by the addition of IC50 concentration of AMA (40 μM). The untreated ADMSCs were used as a negative control. Various analyses were performed to address the geno-protective impacts of NAC.

2.5.1. DAPI/ Phalloidin staining

The MSCs were fixed with 4% formaldehyde for 10 min and then washed several times with PBS. Afterward, the cells were permeated with 0.1% (w/v) Triton X-100 for 5 min, washed again with PBS, and stained with 5μM solution of Phalloidin, at 45 min. then DAPI (200 ng/mL) solution was added at 5 min. after washing, the staining was verified by Cytation™ 5 cell imaging instrument (BioTek, Winooski, USA).

2.5.2. DNA ladder assay

DNA ladder assay by gel electrophoresis was employed to measure DNA single and double strand breaks in the cells. This test was performed to determine the apoptotic cells in oxidative stress and the inhibitory potential of NAC, based on a published protocol [14].

2.5.3. Annexin V/propidium iodide apoptosis assay

ADMSCs were cultured in DMEM-F12 Low glucose medium supplemented with 10 % (v/v) fetal calf serum and 1% penicillin-streptomycin and pretreated with NAC 8 mM at 37°C and 5 % CO2 for 24 h. Then the cells were treated with AMA 40 μM for next 48 h. Apoptotic population of cells were detected using a fluorescein isothiocyanate- (FITC-) annexin V apoptosis detection kit (BD Pharmingen, San Diego, CA) and flow cytometry according to the manufacturer's protocol.

2.6. DCFH-DA assay for oxidative stress assessment

After 24 h of cell culture in 6-well plates and pretreatment with NAC 8 mM, the cells were treated with AMA and then incubated for 12 h. After discarding the supernatant, the cells were washed (×3) with PBS and incubated with 10 μM of dichloro-dihydro-fluorescein diacetate (DCFH-DA) at 37°C for 1 h. The fluorescence was recorded at 485–535 nm using a BD flow cytometry. Finally, the cells were imaged using Cytation™ 5 cell imaging instrument (BioTek, Winooski, USA).

2.7. Assessment of mitochondrial membrane potential (ΔΨm)

The effect of AMA/NAC on mitochondrial membrane potential (Δψm) was measured using

Rhodamine-123 (Rh-123), lipophilic cationic dye. Rhodamine 123 is a fluorescent dye that binds to metabolically active mitochondria. The cells were treated with the NAC for 24 h. Then the cells were treated with AMA for 12 h. The cells were washed with PBS (pH 7.4) and incubated with 5µg/mL rhodamine 123 at 37°C for 15 min. Afterward, the cells were washed with PBS and imaged by Cytation™ 5 cell imaging instrument (BioTek, Winooski, USA).

2.8. Redox signaling regulation by NAC /AMA interplay

Total RNA was extracted from the treated and untreated MSCs using Trizol. The RNA yield and purity were determined by NanoDrop, ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, USA). Then, the cDNA was synthesized according to the literature [15]. Real-time PCR reactions were performed for four major transcription factors including Nrf2 and downstream antioxidant enzymes, RAD54L as regulator of DNA damage repair, HIF1-A (downstream gene for hypoxia-induced resistance and secretome), and mitochondrial sirtuin3 as a gene transcription regulator. In qPCR, the level of expression was calculated based on the PCR cycle number (Ct). The endogenous control B-ACTIN/18S RRNA was used for the normalization of mRNA levels. The Ct values were employed in calculating the relative expression using SPSS software (version 14.0) by the difference in the Ct values of the target RNAs after the normalization to the RNA input level. Relative quantification was represented according to the Pfaffl method [16]. Each reaction was performed in triplicate.

2.9. Western blot analysis

Protein was extracted by RIPA buffer and SDS page was performed according to our laboratory setup. Western blotting was performed based on a protocol set up at the laboratory. The membrane was stained with primary antibodies specific to Nrf2 (sc-365949), SOD2 (sc-137254), VEGF (sc-7269), Casp3 (sc-7272), and B-actin (sc-47778) before being incubated with horseradish peroxidase-conjugated secondary antibody (1:2000). Finally, bands were detected using a Pierce™ ECL western blotting substrate chemiluminescent kit.

2.10. Statistical analysis

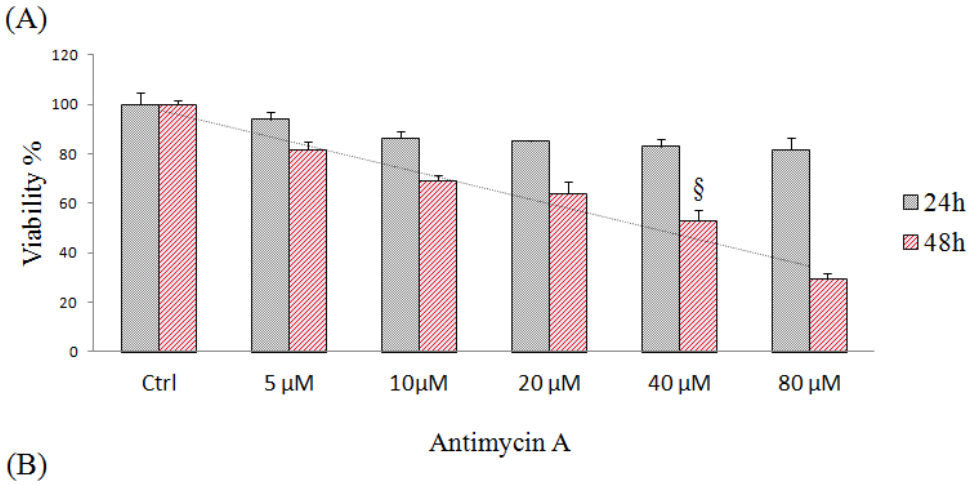
Data were expressed as mean values with standard deviation (SD) from three independent experiments. Statistical significances between groups were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test. The significance level was determined as p-value <0.05.

3. Results

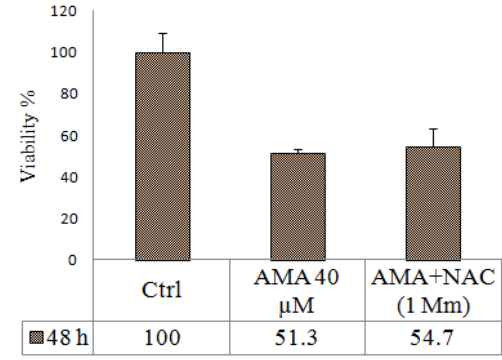
3.1. AMA cytotoxicity and inhibitory effect of NAC

In all studies in which the cells were co-treated with AMA and NAC, a NAC pretreatment was conducted 24 h before AMA treatment. The cells treated with AMA showed CI50 (50% cell viability)

for 40 µM at 48 h (Fig. 1A). Once pre-treated with NAC (8 mM, 24 h), the AMA(CI50)-treated ADMSCs were protected, showing over 77% of viability (Fig. 1B). The microscopic monitoring (data not showed) showed that the 5, 10, 20, 40, and 80 µM concentrations of AMA at 24 h did not change the cell morphology and no apoptotic/necrotic cells were observed, however the cell-cell junction was disrupted and the proliferation was stopped. After 48 h, the cells showed morphological changes and cell death was induced.



(B)



	Ctrl	AMA 40 µM	AMA+NAC (1 Mm)	AMA+NAC (2 mM)	AMA+NAC (4 mM)	AMA+NAC (8 mM)	AMA+NAC (16 mM)	AMA+NAC (32 mM)
48 h	100	51.3	54.7	59.4	68.8	77.5	73.1	60.5

Figure 1. In vitro antioxidant activity of NAC against oxidative stress induced by AMA in MSCs. (A) The MTT assay showed 50% cell viability in the MSCs upon treatment with AMA at a concentration of 40 µM. Different concentrations of AMA for treatment of cells were 5, 10, 20, 40, and 80 µM. (B) The protective potential of different NAC concentrations (1, 2, 4, 8, 16, 32 mM) in co-treatment with 40 µM of AMA. About 8 mM NAC is the optimum concentration for the protective effect. Data show mean values ± SD of at least three independent experiments. §; Represents statistical significance ($P < 0.05$) compared to the control (Ctrl).

3.2. Protective effect of NAC against DNA damages and apoptosis in MSCs

3.2.1. DAPI / Phallacidin staining and DNA ladder assay

These methods are applied for the assessment of genomic DNA fragmentation. These assays provide great plausibility for the measurement of DNA damage as a marker of exposure to a genotoxic agent (i.e., AMA) and the evaluation of geno-protective capacities of the bioactive compound (i.e., NAC). As shown in Fig. 2, in the cells stained with DAPI/ Phallacidin compared with un-treated cells (Fig. 2A-C), nuclear deformation and cytoskeleton disruption were seen in AMA treatment (Fig. 2C), while the pretreatment with NAC could inhibit the chromatin remodeling, the fragmentation of DNA, and cytoskeleton disruption in the AMA-treated MSCs (Fig. 2B).

In the DNA Ladder assay (Fig. 2D), NAC showed a protective effect against DNA fragmentation induced by AMA. The ROS induced by AMA caused a marked increase in DNA fragmentation as compared to the untreated control. Conversely, NAC protected the cells from the AMA-induced toxicity mediated by ROS, showing a decreased DNA damage.

3.2.2. Detection of population of apoptotic cells

Population of apoptotic cells was detected using a fluorescein isothiocyanate- (FITC) annexin V apoptosis detection kit. When the cells were exposed to 40 μM of AMA, the apoptotic cells were reported 73% as compared to the control cells at 48 h time point. However, when the cells were co-treated with NAC (8 μM) and AMA, the frequency of the apoptotic cells were markedly decreased to approximately 37% (Fig. 2E-H).

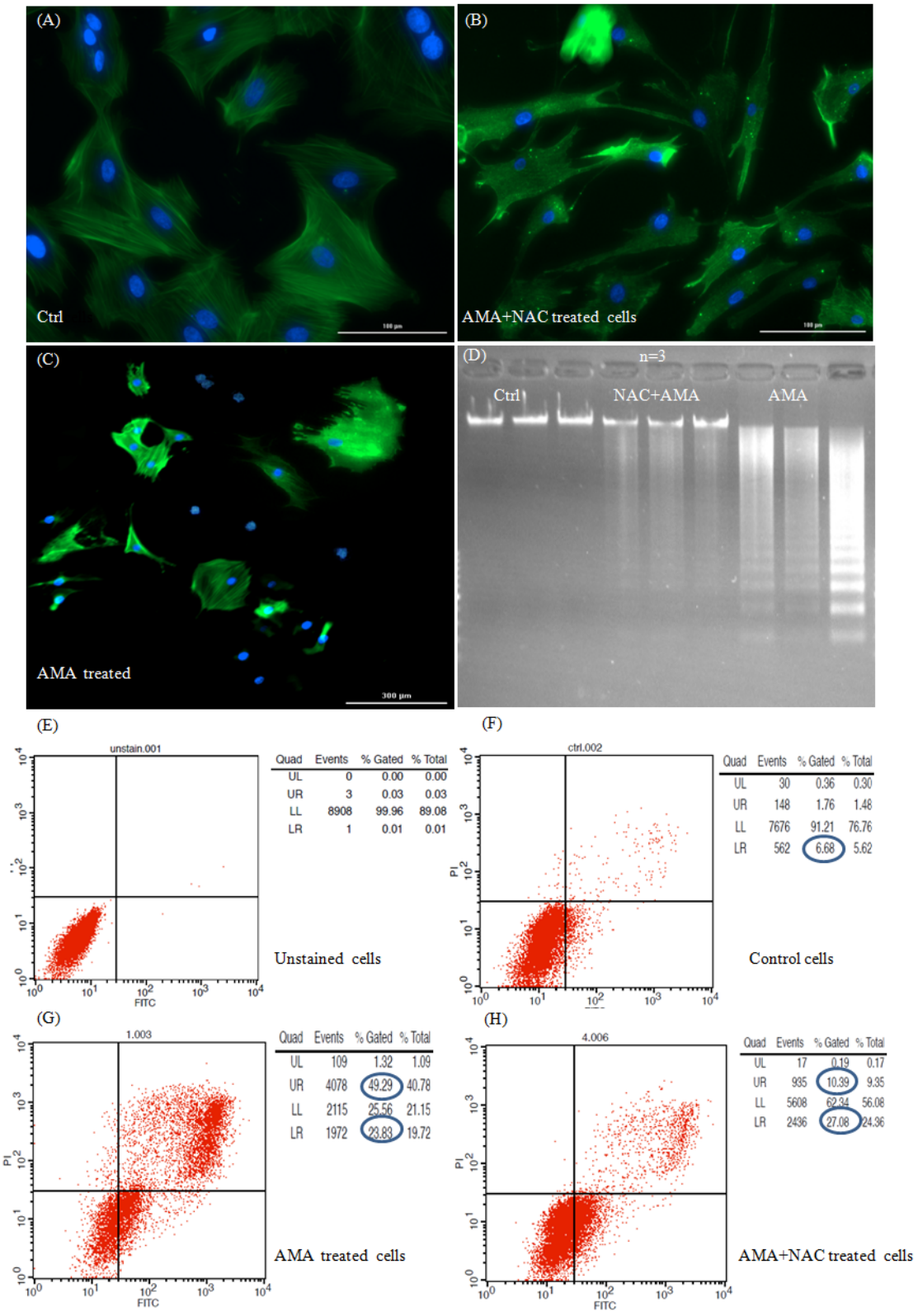


Figure 2. Analysis of the nucleus and cytoplasmic F-actin integrity. Panels A, B, and C respectively

show the DAPI / Phallacidin staining of the untreated cells, the cells co-treated with NAC/AMA, and the cells treated with AMA. Panel D represents the DNA ladder assay in triplicate. Population of apoptotic cells was detected using a fluorescein isothiocyanate- (FITC-) annexin V apoptosis detection kit. When the cells were exposed to 40 μM of AMA, the apoptotic cells were reported 73% as compared to the control cells at 48 h time point. However, when the cells were co-treated with NAC (8 mM) and AMA, the frequency of the apoptotic cells were markedly decreased to approximately 37%.

3.3. Induction of oxidative stress by AMA

The cells were pretreated 24 h with NAC, and then ROS generation was assessed at 12 h after AMA treatment. The florescent microscopic data (Fig. 3A-C) showed that MSCs treated with NAC might alleviate the intensity of ROS as compared to AMA-treated cells. The florescent micrograph of stained MSCs revealed the effect of AMA in –inducing the intracellular ROS generation (Fig. 3E) by flow cytometry analysis. The ROS intensity analysis showed that NAC could significantly reduce the production of ROS as compared to the AMA-treated cells.

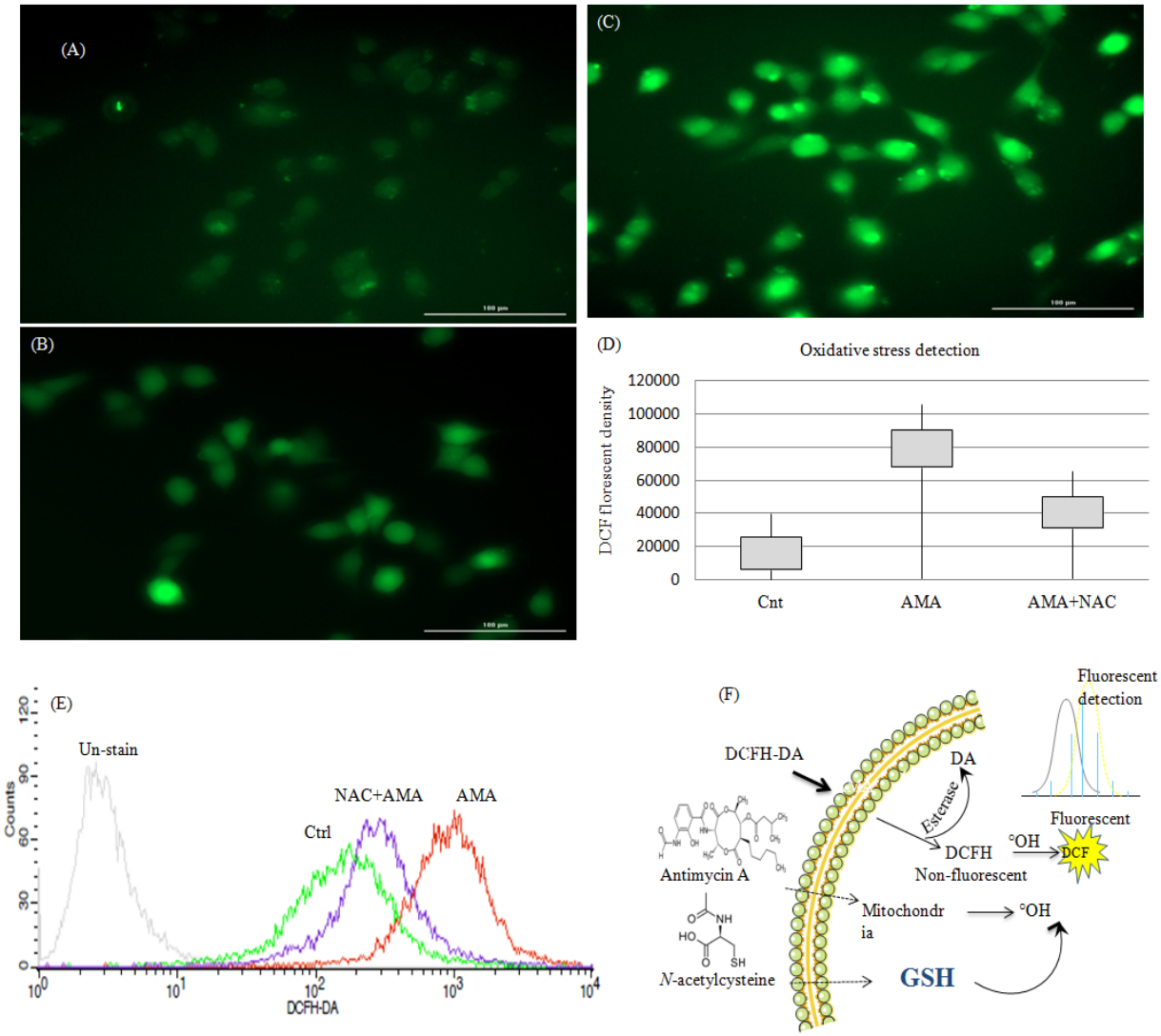


Figure 3. Fluorescent microscopy and flow cytometry determination of oxidative stress within the stem cells. Panels A, B, and C represent the fluorescence intensity in the untreated control cells, the cells treated with AMA and NAC, and the cells treated with AMA, respectively (E); Photomicrographs show intracellular ROS generation in MSCs induced by AMA and stained with DCFH-DA (F); The schematic mechanism of intracellular stress detected by DCFH.

3.4. Rhodamine 123 staining for mitochondrial membrane potential

Untreated MSCs showed high fluorescence, indicating polarized mitochondrial membrane (Fig. 4). The AMA-treated cells that blocked Cytochrom C, showed the disruption of H pumps, and fluorescence intensity was also decreased. The notable results are that NAC although decreased ROS induced by AMA, but it had no effect on mitochondrial polarization, and the cells treated with

NAC/AMA showed low fluorescence, indicating non-polarized mitochondrial membrane.

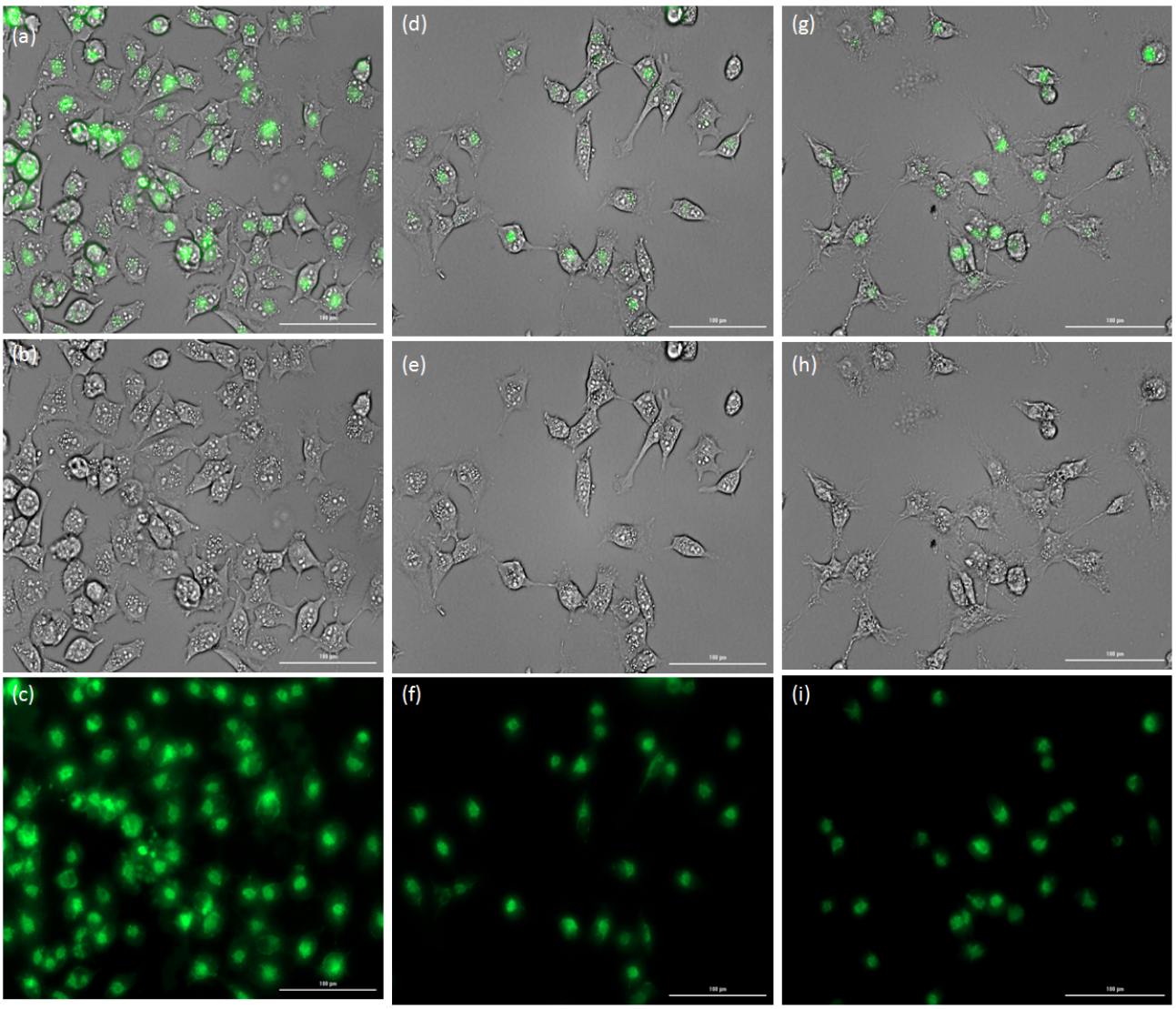


Figure 4. Rhodamine 123 staining for mitochondrial membrane potential. a-c; Untreated MSCs show high fluorescence, indicating polarized mitochondrial membrane (a; merged i
mage, b; light image, & c; the mitochondrial polarization stained with RH 123), d-f; The cells co-treated with NAC/AMA shows protection from apoptosis, but the polarization of mitochondria was disrupted. g-i; The AMA-treated cells showed the disruption of H pumps, and fluorescence intensity was also decreased.

3.5. Nuclear factor erythroid 2-related factor 2 and Redox signaling in AMA/NAC crosstalk

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription activator that binds to the

antioxidant response elements (ARE) in the promoter regions of target antioxidant genes, and is critical for the coordinated up-regulation of genes in response to oxidative stress [17]. As shown in Fig. 5A, an increased level of Nrf2 was seen in AMA-induced oxidative stress as part of cells' defense response. It was expected that Nrf2 will be down regulated slightly by NAC co-treatment, which might have a scavenging impact on intracellular ROS induced by AMA, but it was surprisingly observed that NAC directly affected the cell signaling and had a synergetic effect on Nrf2 expression in normal condition. The downstream antioxidants including Catalase, NQO1, and SOD2 were upregulated in AMA-treated conditions but alleviated via NAC treatment.

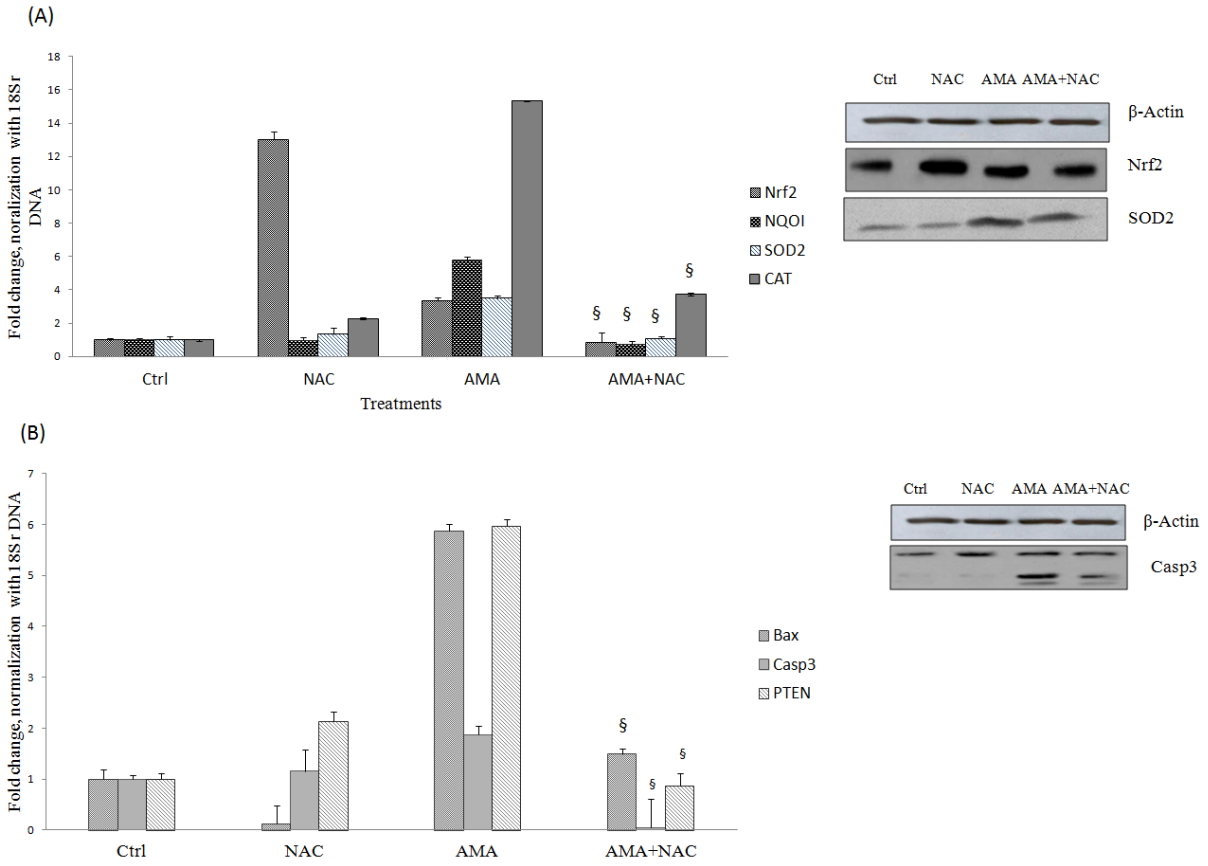


Figure 5. (A), Functional expression of Nrf2 in the MSCs treated with AMA and NAC. (A) RNA and protein profiles of Nrf2 confirming the upregulation under the NAC and AMA treatment. The downstrem antioxidants including Catalase, NQO1, and SOD2 upregulated in AMA-treated condition but alleviated via NAC treatment. (B) The pro-apoptotic and anti-apoptotic gene regulation by AMA

and NAC duel. §: P<0.05 compared to untreated control

3.6. Cellular energy metabolism regulation via Sirt3 and mTOR

Mitochondrial NAD-dependent protein deacetylase activates mitochondrial target proteins through deacetylating key lysine residues. These target proteins are ACSS1, IDH, GDH, SOD2, PDHA1, LCAD, and SDHA, and the ATP synthase subunit ATP5O which contributes to the regulation of the cellular energy metabolism [18]. The other vital protein in this interaction is mTOR, a serine/threonine protein kinase, which is a key regulator of cellular metabolism, growth and survival in response to nutrients, energy, and stress signals. Moreover, mTOR directly or indirectly regulates the phosphorylation of at least 800 proteins involved in the proliferation, differentiation, and bio-function of stem cells [19]. As Fig. 6 A shows the mTOR and Sirt3 were both upregulated in oxidative stress.

3.7. DNA damage and DNA repair pathway

RAD54L, the known protein involved in the homologous recombination and repair of DNA, has been shown to play a leading role in the repair of DNA double-strand breaks under oxidative stress [20]. As Fig. 6B shows AMA induced RAD54L and it could be concluded that AMA induces DNA damage. The binding of this protein to double-stranded DNA induces a DNA topological change, which is believed to facilitate homologous DNA paring.

3.8. The role of oxidative stress and disruption of energy metabolism in MSCs secretome

Hypoxia-inducible factor 1-alpha acts as a master transcriptional regulator of the adaptive response to hypoxia. Under hypoxic conditions, it activates the transcription of over 40 genes such as erythropoietin, glucose transporters, glycolytic enzymes, vascular endothelial growth factor (VEGF), and other genes whose protein products either increase oxygen delivery or facilitate metabolic adaptation to hypoxia [21]. VEGF is a growth factor which is active in angiogenesis, and is one of the vital downstream genes of HIF1-A. The secretome of MSCs importantly affects cell therapy outcome in damaged tissues [22] and as the results showed, NAC could upregulate VEGF in both normal and oxidative stress conditions (Fig. 6C). Therefore, pre-treatment of the MSCs with NAC will protect the transplanted stem cells in hypoxia conditions and enhance the secretome for the repair of tissues.

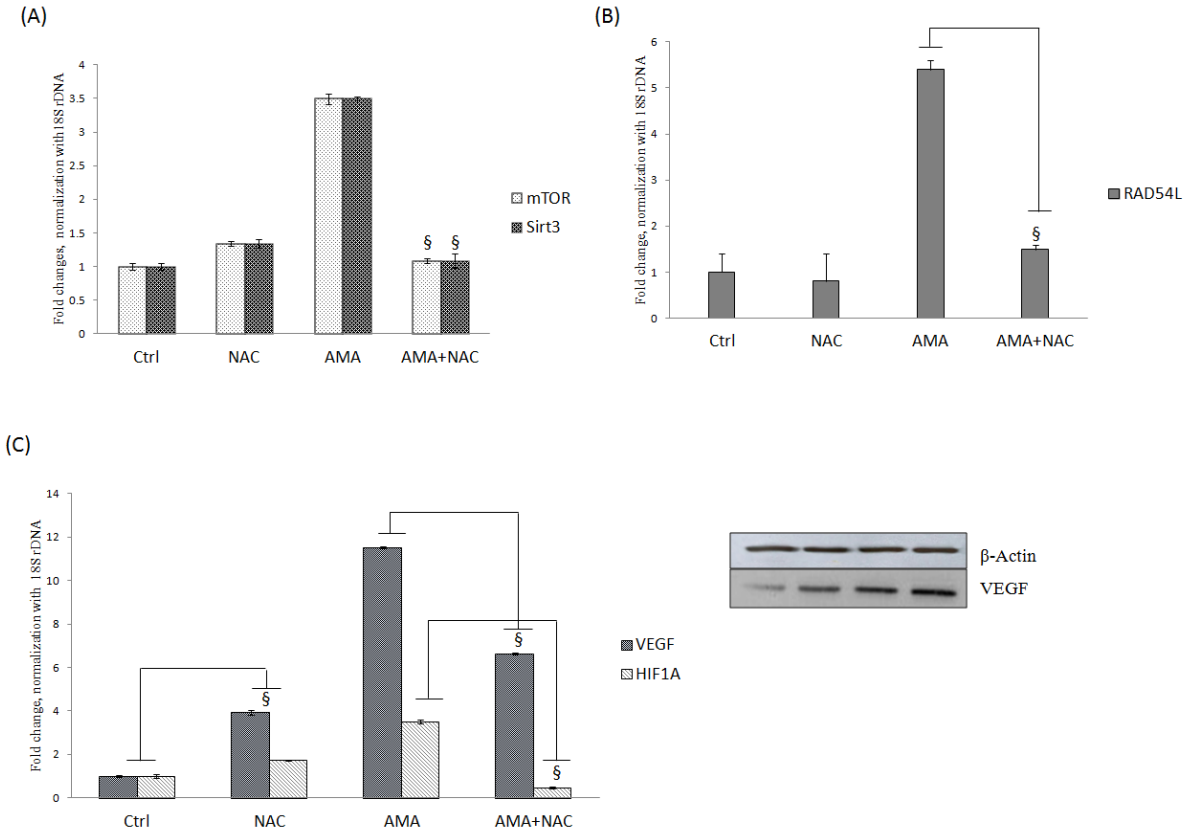


Figure 6. (A); The mRNA profile of Sirt3 and mTOR, the main proteins interplaying in cellular metabolism, growth, and survival in response to nutrients, energy, and stress signals. §: $P < 0.05$ was considered as significant versus control cells, (B); RAD54L, a protein known to be involved in the repair of DNA upregulated under AMA treatment. (C); Profile of HIF1-A gene and its downstream vascular endothelial growth factor (VEGF) §: $P < 0.05$ was considered as significant versus control cells.

4. Discussion

All in all, the cell therapy of damaged tissue had not the expected results, perhaps one of the reasons is that the hypoxia conditions in damaged tissues influence the respiration and subsequently and therapeutically the secretome [18]. In the respiratory chain deficiency, the cells encountered two major problems including oxidative stress and energy deficit. To mimic hypoxia and respiratory deficiency, in this study we used AMA, an inhibitor of cellular respiration, particularly oxidative phosphorylation[23]. AMA binds to the cytochrome c reductase, and causes the disruption of entire electron

transport chain and the formation of proton gradient across the inner membrane of mitochondria [24] (Fig. 7A). This inhibition further results in the formation of toxic superoxide free radical. The rate of superoxide production exceeds the cellular scavenging mechanisms, thereby overwhelming the cell and leading to cell death [20].

NAC is the N-acetyl derivative of the amino acid L-cysteine, and is a precursor for the glutathione synthesis in the cells. Glutathione is majorly known to minimize the lipid peroxidation of cellular membranes and mitochondrial oxidative stress. Another essential role of glutathione relates to its impact on signal transduction and control of gene expression within cells, as well as energy metabolism [25](Fig. 7B).

Previous studies have reported the enhancement of therapeutic efficacy of bone marrow-derived MSCs transplantation in rats with severe acute pancreatitis by the NAC pretreatment [26]. However, the mechanism of NAC protection has not been completely elucidated yet. In the present study, NAC dramatically reduced AMA-induced mitochondrial oxidative stress, and thus effectively protected MSCs. Over cellular studies, DAPI staining and DNA ladder assay revealed that NAC could neutralize the genotoxicity of AMA and protect the cells against apoptosis and DNA damages. Flow cytometry analysis of apoptotic cell population demonstrated that the AMA-treated cells showed 73% apoptosis compared to the control cells (~6% related with unstained cells). However, treatment of the cells with NAC resulted in a markedly decrease in the apoptotic cells (approximately 36%).

To study the AMA and NAC effects on mitochondria and redox homeostasis, we looked at the expression of the nuclear factor-erythroid 2-related factor 2 (NFE2L2, the so-called Nrf2), which is a basic leucine zipper (bZIP) protein. This protein can regulate the expression of antioxidant enzymes that protect the cells against oxidative stress triggered by injuries [27]. In the results, it was anticipated that NAC is obliged to downregulate the Nrf2 gene expression significantly. Considering the scavenging activity of NAC, it was expected that NAC may neutralize the intracellular ROS and in this case, there is no need to employ any cellular antioxidant mechanism to combat free radicals. However, according to the results, NAC exerted a synergetic effect with AMA in upregulating the Nrf2 gene; as a result of which, the cells fought against oxidative stress and survived. In AMA-induced stress, apoptosis rate was increased through significantly elevated levels of pro-apoptotic PTEN, casp3, and Bax gene expression. In conformity with these findings, the expression of these genes was decreased under pretreatment with NAC. These findings suggest a possible role for NAC in encouraging cell survival via activating Nrf2 and prohibiting mitochondria-mediated apoptosis in MSCs. Another cell signalings that we studied in this study were mTOR and Sirt3. Overexpression of Sirt3 in AMA-treated cells increased respiration and decreased the production of ROS. In addition to its known

mitochondrial function, some researchers have proposed that a very small pool of active nuclear Sirt3 exists. This pool is reported to consist of the long form of Sirt3 and has been suggested to have histone deacetylase activity. Sirtuins represent a potential way to achieve this end and warrant further studies in MSCs. Most sirtuins may help enhance our capability in expanding MSCs ex vivo for eventual clinical use. HIF plays a major role in the adaptive regulation of energy metabolism, via triggering a switch from mitochondrial oxidative phosphorylation to anaerobic glycolysis in hypoxic conditions [28]. HIF1-A acts as a master transcriptional regulator of the adaptive response to hypoxia. Under hypoxic conditions, HIF1-A activates the transcription of over 40 genes. HIF also reduces oxygen consumption in mitochondria via inhibiting the conversion of pyruvate to acetyl CoA, suppressing mitochondrial biogenesis, and activating autophagy of mitochondria concomitantly with reduction in ROS production [28].

The secretome of MSCs importantly affects the cell therapy outcome and as the results showed, NAC could upregulate VEGF in both normal and oxidative stress conditions (Fig. 6C). VEGF is a growth factor which is active in angiogenesis, and is one of the vital downstream genes of HIF1-A [22]. The pre-treatment of MSCs with NAC protected the cells in hypoxia conditions and enhanced the VEGF factor.

Furthermore, protein-protein interactions (PPIs) were analyzed in this study using the STRING Database v. 10.5 (http://www.string-db.org), which includes direct and indirect protein associations with redox balance and energy metabolism, collected from different databases. Protein interaction networks were predicted using medium confidence scores (0.40) and clustered using MCL clustering algorithm (inflation parameter: 5) (Fig.7 B). In summary, the present study provided evidence for the possible role of NAC in the protection of MSCs in hypoxia conditions induced by AMA. Our results showed that antioxidants may only affect the cells via directly scavenging the free radicals and indirectly reducing the oxidative stress via modulation of redox signaling, however energy metabolism remains unresolved and we need other strategies to enhance the energy metabolism in hypoxia conditions as well. These results provide new insights into the designing a new strategy to protect the cells not only from hypoxia-induced damages and oxidative stress, but also to enhance the energy metabolism.

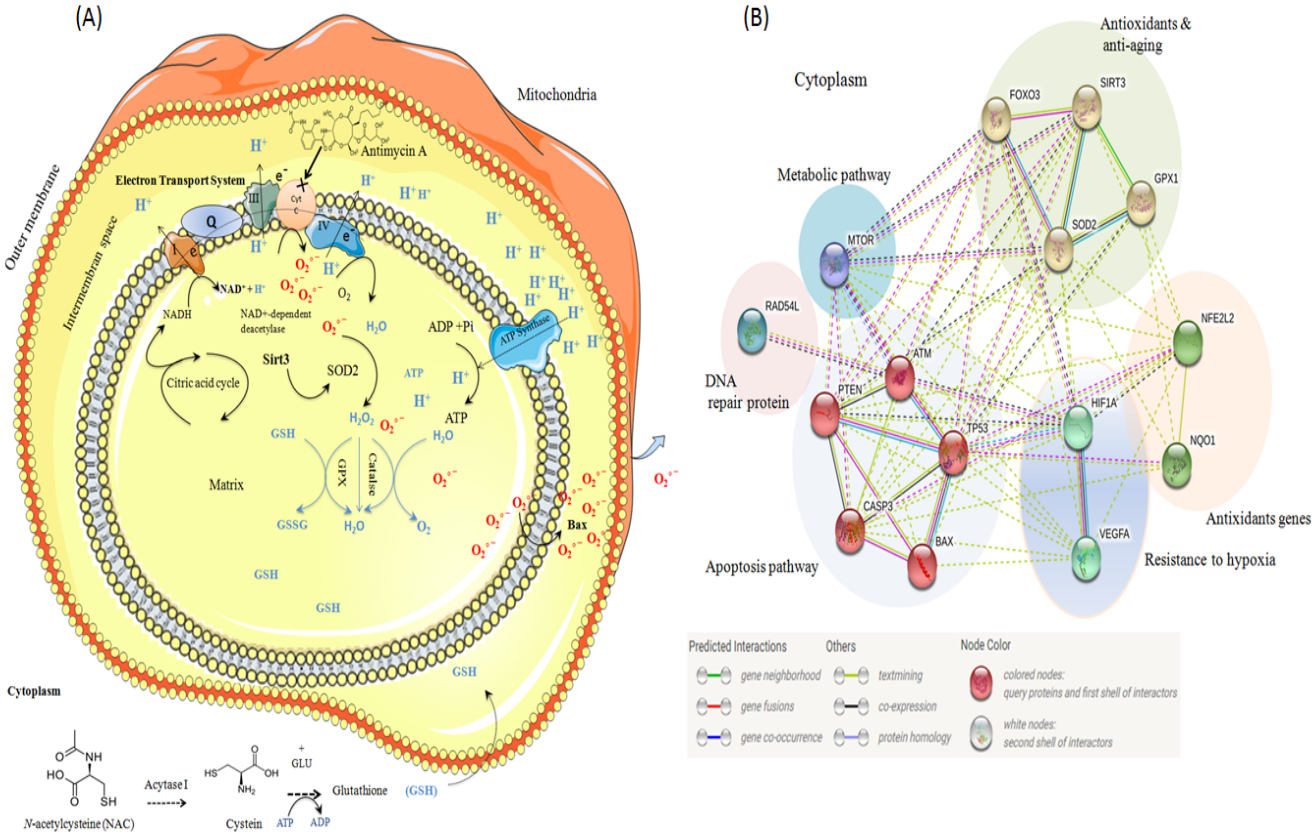


Figure 7. (A); Mitochondria and the duel of NAC and AMA. The NAC treatment of cells increase the pool of GSH in the cytoplasm and GSH directly scavenges the free radicals in the mitochondrial matrix due to the blockage of oxidative phosphorylation cycle. Although the GSH and native antioxidant enzymes (i.e., Catalase and SOD2) could neutralize the free radicals and save the cells from apoptosis, NAC had no effect on the production of ATP. Hence, in monitoring the cells with a microscope, the arrest of cells was observed. GPX: Glutathione Peroxidase, NAD; Nicotinamide adenine dinucleotide.

(B); The protein-protein interactions (PPIs) were analyzed using the STRING Database v. 10.5

(http://www.string-db.org), which includes direct and indirect protein associations with redox balance and energy metabolism, collected from different databases. Protein interaction networks were predicted using medium confidence scores (0.40) and clustered using MCL clustering algorithm (inflation parameter: 5). (https://string-db.org/). Four transcription factors are involved in this crosstalk: 1) Nrf2; Nuclear factor erythroid 2-related factor 2; Transcription activator that binds to the antioxidant response elements (ARE) in the promoter regions of target genes. Nrf2 is important for the coordinated upregulation of genes in response to oxidative stress. 2) Hif1-A; Hypoxia-inducible factor 1-alpha; functions as a master transcriptional regulator of the adaptive response to hypoxia. Under hypoxic conditions, it activates the transcription of over 40 genes, including erythropoietin, glucose transporters, glycolytic enzymes, VEGF, and other genes whose protein products either increase oxygen delivery or facilitate metabolic adaptation to hypoxia. 3) RAD54L; DNA repair and recombination protein. 4) RAD54-like; Involved in DNA repair and mitotic recombination.

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3B Chapter

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ORIGINAL RESEARCH ARTICLE

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Mitochondria-targeted antioxidant mito-TEMPO alleviate oxidative stress induced by antimycin A in human mesenchymal stem cells

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Mitochondria-targeted antioxidant mito-TEMPO alleviate oxidative stress induced by antimycin A in human mesenchymal stem cells

This section describes the second research paper in this thesis. The difference with the first work is using another antioxidant called mito-TEMPO. MitoTEMPO is a mitochondrially targeted antioxidant, a specific scavenger of mitochondrial superoxide. MitoTEMPO is a combination of the antioxidant piperidine nitroxide TEMPO with the lipophilic cation tri-phenylphosphonium, giving MitoTEMPO the ability to pass through lipid bilayers with ease and accumulate several hundred-fold in mitochondria. Inhibition of mitochondrial respiratory chain has been recently found associated with enhancement of stem cell pluripotency. In adittion, have implied that ROS are critical intermediates of cellular signaling pathways.More recent works, however, have repoted that mitochondria oxidative stress lead the stem cells to apoptosis a few hours after transplantation. So, Mito-TEMPO treatmet of stem cells for study of them under hypoxia induced by AMA is the aim of this chapter.

Abstract

The cell therapy of damaged tissue, which is linked to hypoxia condition might fail, in large part due to the emergence of oxidative stress (OS) and/or mitochondrial dysfunctions. Thus, the invigoration of stem cells against oxidative stress could be a reliable strategy to improve the cell therapy outcome. Of various antioxidants, mito-Tempo (mito-T) is one of the potent antioxidants that could target and neutralize the mitochondrial oxidative stress. In this study, for the induction of hypoxia and oxidative stress in mitochondria of the mesenchymal stem cells (MSCs) isolated from human adipose tissue, antimycin A (AMA) was used and then several parameters were analyzed, including cell viability and cell cycle arrest of MSCs exposed to AMA, mito-T, antioxidant potential, redox homeostasis, and signaling pathways in MSCs under oxidative stress. Based on our findings, the treated MSCs were found to impose a high resistance to the OS-induced apoptosis, which correlates with the Nrf2 pathway required to manage OS. Upon the exposure of the MSCs to high oxidative stress conditions using AMA, the cells failed to scavenge. The use of mito-T was found to alleviate the damage induced by oxidative stress through both direct functions of the free radical scavenging and the interplay in terms of cell signaling pathways including the upregulation of the Nrf2 pathway. These findings may pave the way in the stem cell therapy for the hypoxia-mediated tissue damage.

Keywords: Cell cycle; Mesenchymal stem cell; Nrf2 signaling pathway; Oxidative stress

Résumé:

La thérapie cellulaire des tissus endommagés, qui est liée à l'hypoxie, pourrait échouer, en grande partie en raison de l'apparition d'un stress oxydant (OS) et / ou de dysfonctionnements mitochondriaux. Ainsi, la stimulation des cellules souches contre le stress oxydatif pourrait constituer une stratégie fiable pour améliorer les résultats de la thérapie cellulaire. Parmi les différents antioxydants, le mito-tempo (mito-T) est l'un des puissants antioxydants qui pourraient cibler et neutraliser le stress oxydatif mitochondrial. Dans cette étude, pour l'induction de l'hypoxie et du stress oxydatif dans les mitochondries des cellules souches mésenchymateuses (CSM) isolées du tissu adipeux humain, l'antimycine A (AMA) a été utilisée, puis plusieurs paramètres ont été analysés,

notamment la viabilité cellulaire et l'arrêt du cycle cellulaire. Les CSM exposés à l'AMA, au mito-T, au potentiel antioxydant, à l'homéostasie redox et aux voies de signalisation dans les CSM sous stress oxydatif. D'après nos résultats, il a été constaté que les CSM traitées imposaient une résistance élevée à l'apoptose induite par l'OS, ce qui correspond au chemin Nrf2 nécessaire à la gestion de l'OS. Lors de l'exposition des CSM à des conditions de stress oxydatif élevé à l'aide de l'AMA, les cellules n'ont pas réussi à se nettoyer. On a constaté que l'utilisation de mito-T atténuait les dommages causés par le stress oxydatif à la fois par les fonctions directes du piégeage des radicaux libres et par l'interaction entre les voies de signalisation cellulaire, y compris la régulation à la hausse de la voie Nrf2. Ces résultats pourraient ouvrir la voie à la thérapie par cellules souches pour les lésions tissulaires induites par l'hypoxie.

Mots clés : Stress oxydative; Mesenchymal stem cells;

1 | INTRODUCTION

Reactive oxygen species (ROS) have historically been regarded as toxic metabolic byproducts, elevated cellular levels of which are associated with many diseases, including cancer, diabetes, inflammatory diseases, ischemia-related diseases, and neurodegenerative disorders (Cheignon et al., 2018; Incalza et al., 2018; Klaunig and Wang, 2018; Noiri et al., 2018; Robson et al., 2018). More recent works, however, have implied that ROS are critical intermediates of cellular signaling pathways (Hamanaka and Chandel, 2010; Rodriguez-Ruiz et al., 2016). More recently, it has been recognized that ROS originated from mitochondrial sources might involve in cellular signaling (Brand, 2016). Moreover, mitochondrial ROS levels are crucial for the biological functions of stem cells (Zuluaga et al., 2017). Low levels of mitochondrial ROS production are demanded in some cellular processes such as proliferation and differentiation (Hu et al., 2018b; Pashkovskaia et al., 2018). It has been reported that the primary human mesenchymal stem cells (MSCs) may differentiate into adipocytes. As a result, these cells may show enhanced mitochondrial metabolic activities leading to an increased generation of ROS (Tormos et al., 2011). Other studies have highlighted that various factors including ROS may influence the final biological fate of hematopoietic stem cells (HSCs), and the mitochondrial oxidative phosphorylation is not a simple and passive function (Papa et al., 2019). Mitochondrial complexes I and III, and the NADPH oxidase isoform NOX4 are main sources for the production of ROS during the differentiation phenomena of MSCs. Accordingly, ROS are thought to interact with several pathways that might influence the transcription machinery required for the differentiation of MSCs, including the Wnt, Hedgehog, and FOXO signaling cascades (Atashi et al., 2015). In addition, elevated levels of ROS, defined as oxidative stress, may lead to the arrest of the cell cycle in the MSCs and hence possible emergence of apoptosis (Hamanaka and Chandel, 2010). Given that ROS can adversely influence the stem cell properties, it is crucial to attenuate the extent of ROS in order to protect the MSCs during the course of regenerative therapy. ROS can also affect the culture expansion and the longevity of MSCs. Therefore, there exist a lot of demands for the development of methods to protect the MSCs from the oxidative stress and the replicative senescence phenomena.

The mitochondrial functions play crucial roles in oxidative phosphorylation, ATP generation,

and cellular apoptosis (Rodriguez-Ruiz et al., 2018). At the mitochondrial inner membrane, the electron transport chain contributes to the generation of energy, where oxygen acts as the electron acceptor. During oxidative phosphorylation, the electrons leak from the mitochondrial complexes in the electron transport chain can result in the partial reduction of oxygen and the generation of ROS (Paliwal et al., 2018).

In the current study, antimycin A (AMA) was used as an inhibitor of cellular respiration (in particular oxidative phosphorylation in mitochondria) and the production of ROS. It has been reported that AMA can inhibit the growth of various cells, as it may stimulate oxidative stress-mediated cell death (Park and You, 2016). Cellular antioxidant enzymes metabolize toxic intermediates to maintain cellular homeostasis. However, these control mechanisms are not convincing in the dysfunctional mitochondria, and hence, the accumulation of ROS might elicit inevitable oxidative damage in the cells (Paliwal et al., 2018). The mito-Tempo (mito-T) is a hybrid substance (a combination of the antioxidant piperidine nitroxide TEMPO with the lipophilic cation triphenylphosphonium), which can easily cross the lipid bilayers and accumulate in the mitochondria up to several hundred folds. In this investigation, to identify the underlying mechanisms in supporting stem cells from oxidative stress, the human adipose MSCs were treated with mito-T and then the effect of AMA was assessed in terms of mitochondrial functions.

2 | MATERIALS AND METHODS

2.1 | MTT assay for determination of Antimycin A toxicity

Adipose-derived MSCs (ADMSCs) were isolated from adipose tissue and collected through liposuction from the visceral fat of an overweight 37-year old woman at Tabriz International Valiasr Hospital (Tabriz, Iran), which was performed considering all the related ethical issues and obtaining consent from the volunteer. After isolation, AD-MSCs were characterized using cell surface CD markers and adipocyte/lipocyte differentiation potential (data not shown). All experiments were done on passage 3 cells. The cells were cultured in DMEM-F12 low glucose medium supplemented with 10% (v/v) fetal calf serum and 1% penicillin-streptomycin at 37°C and 5% CO2. The cells were detached with trypsin after being reached 70% confluence and seeded at a density of 1.0 × 104 cells/well in 96-well cell culture plates after 24 h. ADMSCs were treated

with different concentrations of AMA (10, 20, 40, 80, and 160 μM) for 24 and 48 h. The cell viability was determined by MTT assay. After incubation, 100 μL MTT reagent (0.5 mg/mL) was added to each well. Cells were incubated at 37˚C for 4 h to allow the MTT to be metabolized. After discarding the supernatant, 2-propanol (200 μL/well) was added to dissolve the formazan crystals. The absorbance of the samples was recorded at a wavelength of 490 nm using a micro-plate reader. The percentage of proliferation was calculated with respect to control.

2.2 | Determination of mito-T neutralizing potential for AMA-induced oxidative stress

Determination of cell viability under stress conditions (AMA, IC50 =40 μM) and with mito-T in differential concentrations (5, 10, 20, 40, 80 μM) was carried out at 24 and 48 h by MTT assay as described in section 2.1. The low concentration of mito-T for the inhibition of IC50 AMA toxicity was determined.

2.3 | Genoprotective effect of mito-T

The MSCs were cultured for 24 h, and then, the stress conditions were induced by the addition of AMA (40 μM) in the presence/absence of mito-T (20 μM), followed by 48-h cell incubation. The untreated MSCs were used as a negative control. Various analyses were performed to address the genoprotective impacts of mito-T.

2.3.1 DAPI

The MSCs were fixed with 4% formaldehyde for 10 min and then washed several times with PBS. Afterward, the cells were permeated with 0.1% (w/v) Triton X-100 for 5 min, washed again with PBS, and stained with DAPI (200 ng/mL) in 5 min. The staining was verified by fluorescence microscopy.

2.3.2 Comet assay

Comet assay, a single cell gel electrophoresis, was employed to measure DNA single and double-strand breaks in the cells. This test was performed to determine the apoptotic cells in oxidative stress and the inhibitory potential of mito-T, based on a protocol published in the Nature Protocols with slight modifications (Olive and Banáth, 2006).

2.3.3 Annexin V/propidium iodide apoptosis assay

In the early stages of apoptosis, some changes occur at the cell surface that are often difficult to be detected. One of these alterations occurred in the plasma membrane is the translocation of phosphatidylserine from the inner side of the plasma membrane to the outer layer, by which phosphatidylserine is exposed on the external surface of the cell. Annexin V is a Ca2+-dependent phospholipid-binding protein with a high affinity for phosphatidylserine (Vermes et al., 1995). Populations of apoptotic cells were detected using a fluorescein isothiocyanate (FITC)-labelled annexin V apoptosis detection kit, BD Pharmingen™ (Becton Dickinson, San Diego, USA) and flow cytometry analysis following the manufacturer's protocol.

2.4 | Oxidative stress assessment

After 24 h of cell culture in 6-well plates, the medium was removed and the cells were washed with PBS (×3). Then, the cells were treated with AMA and mito-T and incubated overnight. After discarding the supernatant, the cells were washed (×3) with PBS and incubated with 10 μM of dichloro-dihydro-fluorescein diacetate (DCFH-DA) at 37°C for 1 h. The fluorescence was recorded at 485–535 nm using a BD flow cytometry. Further, the cells were imaged using Cytation™ 5 cell imaging instrument (BioTek, Winooski, USA).

2.5 | Analysis of cellular DNA contents by flow cytometry: Cell cycle arrest test

Cell cycle phase distribution with cellular DNA contents was carried out using flow cytometry analysis. To this end, the MSCs were seeded into a 6-well plate at a density of 1.0×106 cells/mL and treated with the IC50 concentration of AMA (40 μM) for 24 h in 5% CO2 incubator at 37°C. After 24 h incubation, the cultured cells were harvested, washed (×3) with cold PBS, fixed in 70% ethanol, and treated with RNase A (10 mg/mL). The fixed cells were then stained with propidium iodide (PI) dye followed by incubation for 10 min at room temperature in the dark. The PI fluorescence of individual nuclei was measured using flow cytometer, BD FACS Calibur (Becton Dickinson, Franklin Lakes, USA).

2.6 | Redox signaling regulation by mito-T /AMA interplay

Total RNAs were extracted from the treated and untreated MSCs using Trizol. The RNA yield and purity were determined using NanoDrop, ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, USA). Then, the cDNA was synthesized according to the literature (Asoudeh-Fard et al., 2017). Real-time PCR reactions were performed for redox pathway (Nrf2), and apoptosis pathway (Bax, PTEN, casp3) mRNAs as described previously (Rodriguez-Ruiz et al., 2016). In qPCR, the level of expression was calculated based upon the PCR cycle number (Ct). The endogenous control GAPDH was used for normalization of mRNA levels. The Ct values were used to calculate relative expression using SPSS software (version 14.0) by the difference in the Ct values of the target RNAs after the normalization to the RNA input level. Relative quantification was represented according to the Pfaffl method (Pfaffl, 2001). Each reaction was performed in triplicate.

2.7. Western blot analysis

Protein was extracted by RIPA buffer and SDS-PAGE was performed according to our laboratory setup. Western blotting was performed based on a protocol set up at RCPN. The membrane was stained with primary antibodies specific to Nrf2 (sc-365949), Bax (sc-7480), PTEN (sc-7974), Casp3 (D3R6Y), and GAPDH (sc-32233) before being incubated with horseradish peroxidase-conjugated secondary antibody (1:2000). Bands were detected using a Pierce™ ECL western blotting substrate chemiluminescent kit.

2.8 | Statistical analysis

Data were expressed as mean values with standard deviation (S.D.) from three independent experiments. Statistical significances between groups were analyzed by one-way ANOVA followed by Dunnett's multiple comparisons post hoc test. The significance level was a p-value of <0.05.

3 | RESULTS

3.1 | Cytotoxic impacts of AMA and inhibitory effect of mito-T

To determine the experimental doses of AMA and mito-T, the cytotoxic tests were performed on MSCs using MTT assay and cell morphology assessment. The microscopic monitoring showed

that the concentration (10, 20, 40, 80, and 160 μM) of AMA at 24 h did not change the cell morphology and no apoptotic/necrotic cells were observed. After 48 h, the cells showed morphological changes and cell death was induced. The MTT assay for the cell viability data showed 50% cell viability in the MSCs upon treatment with AMA at a concentration of 40 μM or higher. Upon the microscopic monitoring, cell death was statistically insignificant after 24 h, while it was increased after 48 h based on the MTT analysis. Once treated with mito-T at a concentration of 20 μM, the AMA-treated MSCs were protected from the cell death showing over 80% of viability (Figure 1).

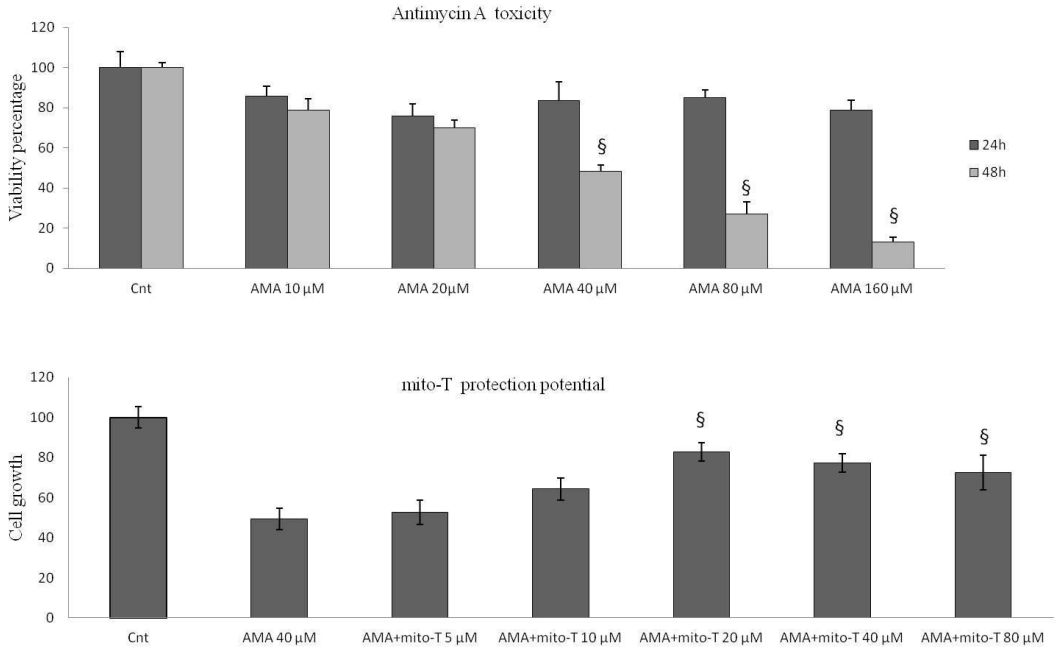

FIGURE 1 *In vitro* antioxidant activity of mito-T against oxidative stress induced by AMA in MSCs. (a) The MTT assay for the cell viability data showed 50% cell viability in the MSCs upon treatment with AMA at a concentration of 40 μM. The different concentrations of AMA for the treatment of cells were 10, 20, 40, 80, and 160 μM. (b) The protective potential of different mito-T concentrations (5, 10, 20, 40, 80 μm) in co-treatment with 40 μM of AMA. About 20 μM mito-T is the optimum concentration for the protective effect. Data show mean values ± SD of at least three independent experiments. § represents statistical significance ($p < 0.05$) compared to control (Cnt). MSCs: mesenchymal stem cells, AMA: antimycin A, mito-T: mito-TEMPO

3.2 | Protective effect of mito-T against DNA damages in MSCs

DAPI staining and comet assay are methods for the assessment of DNA-strand damage. These

assays provide great plausibility for the measurement of DNA damage as a marker of exposure to a genotoxic agent (i.e., AMA) and the evaluation of genoprotective capacities of the compound (i.e., mito-T). As shown in Figure 2, in the comet assay, mito-T showed a protective effect against apoptosis induced by AMA. The ROS induced by AMA showed a marked increase in DNA fragmentation as compared to the untreated control (Figure 2c). Conversely, mito-T protects the cells from the AMA-induced toxicity mediated by ROS, showing decreased DNA damage (Figure 2b). In the DAPI staining analysis (Figure 2d,e,f), nuclear deformation was seen because of the detrimental impacts of AMA on the treated MSCs, while the concurrent use of the mito-T could inhibit chromatin remodeling, the fragmentation of DNA, and the occurrence of apoptosis in the AMA-treated MSCs.

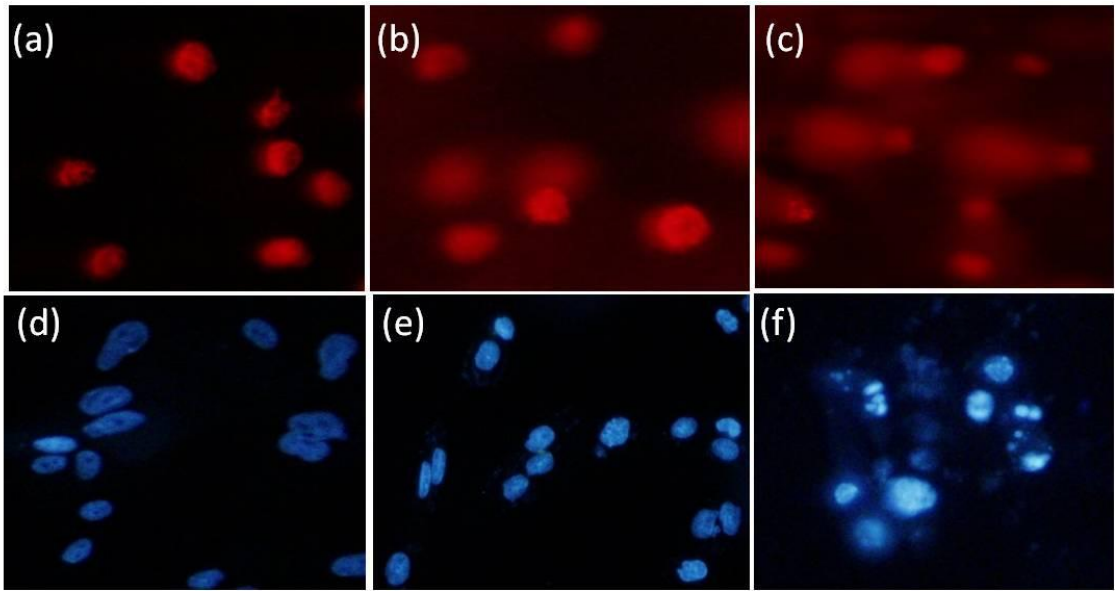

FIGURE 2 Analysis of nucleus integrity. Panels a, b and c respectively show the comet assay of the untreated cells, the cells co-treated with mito-T and AMA, and the cells treated with AMA. Panels d, e and f respectively represent the DAPI staining of the untreated cells, the cells co-treated with AMA and mito-T, and the cells treated with 40 μM of AMA after 48 h. AMA: antimycin A, mito-T: mito-TEMPO [Color figure can be viewed at wileyonlinelibrary.com]

3.3 | Induction of oxidative stress by AMA

ROS generation was assessed at 24 h after AMA treatment. The fluorescent microscopic data (Figure 3a,b,c) showed that MSCs treated with mito-T might alleviate the intensity of ROS as compared to AMA-treated cells. The fluorescent micrograph of stained MSCs revealed the effect

of AMA-induced intracellular ROS generation (Figure 3d) by flow cytometry analysis. The ROS intensity analysis showed that mito-T could significantly reduce the production of ROS as compared to the AMA-treated cells.

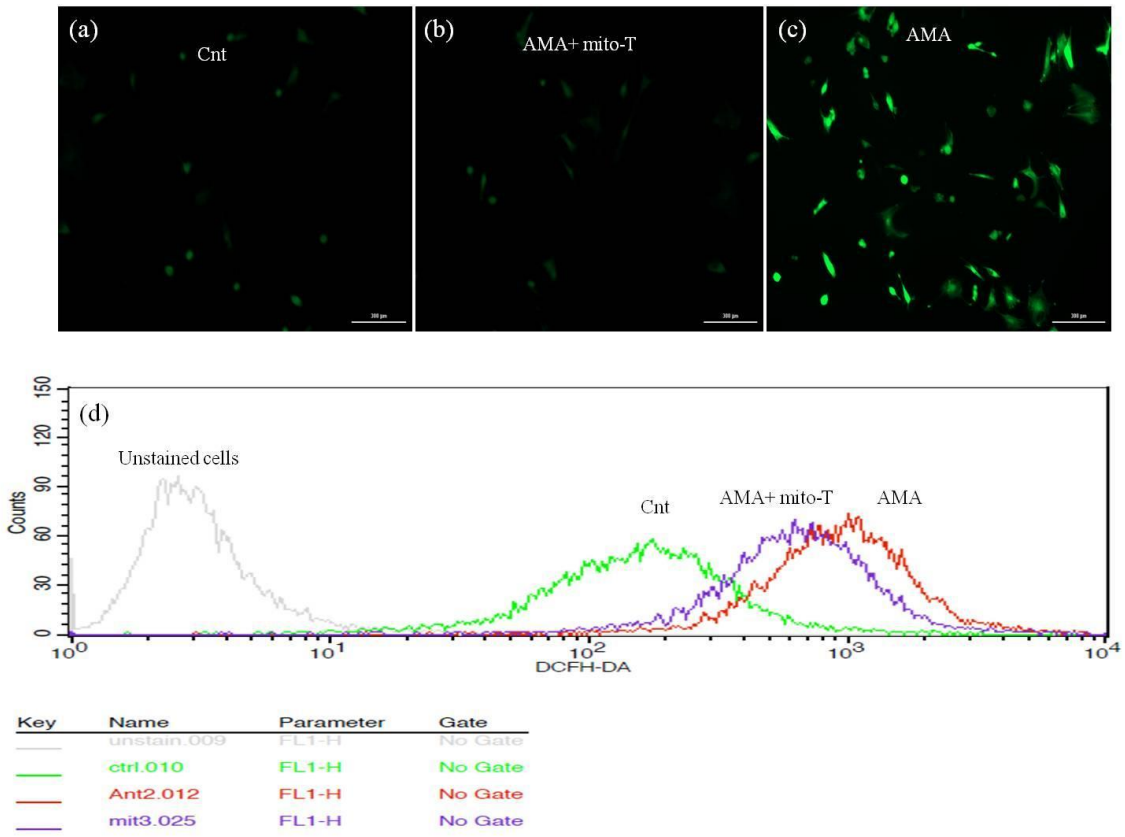

FIGURE 3 Fluorescent microscopy and flow cytometry determination of oxidative stress within the stem cells. The cells were treated with AMA and mito-T, then introduced to 10 μM of DCFH-DA for the analyses. Panels a, b and c represent the fluorescence intensity in the untreated control cells, the cells treated with AMA and mito-T, and the cells treated with AMA, respectively. (d) photomicrographs show intracellular ROS generation in MSCs induced by AMA and stained with DCFH-DA. AMA: antimycin A, mito-T: mito-TEMPO, DCFH-DA: dichloro-dihydro-fluorescein diacetate [Color figure can be viewed at wileyonlinelibrary.com]

3.4 | AMA induces G0/G1 phase arrest

The cell cycle analysis with cellular DNA content was performed by flow cytometry. The apoptotic population of cells was detected using FITC-labeled annexin V apoptosis detection kit. As shown in Figure 4, when cells were exposed to 40 μM of AMA, after 48 h, the treated cells showed 67.64% apoptosis as compared to the control. However, the co-treatment of the cells

with mito-T (20 µM) and AMA (40 µM) resulted in a markedly decrease in the apoptotic cells (approximately 13.71%) (Fig. 4f). Analysis of the cell cycle revealed that in the untreated control cells (Figure 4a), G0/G1 phase arrested cells were observed (2.20%). However, 40 µM of AMA arrested the hypodiploid cells approximately 40% in the G0/G1 phase (Figure 4b), while 20 µM co-treated with mito-T arrested the cells around 19.84% as compared to the control (Figure 4c).

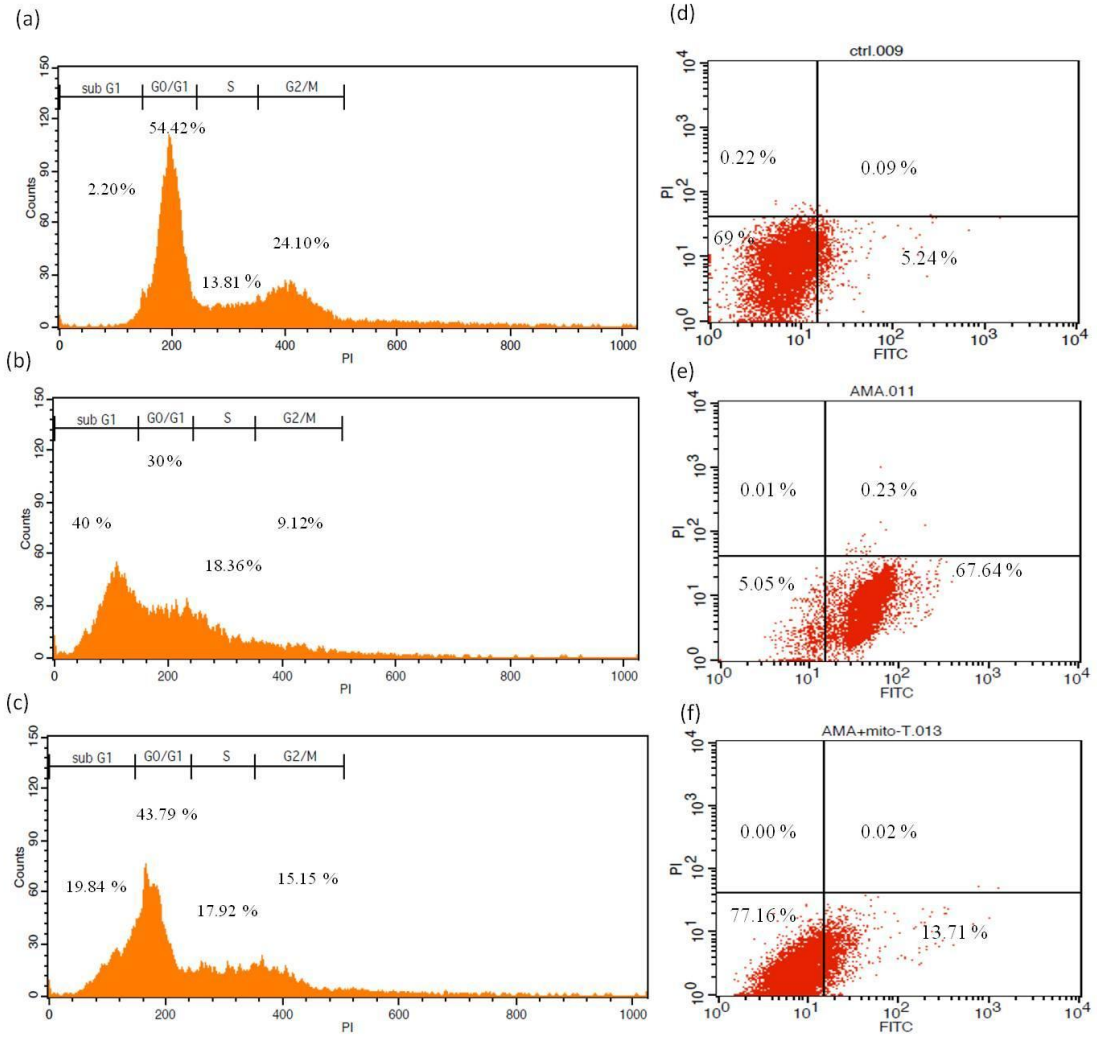

FIGURE 4 The effect of AMA on different phases of the cell cycle. MSCs were treated with IC50 concentration of AMA (40 µM) for 48 h, stained with propidium iodide (PI) and analyzed by flow cytometry. Panels a, b, and c represent the quantitative distribution of MSCs in different phases of the cell cycle in the untreated cells, the cells treated with AMA, and the cells treated with AMA and mito-T, respectively. Panels d, e, and f represent the apoptotic population of the untreated cells, the cells treated with AMA, and the cells treated with AMA and mito-T, respectively. AMA: antimycin A, mito-T: mito-TEMPO [Color figure can be viewed at wileyonlinelibrary.com]

3.5 | AMA induced Nuclear factor-erythroid 2-related factor 2

As shown in Figure 5, an increased level of Nrf2 was seen in AMA-induced oxidative stress as part of the cells' defense response. It was expected that Nrf2 will be downregulated slightly by mito-T co-treatment, which might have a scavenging impact on intracellular ROS induced by AMA, but mito-T had a synergetic effect on Nrf2 expression.

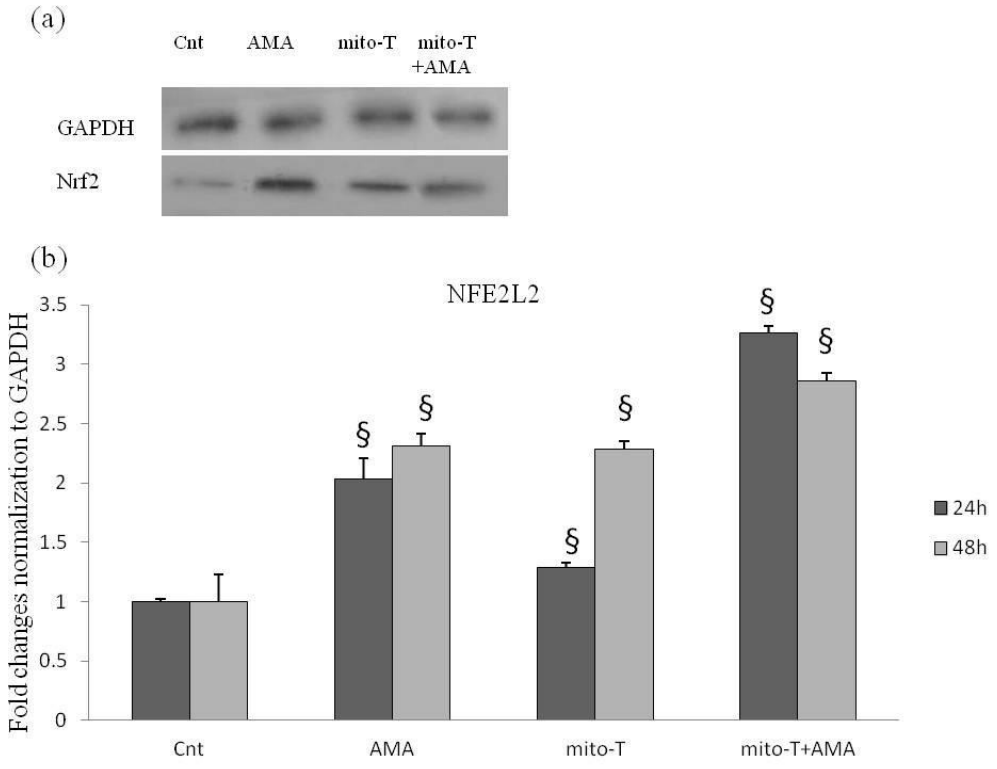

FIGURE 5 The functional expression of Nrf2 in mesenchymal stem cells treated with antimycin A and mito-T. (a) Protein profile of Nrf2 confirming the RNA expression regulation by the treatment. (b) RNA expression in the treated and untreated cells. §: $p<0.05$ compared to untreated control (Cnt). AMA: antimycin A, mito-T: mito-TEMPO.

An increased level of Nrf2 was seen in the AMA-treated cells. It was expected that Nrf2 will be downregulated by mito-T co-treatment, while mito-T intriguingly imposed a synergetic effect on Nrf2 up-regulation.

3.6 | Impacts of mito-T on functional expression of PTEN, Bax, and Casp3

Antimycin A was found to induce the expression of PTEN, Bax, and Casp3 significantly, while

mito-T significantly modulated their expressions (Figure 6, (a) western blotting, (b) RNA expression).

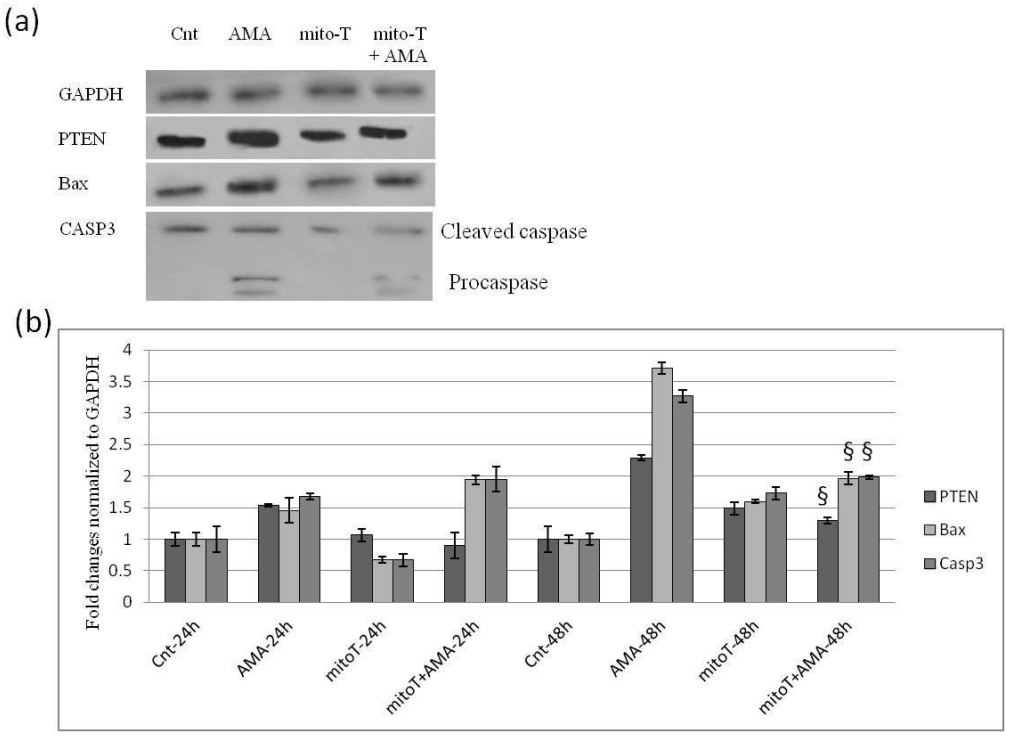

FIGURE 6 The profile of apoptosis gene expression. (a) The protein levels of the pro-apoptosis genes (PTEN, Bax, and Casp3). (b) The expression of mRNAs was analyzed by real-time quantitative RT-PCR and normalized to GAPDH levels. §$P < 0.05$ versus control cells. AMA: antimycin A, mito-T: mito-TEMPO.

4 | DISCUSSION

While free radicals have been reported crucial for normal biology of stem cells, excessive ROS (oxidative stress) adversely influence the stem cell properties and cell therapy outcome (Matsuda et al., 2018). Therefore, it is imperative to control the extent of ROS in order to have a promising regenerative therapy in damaged tissue linked to hypoxia and oxidative stress. In addition, oxidative stress can affect stem cell expansion and longevity (Matsuda et al., 2018). Moreover, it may be possible to control MSC fate in vitro and in vivo by regulating ROS levels surrounding MSCs. Therefore, there is a great need to identify a promising strategy to prevent oxidative stress or pre-invigoration of MSCs (Hu et al., 2018a). One of these strategies is the pre-treatment of stem cells before their transplantation in damaged tissue and hypoxia condition. It could affect

the homing and surviving of transplanted stem cells.

It has been reported that mito-T is a potent mitochondria-targeted antioxidant which could act in ischemic tissues linked with hypoxia-induced oxidative stress (Ding et al., 2017; Du et al., 2017; Du et al., 2019; Li et al., 2018; Liu et al., 2018; Nautiyal et al., 2019; Shetty et al., 2019; Yang et al., 2018; Zhan et al., 2018). In the present study, mito-T, a mitochondria-targeted superoxide dismutase mimetic, dramatically reduced AMA-induced mitochondrial oxidative stress, and thus effectively protected MSCs. In the cellular study, DAPI and comet assay showed that mito-T could neutralize the toxicity of AMA and protect the cells from apoptosis and DNA damages. Flow cytometry study of cell cycle demonstrated that the AMA-treated cells showed 67.64% apoptosis as compared to the control. However, the treatment of the cells with mito-T resulted in a markedly decrease in the apoptotic cells (approximately 13.71%). Analysis of cell cycle revealed that in the AMA-treated cells, G0/G1 phase arrested cells were observed approximately 40%, while co-treatment with mito-T arrested the cells around 19.84% compared to the control.

To study the AMA and mito-T effects on mitochondria redox molecular homeostasis, we looked at the expression of the nuclear factor-erythroid 2-related factor 2 (NFE2L2, the so-called Nrf2), which is a basic leucine zipper (bZIP) protein. This protein can regulate the expression of antioxidant enzymes that protect the cells against oxidative stress triggered by injury (Yamamoto et al., 2018). In the results, it was anticipated that mito-T is obliged to downregulate the NFE2L2 gene expression significantly. Considering the mito-T's scavenging activity, it may neutralize the intracellular ROS and in this case, there is no need to employ any cellular antioxidant mechanisms to combat free radicals. However, according to the results, mito-T exerted a synergetic effect with AMA in up-regulating the Nrf2 gene; as a result of which, the cells fought oxidative stress and survived. In AMA-induced stress, the apoptosis rate was increased through significantly elevated levels of pro-apoptotic PTEN, casp3, and Bax genes. In conformity with these findings, the expression of these genes was decreased under pretreatment with mito-T. These findings suggest a possible role for mito-T in encouraging cell survival via activating Nrf2 and prohibiting mitochondria-mediated apoptosis in MSCs.

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4 Chapter

Nesting and Fate of Transplanted Stem Cells in Hypoxic/Ischemic Injury and the Role of HIF1α/Sirtuins interaction and Other Macromolecular Players

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This section describes the important proteins in the cells, involve in hypoxia adaptation and oxidative stress resistancy. We reviewd the miane protein intractions, have been previously reported in different studies and registred in data banks. Then we discussed about the crosstalk between some molecular players specially HIF1α and sirtuins. In the end we predicted in two conditions, normoxia and hypoxia protein intractions for programming the stem cells fate.

Abstract

The nesting and fate mechanisms of stem cells in the dysfunction niche are just critical question that must be responded to designing a success cell therapy. The fracture site is able to induce senescence or apoptosis based on the surrounding harsh conditions, hypoxia, respiration deficiency (RD), and oxidative stress (OS). RD and disruption in energy metabolism, consequently induction of OS, change the native niche cellular and molecular players. Reactive oxygen species (ROS) has been commonly thought of as toxic to cells and can lead to apoptosis and senescence, moreover, the evidence are that, physiological hypoxia is vital in homing, self renewal, and differentiation of stem cells in niche. For the managing the cell therapy outcome, the key macromolecular players involved in the supporting of surviving and re-adaptation of stem cells in new dysfunction niche must be understood. Hypoxia-inducible factor 1-alpha (Hif1-α) is the master transcriptional regulator of cells response to hypoxia and adaptation of stem cells in new niche. Moreover this protein regulate by interaction with Sirtuins. The Sirtuins are highly conserved NAD+-dependent enzymes that monitor cellular energy status and modulate gene transcription, genome stability, and energy metabolism in response to environmental signals to modulate the homing and fate of stem cells.

here a critical discussion on new insights into the nesting of stem cells in hypoxic ischemic injury, reprogramming of stem cells in new dysfunction niche with the complex macromolecular players and Sirtuins/HIF1 α interactions, to determine the fate will be discussed .

Keywords: Cell signaling, Differentiation, Post translational modification, Niche

1. Introduction

Fast progress in stem cell research has hold great promise for cell therapy and regenerative medicine[1]; yet it is may limited due to the poor survival of stem cells in vivo are linked to anoikis and the loss of extra cellular matrix (ECM), potential immune rejection, upper phisiological hypoxia mediating apoptosis and energy metabolism deficit[2]. Hypoxia preconditioning, antioxidants pretreatments, bio-scafolds designing and hydrogel encapsulation have been revealed as promising strategies to reduce cell apoptosis in vivo while maintaining biological functions of the cells[3]. Moreover, an improved understanding of the relationship between stem cells and their niches will facilitate the engineering of niches in vitro, as well as in vivo manipulation of the dysfunction niches to improve stem-cell-based therapies[4]. If the SC niche is the necessary microenvironment controlling SC fate, a cell therapy trial that does not take into account such component would not be an optimal therapy. The stem cell niche should be active in the restoring area. In such conditions, SC activity would better mimic self-repair occurring in healthy tissues[4]. The interplay between cellular and molecular players in the native niche, invitro system and dysfunction niche determine the behavior and bio-function of stem cells. The process of stem cell therapy is including the isolation from native niches, expansion invitro and then injection in a new and dysfunction niche. The fracture site is able

to induce senescence or apoptosis based on the surrounding strict hypoxia conditions, OS and disrupted energy metabolism [5]. However, stem cells could adapt themselves to the strict condition, hypoxia and physiologically up-regulated ROS in preconditioning and induction of molecular pathways[5].

The preconditioning of cardiac progenitor cells (CPCs) under hypoxia (2%–5% O2) in vitro was found to enhance cell survival in a mouse model of myocardial ischemia–reperfusion injury.[6] MSCs conditioned under hypoxia promoted angiogenesis and neurogenesis in rat ischemic brain models that mimicked stroke.[7] Also, MSCs exposed to hypoxia in vitro, showed the enhanced survival through the up-regulation of Bcl-2 and Bcl-xL, leading to the reduced infarct size and the enhanced heart functions[8]. Major progress has recently been made towards unraveling the complexity of the molecular mechanisms involved in oxygen-mediated signaling. Yet, the major hypoxia-responsive molecular pathway resides in activation of the hypoxia-inducible factor (HIF) transcription complex and Sirtuins. Sirtuins are involved in a broad range of biological functions that includes the regulation of chromatin structure and gene expression, metabolic homeostasis, apoptosis, senescence, DNA repair, and cell differentiation[9]. In addition, sirtuins are sensitive to environmental stimuli and thus act as stress sensors that alarm to the cells to organize the stress response [10]. Because sirtuins act as sensors of environmental stimuli and coordinate the stress response of cells, it is not surprising that deregulation of these macromolecules are associated with hypoxia and energy deficit in damaged tissues [11]. This review was provided a critical discussion on new insights into the nesting of stem cells in hypoxic ischemic injury, and the recent advances in our understanding and an updated integrated view on the cellular and molecular mechanisms by which Sirtuinsh-Hif1α-mediated in the maintenance of surviving and differentiation of stem cells in energy deficit condition with high range of OS.

2. Hypoxia and critical component of the stem cell niche

As mentioned above, an improved understanding of the relationship between stem cells and their niches will facilitate the improvement of stem-cell-based therapies. A ‘stem cell niche’ refers to the local microenvironment within a tissue in which stem cells are maintained in an undifferentiated and self- renewable state and receive stimuli that determine their fate[12]. The niche is a complex and dynamic structure that transmits and receives signals through cellular and acellular mediators[13]. For example, in bone marrow (BM) niches, hemaopoitic stem cells (HSCs) are retained, remain quiescent, respond to external cues, and, if necessary, undergo symmetric or asymmetric cell divisions. Generally, two forms of stem cell niches are evident in the BM microenvironment: the osteoblastic niche that located close to the trabecular bone and the vascular niche, which is associated with the endothelium lining BM sinusoids. It has been postulated that, while osteoblastic niches are populated by HSCs that are more quiescent, endothelial niches contain stem cells that are more advanced in the cell cycle[14].

The natural niche is a highly complicated network, which is composed of proteins, proteoglycans, soluble

signals (growth factors, cytokines, chemokines), physical signals (fibronectin, vitronectin, laminin, collagen, fibrillin) and signals arising from cell/cell interactions . Many of this conserved component may lost in the injury tissues that called dysfunction niche[15].

Oxygen is a one of the critical component of niche[15]. Low oxygen tensions (hypoxia) maintain undifferentiated states of embryonic, hematopoietic, mesenchymal, and neural stem cell phenotypes and also influence proliferation and cell-fate commitment[16]. It has been hypothesized that the presence of low oxygen tensions in stem cell niches offers a selective advantage that is well suited to their particular biological roles[17]. In addition, hypoxia has been shown to activate molecular pathways in multiple stem cell systems that appear to regulate Oct4 and Notch signaling, two important regulators of stemness[18]. Finally, oxygen tensions as low as 1% appear to decrease proliferation and maintain SC pluripotency, while higher oxygen tensions (3%–5%) appear to maintain pluripotency with no effect on proliferation [19]. These results suggest that proliferation, stem cell quiescence, and perhaps even differentiation may be regulated by gradients of oxygen tension supplied by their local niche[20]. The stem cells in local niche sense oxygen and adapt to the tension of oxygen for homeostasis of functions[21]. Oxygen concentration during standard invitro culture of primary human cells is often not adapted to the in vivo situation. Moreover, the physiologic oxygen tension during in vitro culture of human MSC slows down cell cycle progression and differentiation[22]. After transferring MSC from atmospheric oxygen levels of 21% to 1%, HIF-1α expression was induced, indicating efficient oxygen reduction[23]. The main protein involve in the hypoxia condition is HIF1α. The transcriptional reply of a cell to hypoxia is affected by two major mechanism; first, the composition of hypoxia-response elements (HREs), which primarily define how much of the signal of HIF1α is integrated into the transcriptional output of uniqu genes. Second, is the availability of sirtuins cooperating with HIF in the context of HRE[24].

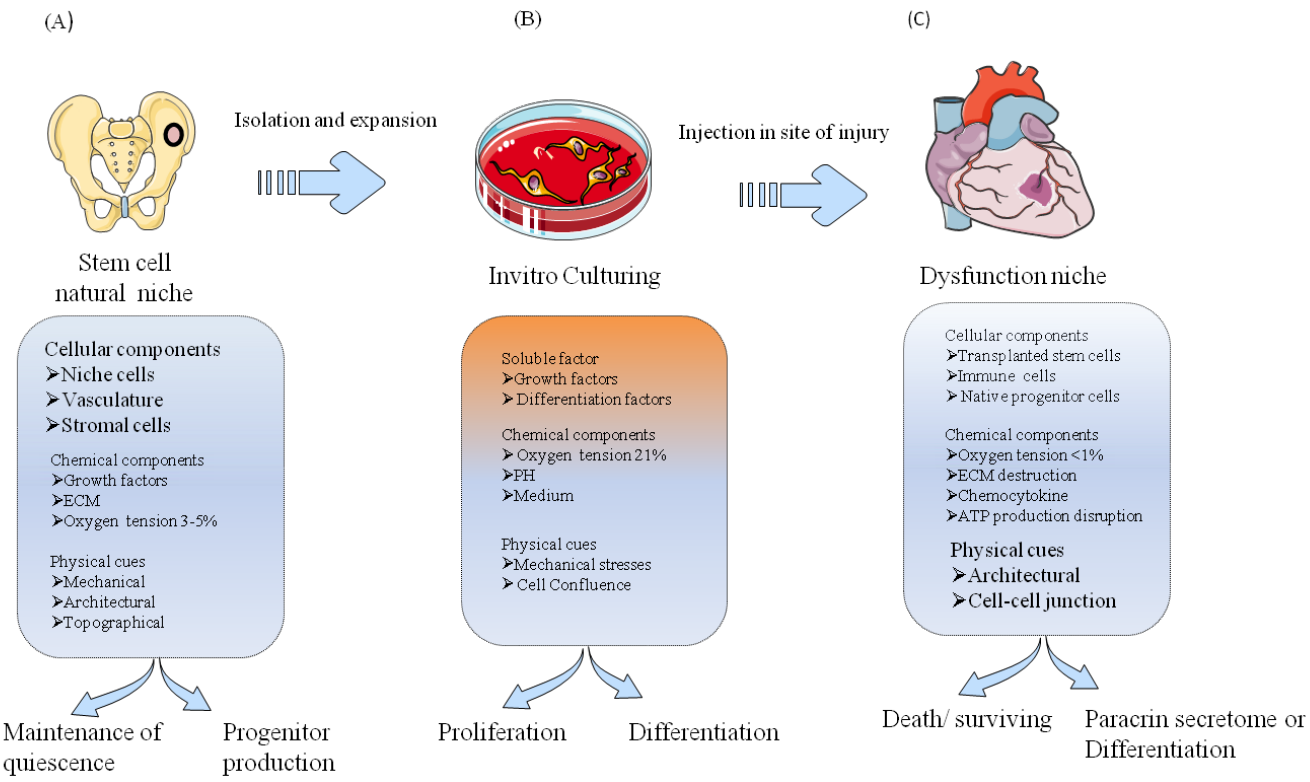

Figure 1 | This schematic depicts a native niche, and injured tissues as dysfunction niche composite, which summarizes known components of previously described, including the stem cell itself, soluble factors, extracellular matrix, vascular network, cell adhesion components in physiological/hypoxia condition. The function of stem cells among the natural niche, invitro culturing and injury tissues were compared (A); the natural niche is a complex and dynamic structure that transmits and receives signals through cellular and acellular mediators. The stem cells habitat in natural niche expose in oxygen with physiological tension. (B); when the stem cells were isolated and cultured invtro, the natural component of niche will be change and will effect to the stem cell proliferation and will inform memory. (C); in the injury site of tissues the hypoxia is strict than physiological hypoxia so, the adaptation of stem cells in this harsh condition need to player of several proteins especially HIF1α and Sirtuins.

3. Stem cells and transplantation in dysfunction niche; no place like home

Deficient niche function in the injury tissues may cause the loss or deregulation of native or transplanted stem cells. Injured tissues, wounds and ischemia involve a series of interactions between cells, chemical signals such as cytokines and growth factors, the extracellular matrix and the vasculature[25]. The cells are maintained by signaling in concert with niche cells – signals include paracrine (e.g. sonic hedgehog), autocrine and juxtacrine signals.

So, the niche component in this damaged site will change. The stem cell itself, stromal cells, soluble factors, extracellular matrix, neural inputs, vascular network and cell adhesion components all will disrupt and change[26]. In this way, the molecular cell signaling involving in the inducing the fate will be affected or

will be go to un-desirable fate.

In the cell therapy, the transplantation of stem cells should be done in dysfunction niche for example heart ischemia injury in which place, the normal function of cardiovascular progenitor cells will lost. The vasculature normally delivers oxygen and nutrients to tissue but, following tissue injury, perfusion is compromised and acute tissue hypoxia is the result[27]. In the hypoxic condition, the cells activate a number of physiological responses, which directly control cell metabolism, cell redox homeostasis, and vascular remodeling. Many examples demonstrated that the cellular response is substantially different at atmospheric O2 concentration when compared to lower O2 concentrations which better approximate the physiologic situation[15].

After transplantation of SCs in tissue injuries, the death of stem cells within the first few days may be done. The main reason is due to the anoikis driven by the loss of cell adhesion, inflammation, respiration chain deficiency linked to the oxidative stress (OS) and disruption of energy metabolism[3]. In this way the hif1α and sirtuins have vital roles in the adaptation of stem cells in the harsh condition. So, the manipulation of stem cells and preconditioning of stem cells to take a memory and induced the hypoxia homeostasis memory is necessary to support the stem cells in hypoxia injury tissues[28].

4. Preconditioning of stem cells to hypoxia for pre-adaptation

There are a number of strategies that have been used to improve cell nesting and engraftment. By far the most studied preconditioning strategy to modify stem cells in culture to develop their nesting capacity is relative hypoxia[29]. The oxygen tension in vitro culture of cells is typically 21%, but MSCs reside in bone marrow niches with oxygen tension is about 5%, and as low as 1% in ischemic tissue[30]. Reducing the oxygen strain to 1% in culture system causes the apoptosis of a number of cells, but it is the successful way to reproduce their native environment in the target tissue where they may be placed, and define the most viable cells collected[30].

Molecular signaling and subsequently the metabolic switching during hypoxia is essential in decreasing the accumulation of oxidative metabolism-derived reactive oxygen species (ROS) and the proliferation and programming to the niche adaptation[31]. Recent studies indicate that differences in metabolic profiles of pluripotent stem cells are closely related to self-renewal and initial cell fate decision of stem cells. Gu and colleagues reported that naive pluripotent stem cells in humans exhibit a high glycolytic flux and pentose phosphate pathway activity[32]. Moreover, metabolic switching induced by hypoxia prevents the excessive generation of ROS and reduces the demand for ATP, both closely associated with maintaining the physiological functions and survival of stem cells. Despite the metabolic switching, chronic hypoxia-induced ROS accumulation leads to mitochondrial apoptosis in embryonic and mesenchymal stem cells[33] .

So, hypoxia preconditioning could induce several macromolecular pleyers to improve the cell therapy output. According to the evidence, hypoxia-preconditioned MSCs exhibit higher transplantation survival rates

and therapeutic potential than normoxia-preconditioned MSCs [34]. Other study showed that hypoxia-preconditioned MSCs exhibit higher concentrations of fatty acids and synthase-mediated lipogenesis, which stimulates cell migration, proliferation, and survival [35]. Additionally, hypoxia induces the hexosamine biosynthesis pathway, thereby increasing O-linked-N-acetyl-glucosaminylation concentrations, which is critical for anti-apoptosis in mouse embryonic stem cells (mESCs) exposed to hypoxia [36].

The protein hypoxia inducing factor alpha (HIF-1a) is a member of the HIF family of transcription factors that are upregulated in low oxygen conditions in the cellular environment [30]. HIF-1a is regarded as the master regulator of hypoxia[37]. Hypoxia also induces the upregulation of a host of other factors in addition to chemokines, including growth factors, and factors involved in cardiac differentiation, with significant increases observed by as little as four hours of hypoxia exposure, and peaks at 8–10 hours [38]. Hypoxia has also been shown to boost the migration of MSCs compared to MSCs grown in normoxic conditions of 21%. Hypoxia preconditioning has a number of beneficial effects in addition to significant upregulation of CXCR4 and CXCR7 in MSCs by activating HIF1α resulting increase in migration, adhesion, engraftment[39-40]. The use of hypoxia preconditioning of stem cells is now into use in clinical trials of several types of cells. The main molecular pathways in adaptation of stem cells in hypoxia condition in injury tissues briefly investigate below.

5. Dysfunction niches and the HIF1α/Sirtuins plays in nesting

The secreted factors and elaborated components of stem cell niche can function to direct stem cell fate decisions. However, the precise signaling pathways may be different for each stem cell type and within each stem cell niche specially the dysfunction niche in injured tissues.

For example, after transplantation of MSCs and recruitment by site of injury, these cells respond to the dysfunction niche in the first of all by expressing angiogenic factors (eg.VEGF)[41]. Then, the putative mechanisms by which transplanted MSCs exert regenerative effects in injured tissues include differentiation, cell fusion, and paracrine effects, such as immunosuppressive and antiapoptotic effects and the stimulation of local progenitor stem cells. But if stem cells couldnot nest and adapted to the harsh condition of injury site the stem cells will death caused by Fas and Fas ligand (FasL) interactions under oxidative condition[42]. In the model of ischemia–reperfusion injury , the interaction between FasL in ischemic heart and Fas in implanted MSCs lead to the apoptosis of transplanted MSCs, whereas the inhibition of this interaction by Fas/Fc treatment improved cell survival and restored heart function[42]. As the above mentioned, preconditioning of stem celle with hypoxia could effect to the surviving with inducing molecular cascade in hypoxia. The main macromolecular is HIF1α, as a master of hypoxia transcription factor, that interact with other proteins and save the stem cells in this new and dysfunction niche. During hypoxia, the intracellular metabolic state is substantially changed, and a metabolic crisis unfolds when oxygen tension drops low enough to deplete ATP. To overcome the metabolic imbalance, cells must induce and activate

HIF-1, because HIF-1 enhances glycolysis by upregulating glucose transporter through lactate dehydrogenase (LDH).

Concurrently, HIF-1 blocks mitochondrial energy metabolism by inducing PDK-1, which inhibits the conversion of pyruvate to acetyl-CoA, and by inhibiting mitochondrial biogenesis via c-Myc repression [43]. HIF-1 also protects hypoxic cells from oxidative damage by replacing cytochrome c oxidase 4-1 with 4-2, which reduces reactive oxygen species generation by efficiently removing electrons [44]. Hypoxic adaptation involves switching energy metabolism from oxidative phosphorylation to anaerobic glycolysis [45], which is mediated by HIF-1 (Figure 2). To date, 80 or more genes have been found to be regulated by HIF-1, which is comprised of HIF-1α and ARNT (also called HIF-1β)[46].

In addition, Metabolic switching during hypoxia is essential in decreasing the accumulation of oxidative metabolism-derived reactive oxygen species (ROS) and the availability of oxygen[47].

Silent information regulators (sirtuins) mediates gene silencing have been identified in mammals, and all catalyze protein deacetylation or adenosine diphosphate (ADP) ribosylation[46]. These enzymes are involved in a broad range of biological functions that includes the regulation of chromatin structure and gene expression, metabolic homeostasis, apoptosis, senescence, DNA repair, and cell differentiation. In addition, sirtuins are sensitive to environmental stimuli and thus act as stress sensors that help to organize the stress response in the cell[10]. Because sirtuins act as sensors of environmental stimuli and coordinate the stress response of cells, it is not surprising that deregulation of these proteins is associated with hypoxia and energy stress in injured tissues [11]. So, in the next section we are going to investigate the crosstalk between sirtuins and hif1α in the adaptation of stem cells in new niche to programming to the targeted tissue repair.

6. Molecular mechanisms to adaptation in hypoxia; HIF1α master regulator of hypoxia homeostasis

Increasing research has suggested that HIF1α expression is elevated during hypoxia[48]. Cellular HIF1α is in a dynamic equilibrium of expression and degradation: when the oxygen concentration increases, HIF1α is hydroxylated and interacts with von Hippel-Lindau tumor suppressor (VHL), which leads to the ubiquitination and degradation. The hydroxylation is suppressed by hypoxia, elevating HIF1α protein level [49] (Figure 2).

HIF-1α is a helix-loop-helix transcription factor of the Per-Arnt-Sim family and is, in its active form, composed of a dimer consisting of an alpha and beta subunit. Genes carrying hypoxia response element (HRE) sequences in their promoter or enhancer are multifactorial and include genes responsible for anaerobic metabolism, angiogenesis and inflammation, and for extracellular matrix deposition and cross-linking[50].

The studies have demonstrated that HIF-1α inducing by hypoxia improves several MSC functions,

including cell adhesion, migration, and proliferation, thereby increasing their therapeutic potential[51]. In addition, the signaling pathways related to several paracrine factors, and proteins interplay in surviving, immune suppression and oxidative stress resistance proteins are upregulated in MSCs with high expressed HIF1α.

Besides, the levels of p38 mitogen-activated protein kinase (p38/MAPK) [52], JNK/SAP [53], AKT[53] , and Notch [32,33], together with fibronectin [54], fibroblast growth factor [55], angiopoietin-1 (ANGPT1) [56], NGF [57], and insulin growth factor [58]signaling pathways, are all induced by HIF-1α. The main HIF1α downstream signaling briefly explained below.

6. 1. Hif1α regulate the angiogenesis

The HIF-1 pathway was found to be a master regulator of angiogenesis. Whether the process is physiological or pathological, HIF-1 seems to participate in vasculature formation by synergistic correlations with other pro-angiogenic factors such as VEGF (vascular endothelial growth factor), or angiopoietins[59]. Angiogenesis, the formation of new vessels from pre-existing vasculature, is an active growth-factor-dependent and hypoxia-induced event that takes place in several organs during the growth and repair of injured tissues. Mesenchymal stem cells (MSCs) have been shown to promote angiogenesis via a mechanism that is potentiated by HIF1α AT hypoxic condition[60]. Over expression of HIF1α in MSCs improves their therapeutic potential by inducing angiogenesis in transplanted tissues. Several studies have shown that HIF1α in crosstalk with hypoxia-activated Akt and mTOR signaling enhance the vascular cell proliferation and angiogenesis[61].

6.2. HIF-1α/GRP78/Akt axis inhibit hypoxia induced apoptosis

It demonstrated that hypoxic preconditioning prevents cell death of transplanted MSCs in a mouse ischemia model[62].Under hypoxia (2% O2), the expression of GRP78 was significantly increased via hypoxia-inducible factor (HIF)-1α. Hypoxia-induced GRP78 promoted the proliferation and migration potential of MSCs through the HIF-1α-GRP78-Akt signal axis[62]. GRP78 plays a key role in cell survival and apoptosis through interaction with protein kinase R-like endoplasmic reticulum kinase (PERK)[63].

6.3. Potential role of the GTPase RhoA and HIF-1α on MSC migration

The studies demonstrate that hypoxia, decreases MSC migration through HIF-1α and RhoA-mediated pathway. The active GTP-bound form of RhoA was reduced in 1% oxygen, whereas activation of RhoA under hypoxic conditions rescued migration[64]. Furthermore, stabilization of HIF-1α under normoxic microenvirnment attenuated cell migration similar to that of hypoxia. These evidences suggest that hypoxia negatively affects MSC migration by regulating activation of GTPases. These results highlight the importance of oxygen in regulating the recruitment of progenitor cells to areas of ischemic tissue damage[64].

6.4. Hypoxia induces a hedgehog response mediated by HIF-1α

Using *in vitro* models of hypoxia, the study reported that the hedgehog response was transient and preceded

by the accumulation of HIF-1α, which is a communicate between hypoxia and hedgehog expression. Indeed, pharmacological inhibition, knockdown or genetic ablation of HIF-1α abolished hedgehog pathway activation. So, hypoxia is translated into a hedgehog response through HIF-1α and this mechanism is likely to be responsible for the hedgehog response observed in various ischaemia models[65].

6.5. The cross talk between HIF1α/ and Notch signaling

Further explore the mechanisms induced by HIF-1α in MSCs, is the Notch signaling that overexpression of HIF-1α in MSCs increased protein levels of the Notch ligands Jagged 1–2 and Delta-like (Dll)1, Dll3, and Dll4 and potentiated Notch signaling only when this pathway was activated[51].

Crosstalk between HIF1α and Notch was resulted in migration and spreading of MSCs, which was abolished by γ-secretase inhibition. However, the HIF-1-induced MSC proliferation was independent of Notch signaling. The ubiquitin family member, small ubiquitin-like modifier (SUMO), has important functions in many cellular processes and increased SUMO1 protein levels have been reported in hypoxia[51]. To investigate the potential involvement of SUMOylation in HIF/Notch crosstalk, the increased SUMOylation in HIF-1-expressing MSCs was reported. Moreover, proliferation and migration of MSCs were reduced in the presence of a SUMOylation inhibitor, and this effect was particularly robust in over expressed HIF1-MSCs[51].

6.6. Hif1 α interaction with Akt/mTOR

The previous studies have shown the regulatory role of the target of rapamycin (TOR) pathway in regulating HIF1 in mammals. Many studies have shown that activating the Akt/mammalian target of rapamycin (mTOR) pathway increases the cap-dependent mRNA translation of HIF1α [66]. In addition to the capacity of mTOR to translationally upregulate HIF1α, mTOR has been reported to increase the stabilization of HIF1α and gene transcription activity of HIF1. However, the effect of hypoxia on mTOR has been debated. It is well documented that mTOR activity is reduced under hypoxic conditions by the tuberous sclerosis protein 1 and 2 (TSC1/2) complex and DNA-damage-inducible transcript 4 (DDIT4), a protein that regulates development and DNA damage response 1 is inactivated under hypoxic conditions[35,67] . In contrast, several studies have shown that hypoxia-activated Akt induces mTOR signaling by enhancing vascular cell proliferation and angiogenesis [68]. The activation of the Akt/mTORC1 pathway by HIF1α enhances the proliferation, migration, and survival of MSCs under hypoxia [35].

6.7. Hif1α regulating stemness via regulating of intracellular calcium levels

The upregulation of intracellular calcium levels is a response observed in many cell types exposed to hypoxia [69]. Recently, hypoxia-mediated calcium upregulation has been closely linked to the stimulation of HIF1α [70]. the study has reported that the release of hypoxia-induced intracellular calcium increases the expression of HIF1α, which is further enhanced by pretreatment with ionomycin, an ionophore [71]. Recently, Kim et al. showed that MSCs primed with both hypoxia and calcium enhanced stemness and the

capacity for immunomodulatory activity, thereby attenuating graft-versus-host disease [72]. This finding suggests that applying calcium to stem cells could be a promising strategy to enhance the efficacy of hypoxia-preconditioned stem cell transplants. Recent gene enrichment analysis data reveal that calcium-regulated calcineurin (CaN)/ Nuclear factor of activated T-cells (NFATc4) signaling is a potential pathway regulating stemness in neural stem cells grown under hypoxic conditions [73].

7. Sirtuin modulate redox stressors and regulate HIF1α

The depletion of energy during ischemia or hypoxic injury tissues and the induction of oxidative stress initiate a cascade of pathways that lead to cell death. Recently, the sirtuin family has gained increasing attention from researchers, due to their involvement in the modulation of a wide variety of cellular functions[74]. There are seven mammalian sirtuins and, among them, the nuclear/cytoplasmic sirtuin 1 (SIRT1) was reported to fine-tune cellular responses to hypoxia by deacetylating HIF-1α and HIF-2α[75]. Immunological analyses revealed that HIF-1α and HIF-2α are acetylated by PCAF and CBP, respectively, but are deacetylated commonly by Sirt1[75].

Substrates of the SIRTs include not only histones, but also diverse other proteins such as p53, NF-κB (in the case of SIRT1), and α-tubulin (in the case of SIRT2) [76]. Functions of the SIRTs seem substrate-specific and localization-dependent, involving regulation of genome stability, stress resistance, cell growth, proliferation, differentiation, and/or metabolic adaptation[77]. At the subcellular level, SIRT1 and SIRT6 are mainly found in cell nucleus, SIRT2 in the cytoplasm, SIRT3, 4 and 5 in the mitochondria and SIRT7 in the nucleolus [78]. Although the SIRTs are generally known as nicotinamide adenine dinucleotide (NAD+)-dependent protein deacetylases, SIRT5 has in addition NAD+-dependent demalonylase and desuccinylase activities[77,79-80]. while SIRT4 and SIRT6 may also have ADP-ribosyltransferase activity [81]. A previous study also reported localization of SIRT5 in cell nucleus and opposite effects of SIRT5 on viability of different cell types[82]. Among the seven sirtuins, SIRT1, SIRT2, and SIRT3 have been extensively studied[79]. Our understanding of the other sirtuins remains much unclear, especially about their functions in the nervous system and implications in neurological/psychiatric disorders.

7.1 Nucleus Sirtuins (SIRT-1 SIRT6 & 7) directly deacetylate HIF-1α

Given that SIRT1 play critical roles in cellular response to metabolic stress and that SIRT1 regulates transcription factors, it reported that SIRT1 modulates cellular adaptation to hypoxia by targeting HIF-1α. The studies demonstrate that SIRT1 directly deacetylates HIF-1α, and thus, inactivates HIF-1α[46]. Furthermore, it shows that along with prolonged hypoxia, HIF-1α acetylation and activity are augmented due to SIRT1 suppression following NAD+ diminution. In addition, the interplay between SIRT1 and HIF-1α was also observed in mouse tissues and in xenografts. These results imply that crosstalk exists between hypoxia- and metabolism-sensing pathways, and that this ensures cellular adaptation to hypoxia.

Sirtuin-7 regulates the level of protein HIF-1α in negatively by a mechanism that does not involve proteasomal or lysosomal degradation, that is independent of prolyl hydroxylation . The effect of Sirt7 was maintained in the presence of the nicotinamide as sirtuin inhibitor and upon mutation of its deacetylase domain, indicating a non-catalytic function. So, the Knockdown of Sirt7 gene led to an boosting of HIF-1α and HIF-2α protein levels and transcriptional activity. Thus, Sirt7 is a negative regulator of HIF signaling [28].

SIRT-6 appears to have vital role in regulating metabolism, and DNA repair. In this context, novel research indicate that SIRT6 is a key regulator of glucose homeostasis. Under normoxia conditions, SIRT6 inhibits expression of glycolytic genes, functioning as a histone deacetylase to co-repress Hif1α. This maintains proper flux of glucose to the TCA cycle. Under hypoxia, SIRT6 is inactivated, allowing activation of Hif1α, recruitment of p300, acetylation of H3K9 at the promoters, and increased expression of glycolytic genes, causing increased glycolysis and reduced mitochondrial respiration[83]. SIRT6 acts as a histone deacetylase to directly repress multiple glycolytic genes SIRT6 also functions as a corepressor of the transcription factor Hif1α Loss of SIRT6 increases glycolysis and diminishes mitochondrial respiration[84].

7.2 The role of mitochondrial Sirtuins in energy metabolism regulation

Recent findings reveal that, a mitochondrial sirtuins function in the control of basic mitochondrial biology including, energy production, metabolism, apoptosis and intracellular signaling[85]. An elegant coordination of metabolism by mitochondrial sirtuins is emerging in which SIRT3, SIRT4 and SIRT5 serve at crucial junctions in mitochondrial metabolism by acting as switches to facilitate energy production during energy adaptation and stress[85]. Till now, rather than satisfy, the results of the study's lead to more questions. How important are changes in mitochondrial acetylation to mitochondrial biology and is acetylation status a read out for sirtuin activity?

Despite the central role for mitochondria in the control of apoptosis, surprisingly little is known about how mitochondrial sirtuins participate in apoptotic programs. SIRT3 plays a pro-apoptotic role in both Bcl2-p53- and JNK-regulated apoptosis[86]. Additionally, cells lacking SIRT3 show decreased stress-induced apoptosis, lending further support for a pro-apoptotic role for SIRT3. SIRT3 is the only sirtuin with a reported association with the human life span. Peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) plays important roles in adaptive thermogenesis, gluconeogenesis, mitochondrial biogenesis and respiration[87]. The results indicate that Sirt3 functions as a downstream target gene of PGC-1α and mediates the PGC-1α effects on cellular ROS production and mitochondrial biogenesis[87]. Thus, SIRT3 integrates the energy metabolism of cells and the generation of ROS . The protein PGC-1α is a main transcriptional coactivator regulating in the expression of the genes involved in energy metabolism. It is the master regulator in biogenesis of mitochondria. This protein interacts with PPAR-γ, which permits the interaction of this protein with multiple transcription factors in nucleues. This protein can regulate the

activities of cAMP response element-binding protein (CREB) and the antioxidant's gene transcription factor, nuclear respiratory factors (NRFs).

7.3. Cytoplasm sirtuin (SIRT2) deacetylate FOXO transcription factors

SIRT2 is a cytoplasmic sirtuin that plays a role in various cellular processes. SIRT2 can regulate caloric restriction-dependent lifespan extension through decreasing the expression level of H4K16Ac during G2/M transition, which indicates that SIRT2 contributes to the alteration of acetylation of histone proteins in cell cycle[88]. The tubulin deacetylase sirtuin-2 regulates neuronal differentiation through the ERK/CREB signaling pathway.[89]. In the other study, it was demonstrated that SIRT2 binds to FOXO3a and reduces its acetylation level. SIRT2 hence increases FOXO DNA binding and elevates the expression of FOXO target genes, p27Kip1, manganese superoxide dismutase (SOD) and Bim[90]. As a consequence, SIRT2 decreases cellular levels of reactive oxygen species. Furthermore, as Bim is a pro-apoptotic factor, SIRT2 promotes cell death when cells are under severe stress. Therefore, mammalian SIRT2 responds to caloric restriction and oxidative stress to deacetylate FOXO transcription factors[90].

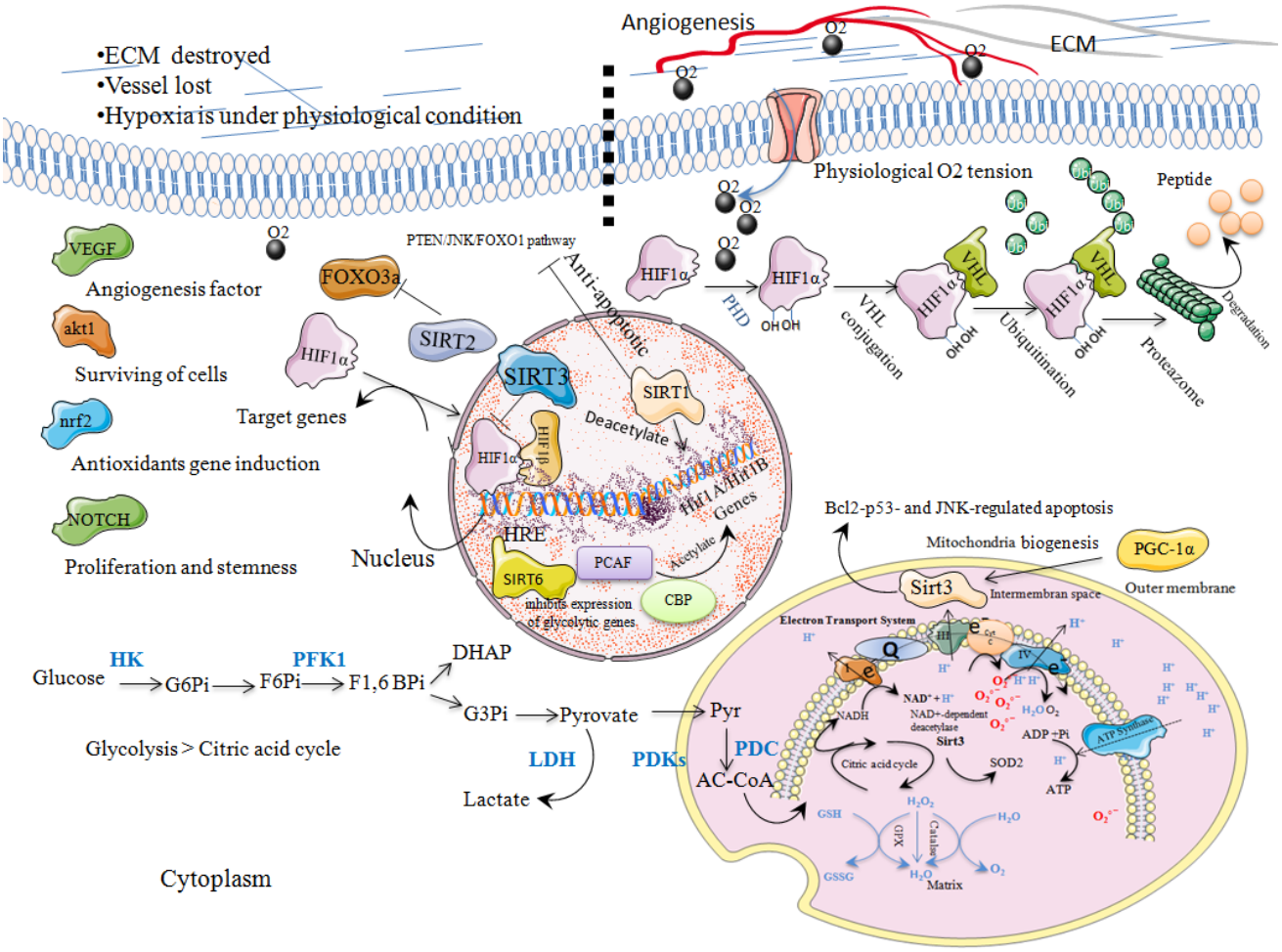

Figure 2 | HIF1/sirtuins mediated adaptation to hypoxia is required to maintain the pluripotency and survival of stem cells under hypoxic conditions and then differentiation. HIF1 activity is controlled by the alpha subunit of HIF1α. The Understanding of the mechanism of HIF1 activity control in the stem cells, will help us to insights into stem cell biology under hypoxia in novel vewe. In the hypoxic condition, the cellular metabolism is substantially altered, and a metabolic crisis unfolds when oxygen pressure drops low enough to deplete ATP. To overcome the metabolic imbalance, cells must induce and activate HIF-1, because HIF-1 enhances glycolysis by upregulating glucose transporter through lactate dehydrogenase (LDH). In glycolysis cycle, lactate dehydrogenase (LDH) shifts the pyruvate to lactate since the pyruvate dehydrogenase complex (PDC) is inactivated by pyruvate dehydrogenase kinases (PDKs). In oxidative glycolysis , PDC converts the pyruvate to β-oxidation increases acetyl-CoA (Ac-CoA), which is further oxidized via tricarboxylic acid (TCA) cycle.The molecular mechanism is not clear but briefly investigated below. HIF1α targets pyruvate dehydrogenase kinase (PDK) one and set in motion the glycolytic shift. Immunological analyses revealed that HIF-1α and HIF-2α are acetylated by PCAF and CBP, respectively, but SIRT1 modulates cellular adaptation to hypoxia by targeting HIF-1α. SIRT2 binds to FOXO3a and

reduces its acetylation level. SIRT2 hence increases FOXO DNA binding and elevates the expression of FOXO target genes, manganese superoxide dismutase (SOD) and Bim. SIRT3 plays a pro-apoptotic role in both Bcl2-p53- and JNK-regulated apoptosis

8. HIF1 manage the surviving/nesting of stem cells and Sirtuins manage differentiation

After transplantation of stem cells in new dysfunction niche that surrounding the harsh and hypoxic condition, the HIF1α help to nesting and surviving of stem cells. For example the Mesenchymal stem cells start to the energy metabolism homeostasis, oxidative stresses neutralizing and in the subsequently secretion of paracrin secretome specially VEGF for angiogenesis[47]. The molecular mechanism is not clear but briefly investigated below. HIF1α target pyruvate dehydrogenase kinase (PDK) one and set in motion the glycolytic shift . After angiogenesis and combat the oxidative stress and nesting in the site of injury, the sirtuins induce the down-stream pathways to support the differentiation[91]. A study demonstrated that SIRT1 regulates differentiation of mesenchymal stem cells by deacetylating β-catenin[91].

SIRT1, SIRT6, and SIRT7 catalyze posttranslational modification of proteins in the nucleus. SIRT1 regulates apoptosis and Nanog expression in mouse embryonic stem cells by controlling p53 subcellular localization[92]. Activation of Sirt1 decreases adipocyte formation during osteoblast differentiation of mesenchymal stem cells[93]. SIRT1 regulates differentiation of mesenchymal stem cells by deacetylating β-catenin[91]. SIRT1 positively regulates autophagy and mitochondria function in embryonic stem cells under oxidative stress[94]. SIRT1 is required for long-term growth of human mesenchymal stem cells[95]. SIRT1 suppresses self-renewal of adult hippocampal neural stem cells.[96]. SIRT1 deacetylates β-catenin to promote its accumulation in the nucleus leading to transcription of genes for MSC differentiation[91]. Peroxisome proliferator-activated receptor γ2 (PPARγ2) is a key element for the differentiation into adipocytes. Activation of the nuclear protein deacetylase Sirt1 has recently been shown to decrease adipocyte development from preadipocytes via inhibition of PPARγ2[97]. SIRT1 deficiency downregulates PTEN/JNK/FOXO1 pathway to block reactive oxygen species-induced apoptosis in mouse embryonic stem cells[98]. SIRT6 safeguards human mesenchymal stem cells from oxidative stress by coactivating NRF2[99]. Also it reported that, SIRT6 regulates osteogenic differentiation of rat bone marrow mesenchymal stem cells partially via suppressing the Nuclear factor-κB signaling pathway[100]. This sirtuin controls hematopoietic stem cell homeostasis through epigenetic regulation of Wnt signaling as well[101]. The protein-protein interactions (PPIs) were analyzed using the STRING Database v. 10.5 (http://www.string-db.org), which includes direct and indirect protein associations with HIF1/sirtuins collected from different databases. Protein interaction networks were predicted using medium confidence scores (0.40) and clustered using MCL clustering algorithm (inflation parameter: 5). (Figure 3).

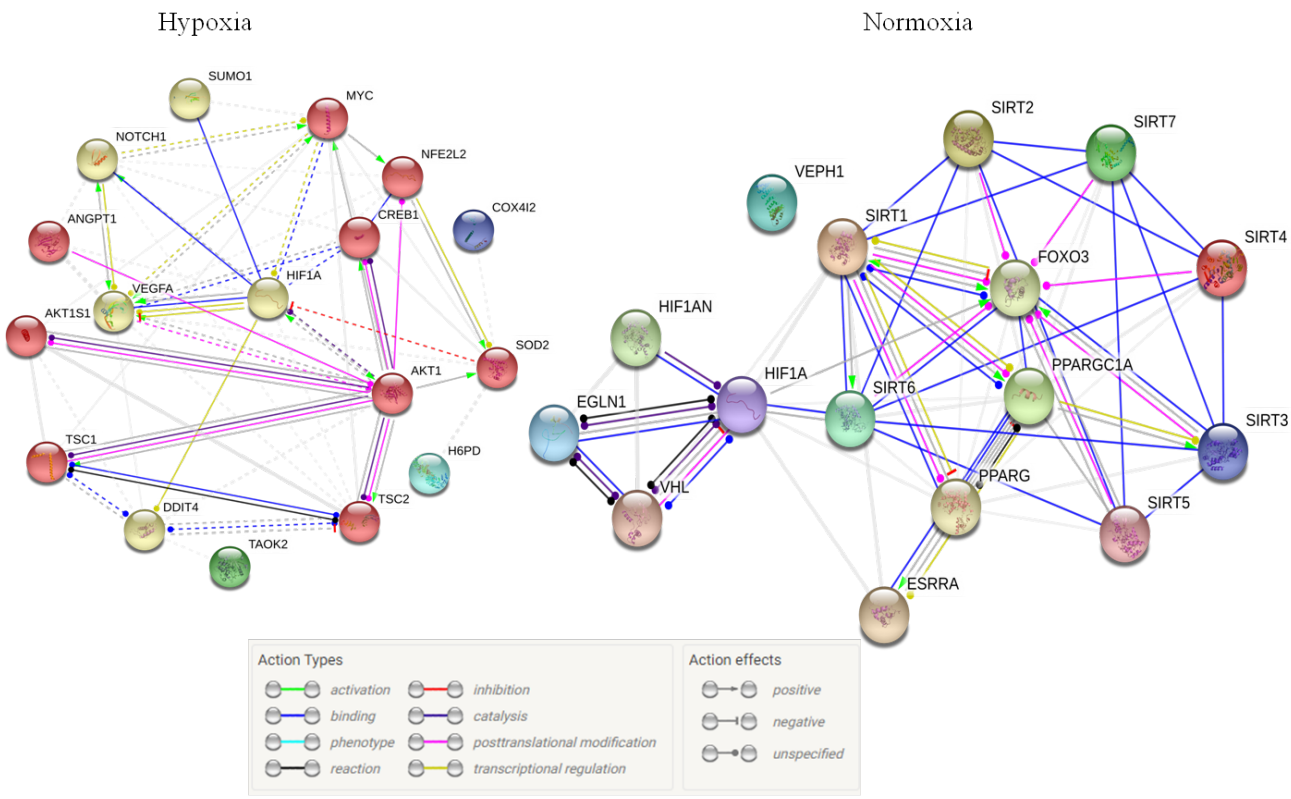

Figure2 | Protein–protein interaction network obtained using STRING software in mesenchymal stem cells over expressing hypoxia inducible factor (HIF-1a) and Sirtuins. The images show the confidence view (http://string-db.org/). Stronger associations are represented by thicker lines.10. The signaling pathways related to several paracrine factors and interleukins are upregulated in MSCs high expressed HIF1α. For example, expression levels of p38 mitogen-activated protein kinase (p38/MAPK) [52], JNK/SAP [53], AKT[53] , and Notch [32,33], together with fibronectin [54], fibroblast growth factor [55], angiopoietin-1 (ANGPT1) [56], NGF [57], and insulin growth factor [58]signaling pathways, are all induced by HIF-1α. In the normoxia sirtuins activate and suppress the Hif1α.

9. Future direction; the role of Hif1α and Sirtuins in stem cells niche engineering

The abilities of MSCs to self-renew and differentiate into various cell types have made these cells a major resource for stem cell-based therapies[102]. The putative mechanisms by which transplanted MSCs exert regenerative effects in injured tissues include cell fusion, and paracrine effects, such as immunosuppressive and anti-apoptotic effects and differentiation or the stimulation of local progenitor stem cells[102]. However, a major obstacle in MSC therapy is the low survival rate after transplantation due to cell death via anoikis, oxidative stress and energy metabolism disruption[103]. As previously described, pre-treatment of stem cells in the hypoxic environment prior to trasplantation to the injured site, can trigger signaling to rescue the cells and ability to nesting and surviving at the site of injury. Have their own paracrine secretome and after angiogenesis, Sirtuins are needed to make a differentiation of stem cells. To improve the survival

and cell adhesion of the transplanted MSCs, various strategies have been investigated, including pretreatment with growth factors or cytokines and designing the biomimic scafolds[104] but also necessary the hypoxic preconditioning[23], and genetic modifications[105] to induce the overexpression of HIF1α/sirtuins as well as treatment with antioxidants[106].

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5A Chapter

Cell and Tissue Research

The role of Piezo proteins and cellular mechano-sensing in tuning the fate of transplanted stem cells

--Manuscript Draft--

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The role of Piezo proteins and cellular mechano-sensing in tuning the fate of transplanted stem cells

The behavior of transplanted stem cells in hypoxia injured tissues can be modulated by not only oxidative stress however by a combination of internal and external signals, including mechanical cues from the surrounding damaged condition. In this chapter we discussed in a review article about the mechanical stress inducing in damaged tissues which way could effect to the proliferation and fate of trasnlanted stem cells. Hypoxia condition of injured tissues much time is incorporate with physiological mechanoinformation destroying. So, for clearance of antioxidants potential in supporting of stem cells in harsh condition, designing nano-scaffolds with distinguish stiffness and maybe formulated with ntioxidants substances may be nesesery.

Abstract

Differentiation of stem cells can be modulated by a combination of internal and external signals, including mechanical cues from the surrounding micro-environment. Although numerous chemical and biological agents have been recognized in regulating stem cell fate, whether or not stem cells can directly sense mechanical signals to choose differentiation into a specific lineage, is yet poorly understood. The success of any stem cell transplantation effort, however, hinges on thorough understanding of the fate of these cells under different signals including mechanical cues.

Piezo proteins, the ion channels activated by membrane tension and mechanical signals, play an important role in translating the information of mechanical forces such as shear stresses and tensile loads, as well as the rigidity and topography of the extracellular matrix to the intracellular signaling pathways, related to

stem cell homing and differentiation.

This review highlights key evidence for the potential of mechanically-gated ion channels expressed by human stem cells, and the mechanotransduction and past mechano-memory in the fate of transplanted stem cells. With this knowledge in mind, by controlling the tissue-specific patterns of mechanical forces in the scaffolds, we may further improve the regulation of homing, the differentiation, and the fate of transplanted stem cells.

Keywords: Biomaterials, Cell Signaling, Mechanotransduction, Piezo Proteins, Regenerative Medicine, Stem Cells

Résumé

La différenciation des cellules souches peut être modulée par une combinaison de signaux internes et externes, y compris des signaux mécaniques provenant du micro-environnement. Bien que de nombreux agents chimiques et biologiques aient été reconnus dans la régulation du destin des cellules souches, la question de savoir si les cellules souches peuvent directement détecter les signaux mécaniques pour choisir la différenciation en une lignée spécifique est encore mal connue. Le succès de tout effort de transplantation de cellules souches, cependant, repose sur une compréhension approfondie du sort de ces cellules sous différents signaux, y compris des signaux mécaniques.

Les protéines Piézo constituent une famille de canaux ioniques excitateurs directement déclenchés par des forces mécaniques. Ces canaux ioniques participent à la mécanotransduction cellulaire et donc à la conversion des forces mécaniques en signaux biologiques. Les signaux mécaniques, jouent un rôle important dans la traduction des informations des forces mécaniques telles que les contraintes de cisaillement et les charges de traction, ainsi que la rigidité et la topographie de la matrice extracellulaire vers les voies de signalisation intracellulaires, liés à l'homing et à la différenciation des cellules souches.

Cette revue met en évidence l'importance des canaux ioniques à déclenchement mécanique exprimés par les cellules souches humaines, ainsi que de la mécanotransduction et de la mécano-mémoire passée dans le destin des cellules souches transplantées. Il est possible d'envisager que le contrôle des forces mécaniques spécifiques des tissus dans les matrices tridimensionnelles utilisées comme support pour l'implantation des cellules souches, permettrait une amélioration ou encore la régulation de l'homing, de la

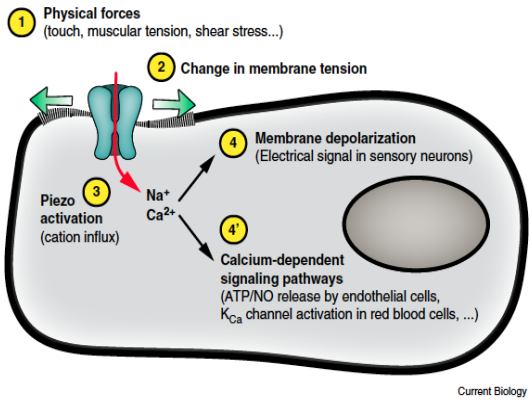

Figure 1. Piezo-dependent mechanotransduction.
Various mechanical stimuli exerted on cells induce changes in plasma membrane tension, eliciting Piezo channel opening. The resulting cation influx can trigger sensory neuron firing or activation of intracellular calcium signaling pathways.

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différenciation et du sort des cellules souches transplantées.

1. Introduction

The idea that mechanical forces may regulate tissue formation and homeostasis was proposed more than a century ago. However, only with recent progress in cellular and molecular techniques, we may have understand how individual cells translate mechanical cues into cellular signals (Eyckmans, et al., 2011) and related biofunctions. Mechanical information of the surrounding cellular micro-environment, elasticity of extracellular matrix, and nanotopography are powerful modulators of stem cell differentiation and lineage choice (Watt and Huck, 2013). Upon transplant therapy, stem cells perceive numerous mechanical signals and develop complex dynamic interactions within the dynamic four dimensional micro-environment of in the damaged tissues or artificial nano-scafolds (Watt and Huck, 2013). Studies illustrate that the transplanted stem cells may have a mechanical memory due to their handling or previous expansion on bioreactors for harvesting the transplanted dose (Yang, et al., 2014). The mechanical properties of the *in vitro* culture environment or stiffness of the nano-scaffolds can influence the cell therapy output (Gilbert, et al., 2010).

Till now, no stem-cell specific mechano-sensory mechanism has been proposed. However, the evidence for supporting a key role of mechanical forces in determining stem cell differentiation are any number of mechanisms, including focal adhesions (Ingber, 2006), changes in membrane curvature or lipid microdomains (Hamill and Martinac, 2001), G-protein-coupled receptors (GPCRs) (Chachisvilis, et al., 2006), mechanosensitive ion channels (Sukharev and Corey, 2004), conformational changes of cytoskeletal proteins (Johnson, et al., 2007), nuclear lamina or nuclear deformations (Lammerding, et al., 2004), and primary cilia (Resnick and Hopfer, 2007).

In tissue environments, somato-sensing by the stem cells could run a complex signaling known as mechanotransduction. Studies have shown that special ion channels are mechanotransducers that quickly convert mechanical forces into cell functions (Vining and Mooney, 2017).

Recently Piezo channels have been recognized as special ion channels mediating mechano-sensing and mechanotransduction. Characterization of Piezo proteins and the related structures have yielded critical insights into the mechanisms of somatosensation, as well as biologic behaviors such as sensing shear stress and tension (Bagriantsev, et al., 2014b). Other roles have been reported for Piezo channels, including participation in cellular development, regulation of cell volume and elongation, migration of cells, and proliferation (Bagriantsev, Gracheva and Gallagher, 2014b). Additionally, these proteins play critical roles in fate determination of neural stem cells (Pathak, et al., 2014), as well as neuronal axon guidance (Koser, et al., 2016). Here, we investigate the role of piezo proteins and the mechanisms adopted by stem cells to mechanical stresses, as well as the way through which this information influences downstream signals.

Ultimately, improvements are needed to better manage the fate of transplanted cells. With in-depth knowledge of the cell mechanotransduction mechanisms, we could design robust bioreactors for the expansion of stem cells, as well as biocompatible nano-scaffolds with specific nano-topography and stiffness to make a control over the homing and differentiation of transplanted stem cells in regenerative medicine.

2. Mechanical forces in stem cell biology

Unlike drugs, which are formulated to act through known mechanisms, cell therapies are alive therapeutics, during which the phenotype and genotype of stem cells may alter; they can undergo undesired differentiation, or undergo necrosis or apoptosis; hence, their therapeutic potential may drastically change. Naturally, the fate of stem cells is influenced by multiple modalities including genetics, epigenetics, different molecular mediators, and cell signaling pathways in order that a cellular response switching between growth, differentiation, and apoptosis could be achieved (Isomursu, et al., 2019).

Recent findings have highlighted the contribution of biomechanical cues to the changes in cell signaling and function, a multistep process known as mechanotransduction (Discher, et al., 2009, Ma, et al., 2018). In the following sections, we analyze different mechanical forces that stem cells may experience in all manufacturing processes, including isolation and expansion, until cell implantation.

2.1 In vitro culture systems and past mechano-memory

Owing to the fact that limited stem cells can be obtained from a donor, one of the main challenges in cell therapy is the expansion of stem cells in bioreactors with no differentiation and alteration of genotype and phenotype (Heo, et al., 2018). The International Society for Cell Therapy provides a guidance for developing autologous and patient-specific manufacturing strategies for the large-scale stem cell production (Eaker, et al., 2013). However, the main critical concern is that stem cells have obtained mechanical information from past physical environments that influences future cell fate decisions (Yang, Tibbitt, Basta and Anseth, 2014). Attaining control over stem cell expansion without differentiation is a great challenge, since these cells induce a complex array of "niche" molecular signals, which regulate their fate (Re'em and Cohen, 2011). In comparison with conventional bench-scale static cultures, large-scale expansion imparts complex shear and hydrodynamic forces on cells. Bioreactors must be able to efficiently expand stem cells using a xeno-free culture system, while maintaining the morphology, immunophenotype, and differentiation potential. The past memory of stem cells cultured in bioreactors, that influences molecular signaling and expressing gene profiles for differentiation or homing in cell therapy, were reported in several studies. Studies on large-scale bioreactors with controlled mechano-stresses are also in progress(Kropp, et al., 2017). Sugimoto et al established a cell culture chamber to control hydrostatic pressure (HP). Using this system they showed that the promotion of osteogenic differentiation by HP is dependent on bone morphogenetic protein 2 (BMP2) expression regulated by Piezo1 in MSCs (Sugimoto, et al., 2017).

Under in vitro conditions, Marrelli et al demonstrated that dental pulp stem cells (DPSCs) are sensitive to various mechanical forces, such as cyclic tensile, fluid flow, and uniaxial mechanical stretch, as well as biomechanical cues provided by micro/nano scale surface topographies (Marrelli, et al., 2018). In other studies, the role of biomechanics in stem cell mechanotransduction pathways have been reviewed (Janaszak, et al., 2016) (Riehl, et al., 2017). Deep understanding of the physical environment that influences the fate of stem cells is needed for designing bioreactors, as well as tissue engineering for clinical therapeutic applications.

2.2. Mechanical homeostasis in tissue micro-environment

Mammalian tissues contain populations of variously specialized phenotypic cells which are tightly modulated based on their roles in tissue homeostasis. These cells include stem and progenitor cells which have the potential for self-renewal and multilineage differentiation and have a significant function during the formation and repair of the tissue (MacQueen, et al., 2013). Stem cells, along with their local microenvironment, namely, niche, communicate by mechanical cues in order to regulate the fate and behavior of the cell and to direct developmental processes. In addition, the physical environment of the pluripotent stem cells can regulate their self-renewal and differentiation (Vining and Mooney, 2017).

The microenvironment of the native stem cell includes the stem cell-attached supporting cells, soluble bioactive factors, and extracellular matrix which acts as the supporting architecture, as well as a container for cellular signaling biomolecules. Cells can detect the biophysical information of the extracellular environment through several channels and receptors with react and adapt to the extracellular milieu in a process of mechanotransduction (Sun, et al., 2016). Hence, to improve the cell therapy outcome, the native mechano and biochemical factors should be mimicked in biomaterial scaffolds or *in vitro* differentiation process.

2.3. Nano-topography and stiffness of scaffolds

Biomaterials are promptly developing for displaying and delivering the stem-cell-regulatory signals in an accurate and near-physiological form and function as powerful artificial microenvironments for inducing the stem-cell fate both *in vitro* and *in vivo*. Further, synthetic materials can affect and even induce the differentiation of the stem cells based on their stiffness and nano-topography (Murphy, et al., 2014). Recent evidence has shown that inherent material properties may be engineered in such a way that stem cell fate decisions are dictated. The design of biomaterials with specific properties evinces a valid approach at modulating and controlling the stem cell environment. Lee et al. reported that directing the stem cell destiny on hydrogel substrates occurs through controlling the cell geometry, matrix mechanics, and adhesion ligand composition (Lee, et al., 2013). Nanoscale topographies were shown to stimulate human MSCs in order to produce bone mineral *in vitro*, in the absence of osteogenic supplements, and by the efficiency comparable to that of the cells cultured in osteogenic media (Dalby, et al., 2007). Furthermore, different mechanical forces with specific matrix stiffness or topography are applied to generate selective differentiation of MSCs

into specific cell phenotypes, as a recent development in the area of tissue engineering (D'angelo, et al., 2010, Engler, et al., 2006, Kilian, et al., 2010). In this respect, literature review revealed that human MSCs responded to hydrogenated amorphous carbon (a-C:H) nanotopographies with groove or grid surface structures, inducing specific changes in their microtubule organization. In particular, the researchers found that the groove nanopatterns had a more dynamic influence, related to the stem cell alignment and elongation. The culture of human bone MSCs on 3D macroporous scaffolds of pullulan/dextran yielded different expression and localization of gap junctional proteins Panx1 and Panx3 when compared to 2D classical culture. Scaffolds allow the improvement of cellular communication within the dense cellular aggregates and support their differentiation towards the osteoblastic phenotype(Guerrero, et al., 2018). Several groups have reported the impact of matrix stiffness on the phenotype and differentiation pathway of the stem cells, showing that stem cells differentiate into different tissues depending on whether they were cultured on elastic moduli in the lower, intermediate, or higher ranges (Ma, Lin, Huang, Li, Wang, Bai, Lu and Xu, 2018). Figure 1 provides guidelines regarding selecting the material for polymer hydrogels related to the engineered 3D mechanical microenvironments for controlling of stem cell's differentiation.

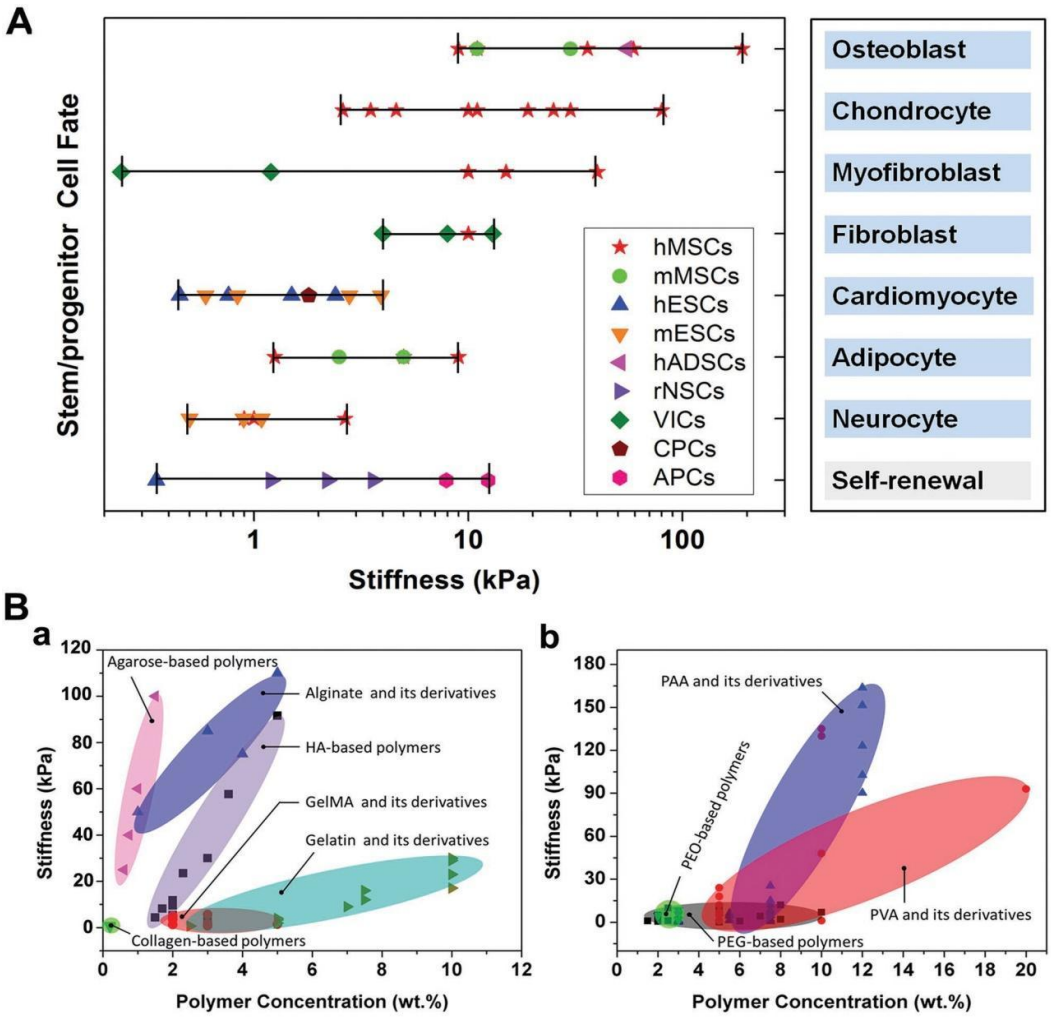

Fig. 1 The effect of engineered 3D mechanical microenvironments stiffness in stem/progenitor cell fate decision. (A) Stem/ progenitor cell fate decision in 3D hydrogel microenvironments with specific mechanical properties (hMSCs: human mesenchymal stem cells; mMSCs: murine mesenchymal stem cells, mESCs: murine embryonic stem cells; hADSCs: human adipose-derived stem cells; CPCs: cardiomyocyte progenitor cells; hESCs: human embryonic stem cells; rNSCs: rat neural stem cells; VICs: valvular interstitial cell; APCs: adipose progenitor cells). (B) Bio-material choice plan: hydrogel stiffness plotted against polymer concentration. Ash by plots describe the probable stiffness ranges for both a) naturally derived and b) synthetic hydrogels (Ma, Lin, Huang, Li, Wang, Bai, Lu and Xu, 2018), Copyright 2019, Wiley-VCH, License Number; 4512370574449.

3. Piezo proteins as the cell's biomechanical sensors

As above mentioned stem cells obtain the mechanical information from previous physical environments or scaffolds that influence future cell fate decisions. Now, how the stem cells process mechanical cues and how such processing causes downstream signaling events that are needed for greater control over the fate of transplanted cells. One of the main biomechanical sensors in the cells are Piezo channels.

Piezo proteins were first recognized as the pore-forming subunits of excitatory mechanosensitive ion channels in 2010 (Coste, et al., 2010). It has been shown that they induce mechanically activated cationic currents in various eukaryotic cell types, linking mechanical forces to cellular signals (Coste, et al., 2012). Piezo proteins enable the cells to sense forces by allowing positively charged ions, including calcium, to leak into the cell in response to mechanical stimuli (Wu, et al., 2017). 3D structures of Piezo proteins shed light on how Piezos employ a variety of mechanical stimuli in channel activation (Guo and MacKinnon, 2017, Saotome, et al., 2018, Zhao, et al., 2018). The cells respond to mechanical stimuli by gating the channels, thereby allowing external ions to pass through the cell membrane into the cytoplasm. Piezo1 proteins as ion channels consist of three bent arms, surrounding a central pore with an extracellular cap (Fig. 2).

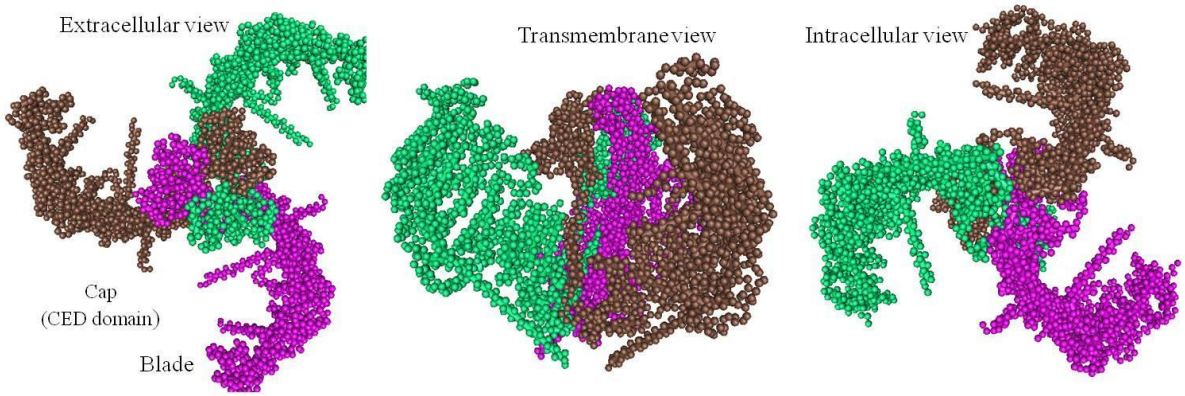

Fig. 2 The molecular structure of mechanosensitive channel Piezo1 in differential view. For Piezo1, both

the cap and the first two extracellular loops near the N terminus are mechanically sensitive.

The cytoplasmic carboxy-terminal domain (CTD) of each Piezo1 molecule extends from the pore to an anchor domain and a 'beam' that connects the arm to the pore. Guo and MacKinnon propose that the curvature of the arms induces deformation of the cell membrane; the membrane would fill the regions between the arms. In contrast, preceding biophysical studies suggested that Piezo channels sense forces directly through the lipid membrane (Cox, et al., 2016, Syeda, 2017). A cellular study was showed that, Piezo1 induces local curvature in a mammalian membrane and forms specific protein-lipid interactions. Piezo1 proteins alter the lipid-regulating proteins expression and influences the local morphology and composition of the bilayer(Buyan, et al., 2019). Clearly, mechanistic comprehension of many aspects of the channel function is still lacking. Despite limited knowledge, all literature converges on the essential mechanosensory role of Piezo proteins in the mechanotransduction of stem cells. Further research is required to identify the mechanisms that control the specific permeation and gating of these channels. The following section focuses on the mechanotransduction processes which the Piezo ion channels require, as well as the molecules and processes that modulate the function of Piezo proteins.

4. Piezo channels and coupling to the mechanotransduction; Role in the stem cell fate

With the increasing knowledge on the signaling pathways in cells, mechanosensing seems to be one of the missing pieces in the puzzle toward the understanding of stem cell fate (cell proliferation, migration, and differentiation) (Poudineh, et al., 2018). In addition, finding the mechanically activated channels and understanding their opening mechanisms could shed the light on the stem cell fate preference. Moreover, since bio-mechanical micro-environment is a demanded niche for tissue engineering, discovering cell mechanosensors can not only explain the mechanisms of differentiation, but also bring about new prospects on the mechanical control of stem cells when delivered to the tissue.

There are four mechanisms through which the bio-mechanical stimuli activate the mechanosensitive channels: the shear stresses and tension in cell membrane; the direct physical cell-cell interaction, the extracellular matrix components; and the intracellular cytoskeleton. These mechanisms may cooperate with each other.

Excitingly, the recent discovery of Piezo1 and Piezo2 in a mouse neuroblastoma cell line as the components of mechanically activated cation channels provides direct evidence that the piezo proteins are one of the core mechanosensors in mammalian cells (Lee, et al., 2019, Totaro, et al., 2018). As multi-pass transmembrane proteins, the contribution of Piezos to integrin activation have been proven. The mechanical stimulation of piezos is then transduced to intra-cellular signaling cascades. By the mechanotransduction cascade, cells translate mechanical cues into biochemical signals, controlling several aspects of cell manners including proliferation and differentiation. How the mechanosensing by Piezo proteins is linked to the

activity of nuclear transcription factors and signaling pathways is summarized in the following section.

4.1. Piezo channels and Cytosolic Ca2+ signaling

Ca^{2+} as an intracellular signal plays a crucial role in cell regeneration and is a potent effector of cellular responses (Gilchrist, et al., 2019). Ca^{2+} transients transduce information through contemporaneous regulation of protein activity and shape cell properties and differentiation fate through execution of specific cell signaling (Marchant, 2019). These attributes strengthen the fundamental contribution of Ca^{2+} -signaling events to reparative physiology, encompassing responses to tissue regeneration. The role of endogenous Ca^{2+} signals in coordination of physiological responses to injury, cell turnover, and tissue replacement has been already reported in the literature.

Of various Ca^{2+}-signaling modulators, mechano-stresses are one of the main effectors. Mechanical force has emerged as athe determinant of the Ccytosolic Ca^{2+} -Ca2+ signaling butsignaling, however, the mechanisms of force sensing and coupling to the mechanotransduction are still unclear. The Piezo knockout phenotypes of the stem cells from the adult Drosophila midgut can be rescued by increasing the concentration of cytosolic Ca^{2+}. Authors have shown that mechanical stress regulates the stem cell differentiation through the stretch-activated ion channel Piezo and therefore, it can be concluded that Piezo channels function through Ca^{2+}signaling pathways (He, et al., 2018). Piezo channel openings lead to Ca^{2+} entry into the cell, which potentially trigger intracellular Ca^{2+} signaling pathways and act as a modulator of differentiation (Pathak, Nourse, Tran, Hwe, Arulmoli, Dai Trang, Bernardis, Flanagan and Tombola, 2014). The increased intracellular calcium functions as a second messenger. In addition, alterations in intracellular free calcium concentration act as a significant biological signal in the mechanotransduction and as one of the first responses against the environmental stress (Uhlén and Fritz, 2010). It has been reported that mechanical shear stress to endothelial cells evokes Piezo1-dependent currents with calcium influx, followed by the activation of calpain-2 activity (Bagriantsev, et al., 2014a). Calpains are calcium-regulated cysteine proteases, the activity of which has been reported in a wide range of cellular reactions, including apoptosis, cell cycle regulation, and cell differentiation. Calpain is usually present in an inactive form and is activated by calcium ions (Yajima and Kawashima, 2002). The ability of calpain to alter, by limited proteolysis, the activity or function of numerous cytoskeletal proteins and enzymes, as well as receptors suggests its contribution to many Ca^{2+} -regulated cellular functions (Yajima and Kawashima, 2002). Calpain 1 and calpain 2 were found to distinctly play a role in neural stem cell fate decision (Santos, et al., 2012). The essential, positive or negative roles of calpain in the differentiation of myoblasts, osteoblasts, chondrocytes, or adipocytes, derived from common MSCs have been displayed (Yajima and Kawashima, 2002). Overexpressed Fam38 is localized to the endo-plasmic reticulum, where it regulates calcium release and promotes □1 integrin conversion to the high affinity state. The increased release of Ca^{2+} from the endoplasmic reticulum (ER) activates different downstream signaling pathways and leads to the activation of the calcium-dependent

protease calpain (Fig. 3) (McHugh, et al., 2010).

4.2. Piezo channel activates ADAM10 sheddase to regulate Notch1

Notch homolog 1 translocation-associated (Notch1) pathway is one of the most established mediators of short-range signaling interactions by which physically adjacent cells control each other's fate. As an evolutionarily conserved single-pass transmembrane receptor, Notch1 participates in a variety of developmental processes. Notch signaling commonly regulates the proliferation and differentiation of stem and progenitor cells (VanDussen, et al., 2012). It also regulates stem cell numbers *in vitro* and *in vivo* with the activation of cytoplasmic cell signals, including the serine/threonine kinase Akt, the transcription factor STAT3, and mammalian target of rapamycin (Androutsellis-Theotokis, et al., 2006). There is evidence that activated Notch 1 and Notch 3 promote the differentiation of progenitor cells into astroglia (Tanigaki, et al., 2001). Piezo1 channel opening in response to shear stress led to the activation of a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10), a Ca^{2+}-regulated transmembrane sheddase that mediates S2 Notch1 cleavage (Beech, et al., 2019).

4.3. Interaction between Piezo1 and the cytoskeleton

Piezo2 controls the formation of actin-based stress fibers and the orientation of focal adhesions in complex ways (Pardo-Pastor, et al., 2018). Force transduction either through cytoskeletal tethers or through the membrane can gate the Piezo channels (Nourse and Pathak, 2017). Moreover, several studies have demonstrated that Piezo1 gates with lipid tension (Cox, Bae, Ziegler, Hartley, Nikolova-Krstevski, Rohde, Ng, Sachs, Gottlieb and Martinac, 2016, Lewis and Grandl, 2015). Interesting dynamics between Piezo1 and the cytoskeleton were also reported (Gottlieb, et al., 2012). For example, external mechanical forces are probably transmitted and recognized at focal adhesion zones (FAZs), representing the cytoskeletal structures that link the extracellular matrix (ECM) to the intracellular actin cytoskeleton (Nourse and Pathak, 2017). Integrins are one of the major components involved in mechanotransduction connected to the Piezo channels. Integrins of adherent stem cells (i.e., MSCs) are regarded as direct mechanosensors that physically connect the ECM to the cytoskeleton, thereby acting as signaling receptors. The molecules such as myosin II, the Rho/ ROCK system (Kim, et al., 2009), and vinculin (Holle, et al., 2013) discovered to date are mostly cytoskeletal proteins. Although cytoskeletal proteins, as well as cellular contractility regulators are required for producing the traction forces and perceiving the environmental mechanics, the molecular mechanisms which connect these signaling molecules to intracellular pathways, intergrin-dependent signaling, and MAPKs activation that influences differentiation have not been elucidated yet (Gao, et al., 2017, Gao, et al., 2016, Liu, et al., 2019).

4.4. Piezo and RhoA- ROCK pathway

Some studies have highlighted that substrate rigidity regulates the magnitude and frequency of the calcium oscillations by the RhoA pathway in human MSCs (Kim, Seong, Ouyang, Sun, Lu, Hong, Wang and Wang, 2009). In addition, Piezo2-mediated Ca^{2+} influx are supposed to trigger RhoA in order to control the formation and orientation of the stress fibers, along with the focal adhesions. On the other hand, recruiting the Fyn kinase to the cell leading edge, as well as calpain activation is considered as one possible mechanism for the Piezo2-mediated activation of the RhoA. Calpain is another signaling molecule that may involve Piezo2-induced Ca^{2+} signals in the activation of RhoA. Calpains are Ca^{2+}- dependent intracellular proteases that regulate FA dynamics (Franco, et al., 2004). Some studies have associated the activation of RhoA with integrin signaling in human MSCs (Meyers, et al., 2004). RhoA is demanded for the tyrosine phosphorylation and full activation of FAK (Clark, et al., 1998). The small GTPase, RhoA, regulates the formation of actin stress fiber and has been implicated in the lineage commitment of human MSCs. The Rho subfamily of Ras-associated small GTPases is predominantly distinguished by its regulation of the actin cytoskeleton. Activation of RhoA results in the activation of Rho-kinase (ROCK); Hence, ROCK can phosphorylate LIM-kinase (LIMK). LIMK, in turn, phosphorylates cofilin, a small actin-binding protein that promotes F-actin depolymerization. The Ca^{2+} oscillation was shown to be dependent on ROCK (Fig. 3). Consistently, the Ca^{2+} oscillation was inhibited either by a constitutive active mutant of RhoA (i.e., RhoA-V14) or a dominant negative mutant of RhoA (RhoA-N19) (Kim, Seong, Ouyang, Sun, Lu, Hong, Wang and Wang, 2009). Further, RhoA and cytoskeletal disruption mediate the decreased osteoblastogenesis while increasing the adipogenesis of human MSCs (Meyers, et al., 2005). Furthermore, other experiments demonstrated that the human MSCs cultured on gels with low elastic moduli displayed low RhoA activities (Kim, Seong, Ouyang, Sun, Lu, Hong, Wang and Wang, 2009). Moreover, MAPK signaling acts as a downstream effector of RhoA, suppresses the activation of PPARg, and upregulates the Runt-related 2 (Runx2) gene that is a master osteogenic gene (Shi, et al., 2011).

4.5. Piezo1–YAP/TAZ connection in stem cell differentiation

Dupont et al reported YAP (Yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif) as the nuclear relays of mechanical signals which are exerted by ECM rigidity and cell shape (Dupont, et al., 2011). This regulation requires Rho activity and the tension of actomyosin cytoskeleton (Dupont, et al., 2011). On the other hand, nucleo–cytoplasmic localization of the mechano-reactive transcriptional coactivator Yap is influenced by the Piezo1 activity. The nucleo-cytoplasmic localization of Yap alone or in combination with its partner Taz controlled the choice of the MSCs lineage (Dupont, et al., 2011). Further mechanistic basis of the Piezo1–Yap/Taz connection in the differentiation of stem cells has been elucidated in the observations of neuronal–glial lineage specification (Pathak, et al., 2014).

The requirement of YAP/TAZ for MSCs differentiation has also been applied in the study of YAP/TAZ

function and regulatory inputs, including Wnt (Azzolin, et al., 2012, Hong, et al., 2005), distinct from mechanical cues. Moreover, mechanical cues are recognized in the regulation of the activity of ion channels, including mechanoresponsive Piezo channels.

The role of Piezo1 channel in mediating shear stress or stretch-induced calcium currents was discovered; however, its role in mediating spontaneous calcium currents in a manner dependent on cytoskeletal tension was recently shown. Additionally, such currents are necessary in human neural stem cells for regulating the choice between astrogenesis and neurogenesis against matrix stiffness and influencing YAP nuclear localization (Pathak, Nourse, Tran, Hwe, Arulmoli, Dai Trang, Bernardis, Flanagan and Tombola, 2014). This may demonstrate the requirement of Piezo for integrin activation and cell adhesion (McHugh, Buttery, Lad, Banks, Haslett and Sethi, 2010), or a more direct effect of calcium signaling on YAP/TAZ. This effect is not fully understood yet (Kim, et al., 2009).

According to a report by Guo et al, kindlin-2 regulates MSCs differentiation through the mechanosensitive transcription co-regulators YAP1 and TAZ (Guo, et al., 2018). Kindlin-2 is a component of ECM and is required for proper control of cell shape change. A major task of kindling-2 is to regulate the activation of integrins. The knockout of kindlin-2 in MSCs induces adipogenesis and inhibit osteogenesis *in vitro* and *in vivo* (Guo, Cai, Chen, Wang, Wang, Cui, Yuan, Zhang, Liu and Deng, 2018) (Fig. 3). Kindlin-2 physically relates to myosin light-chain kinase in response to mechanical cues and develops myosin light-chain phosphorylation. However, the knockout of kindlin-2 prohibits RhoA activation and decreases myosin light-chain phosphorylation, stress fiber formation, and focal adhesion assembly (Guo, et al., 2018).

The prominent regulatory roles of YAP and TAZ in the cell proliferation and survival, as well as controlling organ growth, stem cell self-renewal, and cell differentiation have been verified [25-27]. These proteins also shuttle between the cytoplasm and the nucleus and contribute to the regulation of gene expression [30,31]. The regulation of Wnt signaling in the cytoplasm is another additional function of these proteins [32,33]. The serendipitous discovery that the F-actin cytoskeleton can regulate the YAP/TAZ, along with their connection with ECM stiffness and geometry sensing, was independently inspected in several studies. Furthermore, as a transcriptional modifier, TAZ contributes to the differentiation of MSCs through increasing the osteoblast differentiation while simultaneously damaging adipocyte differentiation (Fig. 3). Differentiation of MSCs into osteoblasts is critically rendered by Runx2 (Hong, Hwang, McManus, Amsterdam, Tian, Kalmukova, Mueller, Benjamin, Spiegelman and Sharp, 2005). TAZ acts as an endogenous coactivator of Runx2 and on the contrary, differentiation of stem cells into adipocytes depends on PPARg-signaling pathways that are straightly inhibited by endogenous TAZ (Hong, Hwang, McManus, Amsterdam, Tian, Kalmukova, Mueller, Benjamin, Spiegelman and Sharp, 2005).

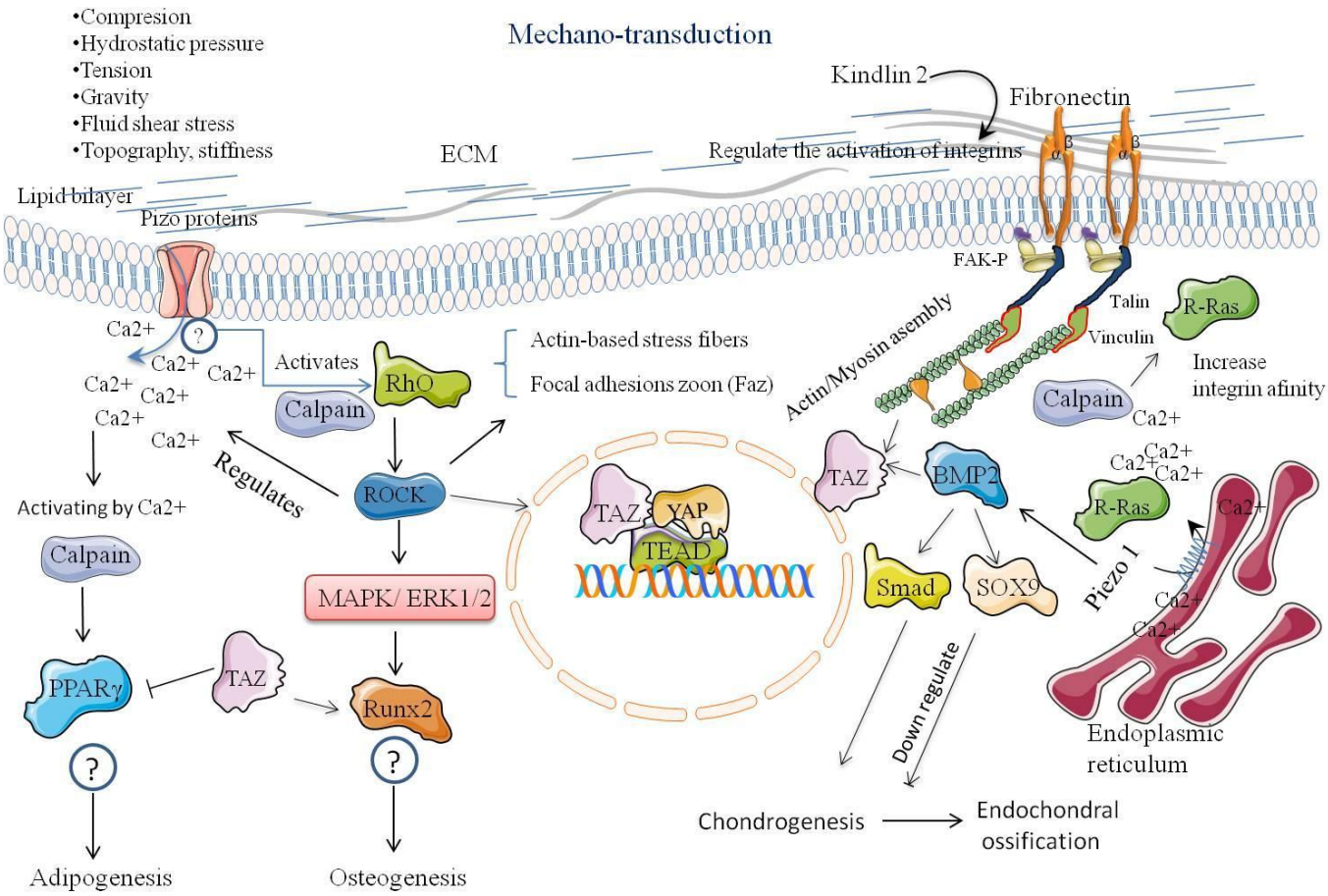

Fig. 3 Proposed schematic diagram for mechanical related signaling pathways in the MSCs differentiation. different mechanical stimuli exerted on cells induce changes in plasma membrane tension, which elicit Piezo channel opening. Piezo channels are the major regulators of calcium in the cells, which have a strong connection with cell signaling involved in the proliferation and differentiation of stem cells. Piezo/Fam38 is localized to the endo-plasmic reticulum, where it increased Ca2+ release from the ER, activates numerous downstream signaling pathways, and in particular, causes the activation of the calcium-dependent protease calpain and promotes b1 integrin conversion to the high affinity state. Piezo2-mediated Ca2+ influx activates RhoA in order to manage the formation and orientation of the stress fibers and focal adhesions. One possible mechanism for the Piezo2-mediated activation of RhoA is the recruitment of calpain. Calpain is another signaling molecule that may involve Piezo2-induced Ca2+ signals in the activation of RhoA. RhoA adjusts mechanical feedback by activating Rho-associated protein kinase 1 (ROCK). Fam38A expression relocalizes R-Ras to the ER, stimulating Ca2+ release and calpain activation. The combination of calpain activity with any active R-Ras causes the increased integrin affinity, and ligand binding, as well as cell adhesion. Furthermore, MAPK signaling acts as a downstream effector of RhoA, suppresses the activation of PPARg, and up-regulates the Runt-related 2 (Runx2) gene that is a master osteogenic gene. Differentiation of MSCs into osteoblasts is critically rendered by Runx2. TAZ acts as an endogenous coactivator of Runx2 and on the contrary, differentiation

of stem cells into adipocytes depends on PPARg-signaling pathways that are straightly inhibited by endogenous TAZ. Sox9 augments BMP2-induced chondrogenic differentiation via down-regulating Smad7 in MSCs (Franco, Rodgers, Perrin, Han, Bennin, Critchley and Huttenlocher, 2004, Guo, Cai, Chen, Wang, Wang, Cui, Yuan, Zhang, Liu and Deng, 2018, He, Si, Huang, Samuel and Perrimon, 2018, Holle, Tang, Vijayraghavan, Vincent, Fuhrmann, Choi, Álamo and Engler, 2013, Hong, Hwang, McManus, Amsterdam, Tian, Kalmukova, Mueller, Benjamin, Spiegelman and Sharp, 2005, Katsumi, et al., 2004, Pardo-Pastor, Rubio-Moscardo, Vogel-González, Serra, Afthinos, Mrkonjic, Destaing, Abenza, Fernández-Fernández and Trepat, 2018, Viti, et al., 2016).

4.6 The crosstalk between Piezo channels and other cell signaling

For clear the role of Piezo channels in fate of stem cells, protein-protein interactions (PPIs) were analyzed using the STRING Database v. 10.5 (http://www.string-db.org), which includes direct and indirect protein associations with Piezo channels, collected from different databases. Protein interaction networks were predicted using medium confidence scores (0.40) and clustered using MCL clustering algorithm (inflation parameter: 5). As shown in Figure 4, in addition to the physical cue and mechanotransduction pathway interaction with piezo proteins, there are many other signaling networks, including differentiation, proliferation, and stemness maintenance pathways. Figure 4 shows the link between mechanotransduction and stimulus-response decisions including differentiation genes.

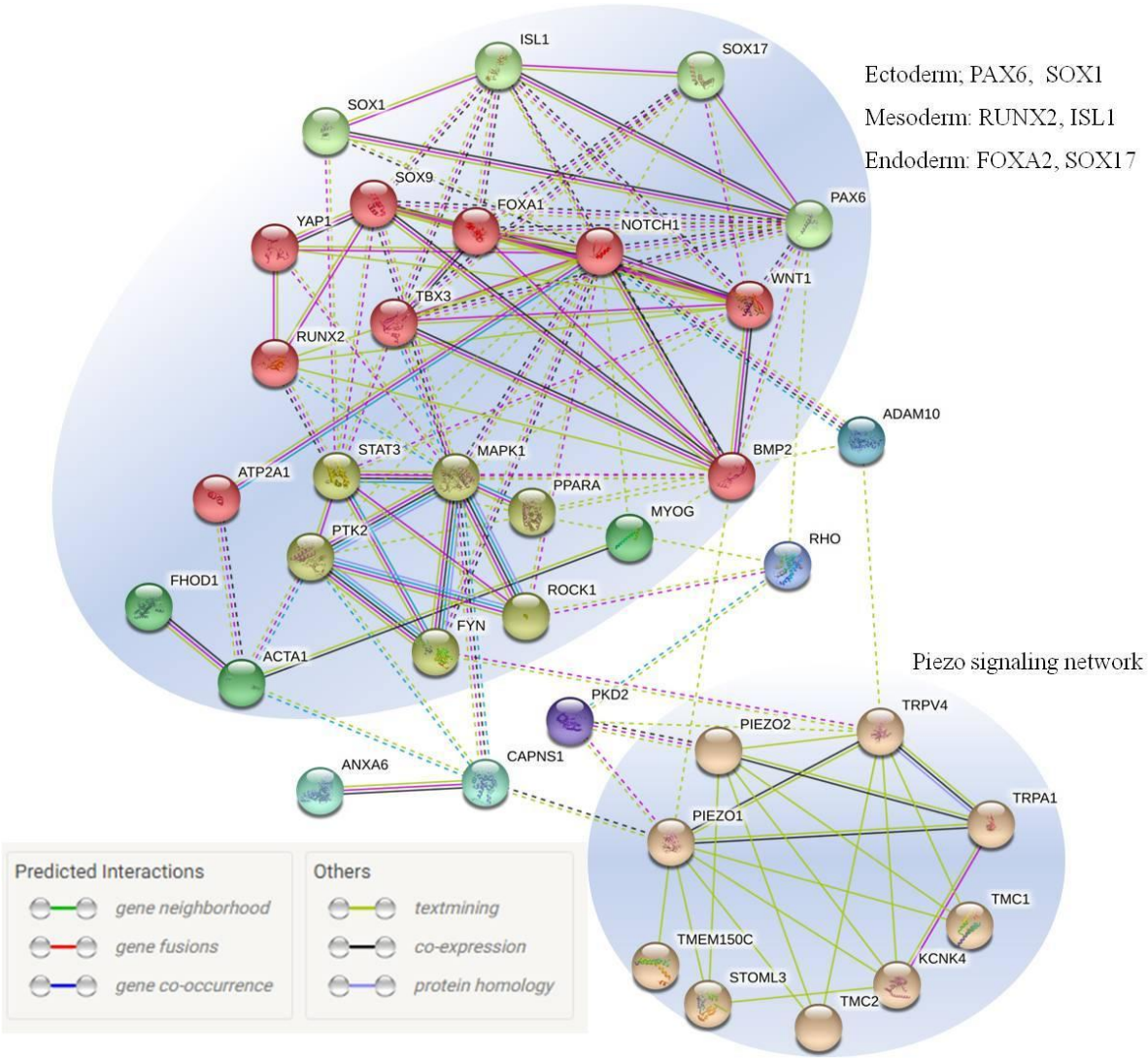

Fig. 4 The mechanotransduction stimulate by piezo chanels in coupling with direct and indirect protein associations in determination of stem cells fate (differentiation to the ectoderm, mesoderm and endoderm). The interaction network was prepared using the STRING Database v. 10.5 (http://www.string-db.org), collected from different databases. The medium confidence scores was 0.40 and MCL clustering algorithm (inflation parameter: 5) was used. Functional interaction proteins have been predicted for Piezo1 and Piezo2, including inhibiting proteins such as PKD2 gene, ATP2A gene, and ANXA6. The potentiating proteins such as STOML3 and transmembrane protein 150C TMEM150C. The other important proteins that play a key role in coupling the mechanotransduction module to the pore module signaling in determination of stem cell fate are Rho, PKD2, ADAM10, CAPNS1, and BMP12. Also, Piezo channel activates ADAM10 sheddase to regulate Notch1. Polycystin-2 (Rho) ; functions as a cation channel involved in fluid-flow mechanosensation. Functions as outward-rectifying K(+) channel, but is also permeable to Ca(2+). May contribute to the release of Ca(2+) stores from the endoplasmic reticulum. Together with TRPV4, forms mechano-sensitive channels. Calpain small subunit 1(CAPNS1) is the regulatory subunit of the calcium-regulated non- lysosomal thiol-protease which catalyzes limited proteolysis of substrates involved in cytoskeletal remodeling

and signal transduction. Neurogenic locus notch homolog protein 1(NOTCH1) functions as a receptor for membrane-bound ligands Jagged1, Jagged2 and Delta1 to regulate cell-fate determination. Annexin A6(. ANXA6) may regulate the release of Ca(2+) from intracellular stores

Functional interactive proteins have been identified for Piezo1 and Piezo2, including inhibiting proteins such as Polycystin-2 (PKD2 gene)(Peyronnet, et al., 2013), sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (ATP2A gene) (Zhang, et al., 2017), and annexin A6(ANXA6) (Raouf, et al., 2018)and potentiating proteins such as Stomatin Like 3 (STOML3) (Qi, et al., 2015) and Transmembrane Protein 150C TMEM150C(Anderson, et al., 2018).

The other important proteins that play a key role in coupling the mechanotransduction module to the pore module signaling in the determination of stem cell fate are Rhodopsin (Rho), Polycystin-2 (PKD2), a Disintegrin and metalloproteinase domain-containing protein 10 (ADAM10), Calpain small subunit 1(CAPNS1), and Bone morphogenetic protein-12 (BMP12). Unraveling the regulatory mechanism of these genes in Piezo1 might have implications for how we target the protein interaction of Piezo channels for designing robust nano-scaffold for cell therapy and *in vitro* expansions in bio-mechanical conditions.

5. Conclusion and future direction

Important and extended studies have revealed that the output of stem cell therapy *in vivo* is dependent on the elasticity and stiffness of scaffolds or in vitro culture mechanical stresses. This implies that cells remember past and present mechanical micro-environments and this memory may influence their fate, even after transplantation into the damaged tissues. To determine whether or not stem cells possess such a mechanical memory, several studies assayed stem cells behavior during culture on supraphysiologically stiff conditions (Yang, Tibbitt, Basta and Anseth, 2014). These studies are based on the principle of cellular mechanotransduction: the hypothesis that cells sense and integrate mechanical cues from the ECM, which eventually direct gene expression and cell fate decisions (Guilak, et al., 2009). A seminal work in this area demonstrated that culture geometry or modulus alone influences cell proliferation, angiogenic sprouting, and stem cell differentiation (Yang, Tibbitt, Basta and Anseth, 2014). The connection between differentiation signaling and mechanotransduction pathways is emerging as a key in determining cell fate but remains largely unexplored.

This article highlights key evidence for the role of Piezo proteins, as well as doing the mechanistic recognition of how the stem cells process mechanical cues and how such processing causes downstream signaling events that are needed for greater control over the fate of transplanted cells.

Expanding large numbers of cells for transplantation is regarded as one major bottleneck in bringing the stem cell therapies to the clinic (Heathman, et al., 2015). Cells should be cultured under completely-defined

conditions to meet the regulatory requirements for being used in human therapies while not attaining unwanted differentiation or past memory effect and, finally, the aims for consistent therapeutic efficacy could be fulfilled (Yang, Tibbitt, Basta and Anseth, 2014).

Furthermore, novel smart biomaterials with programmed mechano-forces and biochemicals must be designed for the delivery and homing of stem cells to control the fate of transplanted stem cells (Perestrelo, et al., 2018). To manipulate mechanical forces at molecular and cellular scales new methods are required, which can combine the theory and techniques obtained from the ion channel biophysics with cell biological principles. Therefore, a smart control over mechanical stresses for a successful cell therapy deems necessary.

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REVIEW ARTICLE

The role of Hippo signaling pathway and mechanotransduction in tuning embryoid body formation and differentiation

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The Role of Hippo Signaling Pathway and Mechanotransduction in Tuning Embryoid Body Formation and Differentiation

For the study of the role of antioxidants in oxidative stress and hypoxia adapation of stem cells and with considering of stem cell native nich components, we resulted that other component of nich must be considred in the exprements to getting real results. In the monolayer cell culture we could not prepare the ideal components. In this chapter we explaned about the emberyoid bodies and the molecular mechanism must be considred in the formation and differentiation.

We investigate the metabolism shifting and hypoxia in core of emberyoid body that could be a gold model to study of hypoxia and antioxidants interplay. In contrast to monolayer cultures with monolayer cultures, the inducing hypoxia and investigation of pre-treatments can be precisely and homogeneously controlled. It has been estimated that oxygen transport is limited in cell aggregates larger than approximately 300 µm in diameter due to such physical restrictions.

Abstract

Embryoid bodies (EBs) are the three-dimensional aggregates of pluripotent stem cells that are used as a model system for the *in vitro* differentiation. EBs mimic the early stages of embryogenesis and are considered as a potential biomimetic body in tuning the stem cell fate. Although EBs have a spheroid shape, they are not formed accidentally by the agglomeration of cells; they are formed by the deliberate and programmed aggregation of stem cells in a complex topological and biophysical microstructure instead. EBs could be programmed to promisingly differentiate into the desired germ layers with specific cell lineages, in response to intra and extra biochemical and biomechanical signals.

Hippo signaling and mechanotransduction are the key pathways in controlling the formation and differentiation of EBs *in vitro*. The activity of the Hippo pathway strongly relies on cell-cell junctions, cell polarity, cellular architecture, cellular metabolism, and the mechanical cues in the surrounding microenvironment. Although the Hippo pathway was initially thought to limit the size of organ by inhibiting the proliferation and the promotion of apoptosis, the evidence suggests that this pathway even regulates stem cell self-renewal and differentiation. Considering the above-mentioned explanations, the present study investigated the interplay of the Hippo signaling pathway, mechanotransduction, differentiation, and proliferation pathways to draw the molecular network involved in the control of EBs fate. In addition, this study highlighted several neglected critical parameters regarding EB formation, in the interplay with Hippo core component involved in the promising differentiation.

***Keywords*:** Biomimetic scaffolds, Cell signaling, Stem cells, Tissue engineering

1 Introduction

Embryoid bodies (EBs) are three-dimensional aggregates, which are formed in suspension culture by pluripotent stem cells (PSCs), including the embryonic stem cells (ESCs) and the induced pluripotent stem cells (iPSC)(Silva et al., 2019).

EBs differentiation is a common platform for generating specific cell lineages from PSCs (Silva et al., 2019). However, the formation and differentiation of EBs are not yet completely controlled, probably due to the insufficient knowledge about the biochemical and biophysical parameters of the EBs and its microenvironment. The spheroid shape, morphology, and microstructural characteristics of the EBs are critical for their whole function and physiological relevance. Although EBs have a spheroid shape, they are not formed accidentally by the agglomeration of cells; they are formed by the intentional and programmed aggregation of stem cells with potential capacities for differentiation (Rungarunlert, Techakumphu, Pirity, & Dinnyes, 2009). The evaluation of EBs in terms of the morphology and potential for differentiation

indicates that they are typical in microstructure and can develop various cell lineages. They are also able to shape into tissue-like structures (Knight & Przyborski, 2015). Further, EBs recapitulate many aspects of cell differentiation during early embryogenesis and thus can play an important role in differentiating PSCs into a variety of cell types *in vitro* (Rico-Varela, Ho, & Wan, 2018). Likewise, the molecular pathways and cellular events within EBs recapitulate many aspects of embryo development and hence result in the differentiation into the cells of three embryonic germ layers (i.e., endoderm, mesoderm, and ectoderm), similar to the *in vivo* gastrulation (Figure 1, Panel A-B) (Itskovitz-Eldor et al., 2000). EB formation involves the establishment of complex interactions and paracrine signaling between cells (Discher, Mooney, & Zandstra, 2009). The new bioengineering methods of EB production, mimicking the *in vivo* EB formation conditions, must allow the biomimetic fabrication of spatially defined 3D cultures controlling the biomechanical and cellular interactions within the EB microenvironment. There are several methods for inducing the formation of EBs from ESCs and iPSCs basically including static suspension, hanging-drop, multi-well culture and bio-printing (Ouyang et al., 2015). Static suspension methods inoculate the ESCs suspension onto the non-adhesive surfaces to allow the cells to spontaneously aggregate into spheroids. The hanging drop method suspends ESCs on the lid of a dish and EBs form at the bottom of the drops through aggregation. These methods are easy to operate; however, control over the EBs size and shape is limited due to the probability of accidental encounter between the ESCs (Ferguson & Subramanian, 2018; Ouyang et al., 2015). Not any cell signaling pathway involves in this reconstruction, and the differentiation could be affected by accidental mechanical and shear stresses (Lei, Deng, & Duan, 2016). In addition, the number of cells serves a critical role as it determines the delivery of oxygen, access to nutrients, and cellular interactions, all of which influence the fate of stem cells (Kurosawa, 2007). Moreover, the past memory of ESC/iPSs and methods of EB formation, as well as external and internal signals could affect the differentiation and the fate of EBs (Boraas, Guidry, Pineda, & Ahsan, 2016; Vining & Mooney, 2017). Recently, alternative methods of EB formation have been developed that allow for highly accurate control of EBs size, shape, and external biochemical and biomechanical signals, resulting in reproducibly produced homogeneous EBs with the simulation of cell signaling pathways (Sabra et al., 2017; Sato, Idiris, Miwa, & Kumagai, 2016; Son et al., 2017). Now, a fundamental question arises regarding whether the size of EBs, uniformity, and topological microstructure are determined by accidentally spherical agglomerated cells or the cell aggregate under a programmed process and elaborate molecular mechanism. Two molecular signaling pathways including the Hippo pathway and mechanotransduction are the keys to the question. The first pathway is involved in restraining cell proliferation and inducing apoptosis (Zhao, Tumaneng, & Guan, 2011). Additionally, it has a vital role in tissue-specific progenitor and stem cell self-renewal and expansion (Ramos & Camargo, 2012). Hippo signaling is also a conserved network in evolution, as it plays a fundamental role in cell fate regulation by inhibiting the activity of the Yes-associated protein (YAP) and TAZ

(Ramos & Camargo, 2012). Various upstream inputs, including biomechanical and biochemical cues, regulate Hippo signaling and allow it to have a main role as a mechano-sensor and an integrator of growth control signals. Several proteins of this pathway localize cell-cell junctions and contribute to the regulation of Hippo signaling by cell contacts and polarity, and the cytoskeleton (Misra & Irvine, 2018). Mechanotransduction is considered as the other important mechanism. Fundamental insights into the mechanobiology of EBs also inform the design of bio-scaffolds to support the programming of the differentiation of EBs (Shao, Sang, & Fu, 2015). These two modulators are usually studied separately in embryogenesis and EB genesis. Therefore, this review investigated the roles of Hippo-signaling and mechanotransduction in terms of the size and homogeneity of these small micro-tissues (EBs), and understanding the controlling mechanisms of EB formation and differentiation. In addition, possible connections between mechanotransduction, Hippo signaling, differentiation proteins, and their relevance in modulating the cell fate in other contexts were pinpointed.

2 | EBs Mimic Embryogenesis in Vitro

Unlike the previous belief, EB differentiation is not unintentional; it mimics the natural process of embryogenesis (Shkumatov, Baek, & Kong, 2014). Embryogenesis is a native model for EBs to mimic its mechanobiology, differentiation process, and the key milestones of gastrulation. Multiple mechanical transitions, uterine environment, or embryonic tissues themselves shape the differentiation, including tight cell junction, cell polarization, cortical tension, and blastocoel pressure induced by accumulated fluid that controls both the size and the fate within the early blastocyst (Chan et al., 2018). Further, after zona hatching and implantation, the development of epiblast (EPI) against the walls of the uterine crypt forces elongation antimesometrially with tension focused at the tip of the embryo. Mechanical tension at the tip is believed to help the egress of EPI cells into the visceral endoderm (VE), where they would form distal visceral endoderm (Hiramatsu et al., 2013), the details of which are illustrated in Figure 1. The mentioned mechanical cue may not involve in EB differentiation but other accidentally or targeted mechanical stresses may affect in vitro differentiation and expansion processes. Furthermore, gastrulation is a highly controlled procedure, however our evidence is not enough to prove whether EBs pose the gastrulation process or direct differentiation. Several studies have convincingly demonstrated that gastrulation and axis formation may occur in EBs (Maranca-Hüwel & Denker, 2010; ten Berge et al., 2008). *In vitro* formation of the three germ layers suggests that gastrulation takes place in EB formation with the self-organization of differentiating cells. Moreover, the aggregation of embryonic stem cells in EBs turned out to be a differentiation signal. The analysis of gene expression during differentiation indicates that EBs undergo a process of development resembling gastrulation in triploblastic embryos (C. Fuchs et al., 2012). The regional expression of specific

embryonic markers specific to different cellular lineages, namely, neurofilament (ectoderm), ζ-globin (mesoderm), and **α**-fetoprotein (endoderm) was found in human EBs (Itskovitz-Eldor et al., 2000). Although gastrulation is not demonstrated in all experimental studies, controlling the micro-structure, intracellular mass polarization, and cell junction, especially mechanical properties and spatial coordination of EBs in order to ensure the signaling pathway required for proper differentiation, are considered essential.

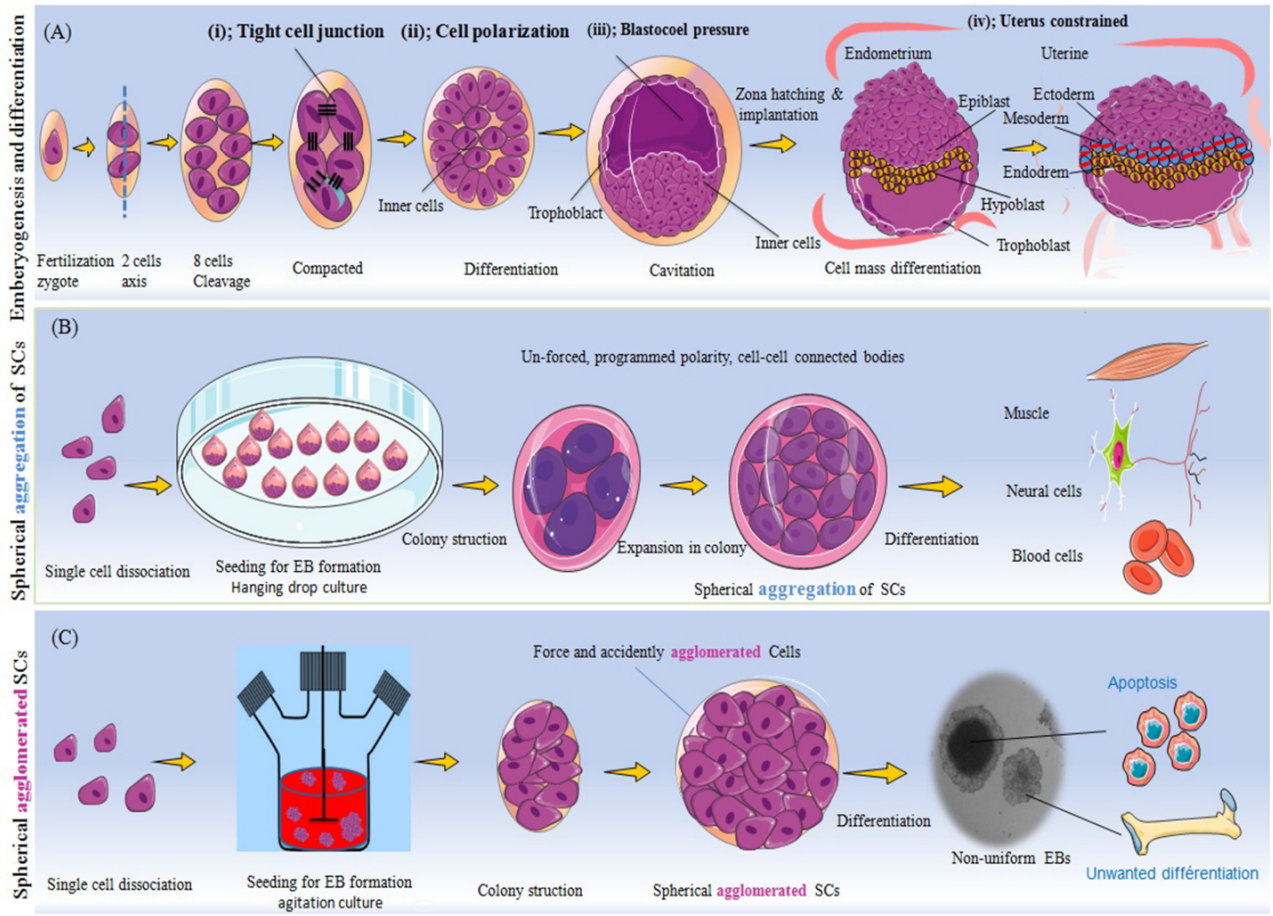

FIGURE 1| The effect of mechanical forces, cell polarity, and cell arrangement, as well as cell-cell junctions in the formation and differentiation of embryoid bodies (EBs), Panel A: In vivo mechanical cue in embryogenesis and gastrulation process including (i) Tight junction, (ii) Blastocoel pressure, (iii) Cell polarization, (iv) Uterus constrained that affects differentiation; Panel B: Shows that EB is formed by the re-arrangement and the compaction of stem cell aggregates. Consequently, the correct and promising differences

are related to EB sizes, uniformity, cell-extracellular matrix and cell-cell interactions, cell polarity, and non-accedental spatial coordination [64-66]; Panel C: Demonstrates that the forced agglomeration of stem cells, especially in agitation culture system may form defective EBs. These aggregated cells will go astray and will undergo the apoptosis or unwanted non-controlled differentiation.

3 | Multi-parameter Effects in EB Formation and Differentiation

There are several critical and some neglected parameters that directly affect the formation and differentiation programs including the size, uniformity, non-forced aggregation, and spatial coordination of EBs. Given the role of these parameters in the molecular pathways of formation and differentiation including Hippo signaling, and mechanotransduction, in the first, we discuss the mentioned parameters and the methods for controlling them.

3.1 | The Number of Cells and Inoculums Density

The defined number and spatial coordination of different cell-cell interactions involved in EB formation are considered to influence the size and uniformity of EBs and their synchronous differentiation (Sarvi, Arbatan, Chan, & Shen, 2013). If it is assumed that EB formation is dependent on the accidental contact of the stem cells with one another, the high density of seeded cells leads to the formation of a greater number of EBs compared with low densities of seeded cells (Sarvi et al., 2013). However, the forced aggregation of the high numbers of the stem cells into EBs leads to the formation of giant-sized EBs and generates tension within the internal cell mass. These are critical parameters that directly affect the differentiation pathway (i.e., the Hippo pathway and mechanotransduction). The size of EBs modulates the diffusion of the nutritive elements and the oxygen, and subsequently the cell secretome will change, and the disposition and the polarity of the cells will be affected. In this scenario, spatial coordination plays an important role in translating the mechanical forces into the intra- and extracellular biochemical cues through activating the signaling pathways (Sarem, Otto, Tanaka, & Shastri, 2019). The other factor is hypoxia in the core layer of EBs that could contribute to the change of metabolism and micro-environmental pH (Figure 2). The changed metabolism linked to the Hippo pathway involves in controlling the differentiation pathways (Liu, Tsai, Yuan, Li, & Ma, 2017). From another view, the low density of the stem cells must be considered if the aggregation of the stem cells is not accidental, but rather a dynamic smart spatial coordination process. The research specified that the defined inoculum density of the stem cells fosters robust and reproducible differentiation (Ng, Davis, Azzola, Stanley, & Elefanty, 2005).

3.2 | The Hydrodynamic Conditions

Stem cells react to a range of environmental cues *in vitro* to either self-renew or regulate differentiation. These cues include both exogenous and endogenous biochemical factors, cell junctions, cell-matrix interactions, and mechanical stimuli (Barui & Datta, 2019; Perestrelo, Correia, Ramalho-Santos, & Wirtz, 2018). The improvement of differentiation efficiency of EBs by controlling the liquid flow in the culture system is a requisite (Kato, Matsumoto, & Kino-oka, 2019). The aggregated cells can be disrupted by fluid flow in EBs, leading to the dissociation of the cells. Although fluid flow is known to influence individual stem cells, changes in EBs fate in shear stresses have received little attention (Kato et al., 2019). In addition, the culture systems keep a balance between the aggregation of ESCs necessary for EB formation and the prevention of EB agglomeration. Agglomeration of EBs was sequentially reported to have negative effects on proliferation/ differentiation of stem cells and cause extensive cell death (Kinney, Sargent, & McDevitt, 2011).

3.3 | EB Size

Stem cells aggregate in a micron-scale tissue, recreate biochemical and biophysical microenvironment that is defined by the intercellular adhesions and polarity, extracellular matrix, and signaling between the neighboring cells (Silva et al., 2019). EBs are typically produced from suspension cultures, resulting in heterogeneous micron-scale structures with a wide range of sizes and shapes. The EBs differentiation potential has been shown to be strongly influenced by the resultant size of the aggregate. The size of EBs controls the phenotype of individual cells, cell-cell interactions, deposition of extracellular matrix, as well as the diffusion of soluble growth factors, oxygen and nutrients, and thereby controlling the overall model behavior (Zhang & Xia, 2012). The key role of the EB size in the differentiation process could be explained in part by the diffusion of nutrients (e.g., oxygen and glucose) and cellular-signaling gradients within the aggregates. In this regard, it is preferred to use the EBs with uniform and precisely controlled sizes to better differentiate them into the specified lineages. In this respect, instead of suspension culture technique, a range of novel methods have been developed for the formation of EBs among which, the hanging-drop method allows one to control the EB size (Yamada et al., 2002). Moreover, the use of microwell array technique showed the control of size and shape of EBs. Hydrogel-based microwell platform showed that cardiogenesis was enhanced in larger EBs (~450 μm in diameter), and in contrast, endothelial cell differentiation was increased in smaller EBs (~150 μm in diameter)(Hwang et al., 2009). However, the number of EBs in microwell array is rather limited due to the involvement of a two-dimensional setting (Moeller, Mian, Shrivastava, Chung, & Khademhosseini, 2008). In addition, the size of the EBs could be readily controlled by changing the time of culture or using scaffolds with different pore sizes.

For example, micropatterning methods provide external forces or confined geometry to control the

homogeneity and diameter of EB but the force for aggregation or dispersing with the aim of a promising uniformity could affect the molecular signaling and differentiation. Further, using a method that disaggregates ESC colonies into several pieces of exactly desired sizes, the results of an attempt to improve the efficiency of differentiation by depositing the known numbers of ESCs, showed that it was impossible (Ng et al., 2005). Recently, EBs with a spatial pattern and controlled size have been engineered using laser-based 3D bioprinting (Kingsley et al., 2019). By adjusting the beam diameter of the laser, the authors could obtain size-controlled microbeads further processed into microcapsules that would constrain the final aggregate geometry.

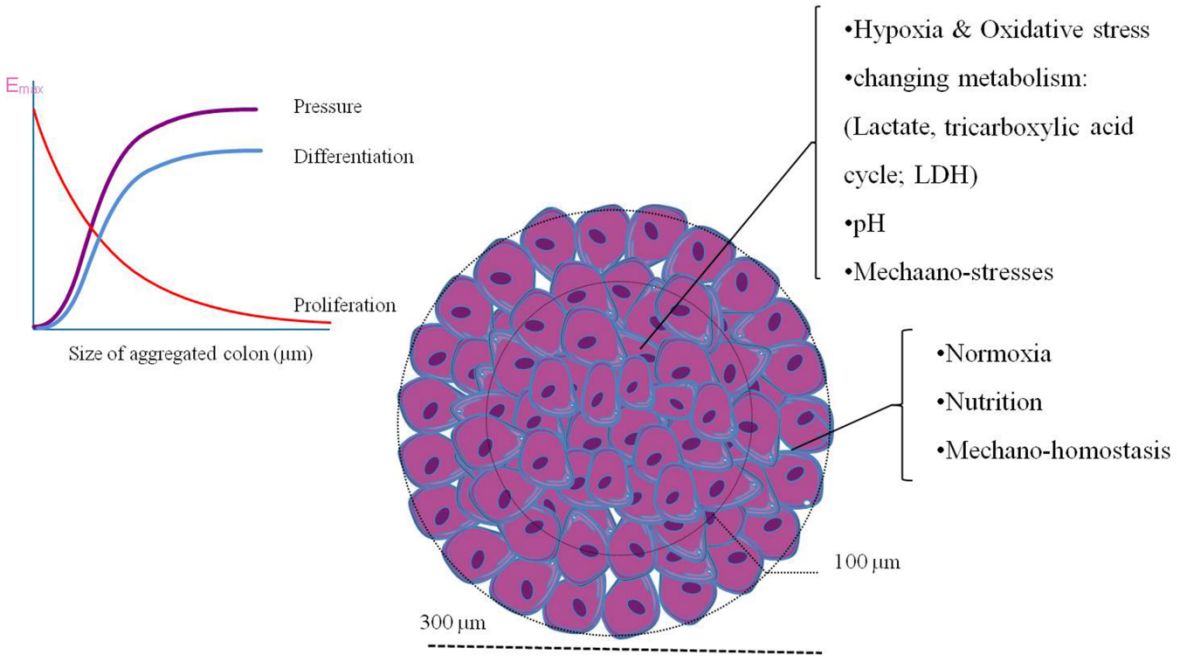

FIGURE 2| The micro-scale changes in the transport of nutrients, oxygen, and stem cell metabolism changes. The intensity of outer layers represents the concentrations of oxygen and nutrients within the populations of single cells. In addition, the concentration of nutrients and oxygen gradients arises in spheroid EBs, with diffusive limitations in large aggregates. Further, the alteration of metabolic pathways in the core of EBs leads to a change in the local concentrations of nutrients and metabolites, thereby affecting the proliferation and differentiation. In the hypoxia conditions, the changes in metabolism may lead to a change in the pH level and production of some metabolites. Finally, all metabolites and biomechanical pressure influence the differentiation.

3.4 | Asymmetric Division of Stem Cells

A hallmark of stem cells is the capacity to simultaneously do self-renewal and generate differentiated progeny (Knoblich, 2008). Furthermore, an essential characteristic of the stem cells is their capacity for

asymmetric division; in other words, daughter cells may either retain the stem cell identity or initiate differentiation. Moreover, the intricate balance between these two cell fates is maintained by exactly controlled symmetric and asymmetric cell divisions. For example, asymmetric cell divisions are responsible for the formation of the body axis during the development. The mitotic process of asymmetric cell division leads to the polarization and asymmetric separation of essential components for the determination of cell fate so that, once cell division is completed, one daughter cell receives proteins, RNA, and the additional molecules that continue the undifferentiated program, whereas the other cell receives lineage commitment factors (E. Fuchs & Chen, 2013). The balance between self-renewal and differentiation in the stem cells is controlled by a variety of biophysical and biochemical cues. Although the role of biochemical factors in stem cell asymmetric division is widely studied, the effect of biophysical cues in stem cell self-renewal is not comprehensively understood (Barui & Datta, 2018). The forced agglomeration of the stem cells influences the aggregated cells via cell-cell and cell-matrix interactions, as well as the asymmetric or symmetric division of the stem cells, thereby affecting the differentiation of EBs (Morrison & Kimble, 2006).

4 | The Molecular Cascade Involved in the Control of EB Formation and Differentiation

Recently, several studies have shown that EBs mimic the key milestones of mammalian embryogenesis and gastrulation. Similarly, EBs normally aggregate and grow to a specific size, which depends on poorly understood signaling mechanisms that coordinate and orchestrate the pattern and timing of cell proliferation and differentiation (Theka et al., 2019). Two major signaling pathways contribute to the stem cells and EB proliferation and differentiation, namely, mechanotransduction in an interplay with Hippo core signaling (He, Si, Huang, Samuel, & Perrimon, 2018; Park, Shin, & Park, 2018).

The study of the mechano-geometrical contributions to early mammalian development helps to understand EBs controlling and programming to our promising specific cells. Multiple mechanical transitions shape the embryo throughout the key milestones of development. Considering the increasing knowledge on signaling pathways in the cells, mechanosensing seems to be one of the missing pieces of the puzzle toward understanding the stem cell fate (i.e., cell proliferation, migration, and differentiation). By mechanotransduction cascade, cells translate mechanical cues into biochemical signals controlling several aspects of cell manners, including proliferation and differentiation. Piezo proteins, namely, the ion channels activated by membrane tension and mechanical signals, play an important role in translating the mechanical stress with the past memory, and inside of EBs mechano-information to intracellular signaling pathways, related to stem cell differentiation (He et al., 2018). If the aggregation of the stem cells to the spatial construction of EBs is non-accidental, Hippo signaling cascade controls the size through regulating cell proliferation and

apoptosis (Park et al., 2018). The next section focuses on Hippo cell signaling and its interplay with bio-mechanical cues in the formation and differentiation of the stem cells inside the EBs.

4.1 | Core Hippo Pathway Components and Their Adaptor Proteins

The Hippo signaling cascade, also known as the Salvador/Warts/Hippo pathway, controls the organ size through regulating cell proliferation and apoptosis. However, the rising evidence suggests that the Hippo pathway may also regulate the stem cell and progenitor cells' self-renewal and proliferation. The pathway takes its name from the protein kinase Hippo (Hpo), which is one of its key signaling components (Karaman & Halder, 2018). It promotes the activation of large tumor suppressor (LATS) kinases, which control gene expression by inhibiting the activity of transcriptional coactivator proteins, namely, Yes-associated protein (YAP) and TAZ. The regulation of the Hippo core includes the sequence of interactions and a phosphorylation cascade involved in the activation of LATS proteins. Hippo (Mst1/2 homologues of Hippo) is activated by phosphorylation within its kinase domain, which is mediated by the auto-phosphorylation or the action of Tao1. Additionally, Hippo phosphorylates Ser residues in its linker region, which recruits MOB. MOB is then phosphorylated by the Hippo and phosphorylated MOB recruits the LATS (Misra & Irvine, 2018). Next, LATS is phosphorylated by Hippo and autophosphorylated to generate the active LATS. This complex can be deactivated by the SLMAP-mediated recruitment of the striatin-interacting phosphatase and kinase PP2A phosphatase complex. This recruitment is inhibited by Salvador (Figure 3). In addition, inactivation of YAP protein leads to the silencing of its target genes, which include anti-apoptotic factors such as cyclin E, DIAP1, and the miRNA. Thus, the Hippo pathway controls tissue size by antagonizing YAP/TAZ function.

4.2 | Upstream Factors Influencing the Activation of the Core Hippo Pathway

The activity of the Hippo pathway is strongly dependent on different parameters such as cell junctions, cellular architecture, and the mechanical properties of the microenvironment (Karaman & Halder, 2018). In addition, several upstream factors influence the activation of the core Hippo pathway kinases and YAP (Misra & Irvine, 2018). Upstream biochemical cues and crosstalk from the other major signaling pathways include Ds–Fat signaling, epidermal growth factor receptor (EGFR), **wingless-related integration site** molecule (Wnt), bone morphogenetic protein (BMP), Hedgehog, and G-protein-coupled receptor, as well as integrin signaling, cell polarization, and cellular metabolic pathways. Further, upstream physical cues include F-actin levels, the tension in the cytoskeleton, cell-cell contact with focal adhesions, and mechanical stress (Figure 3).

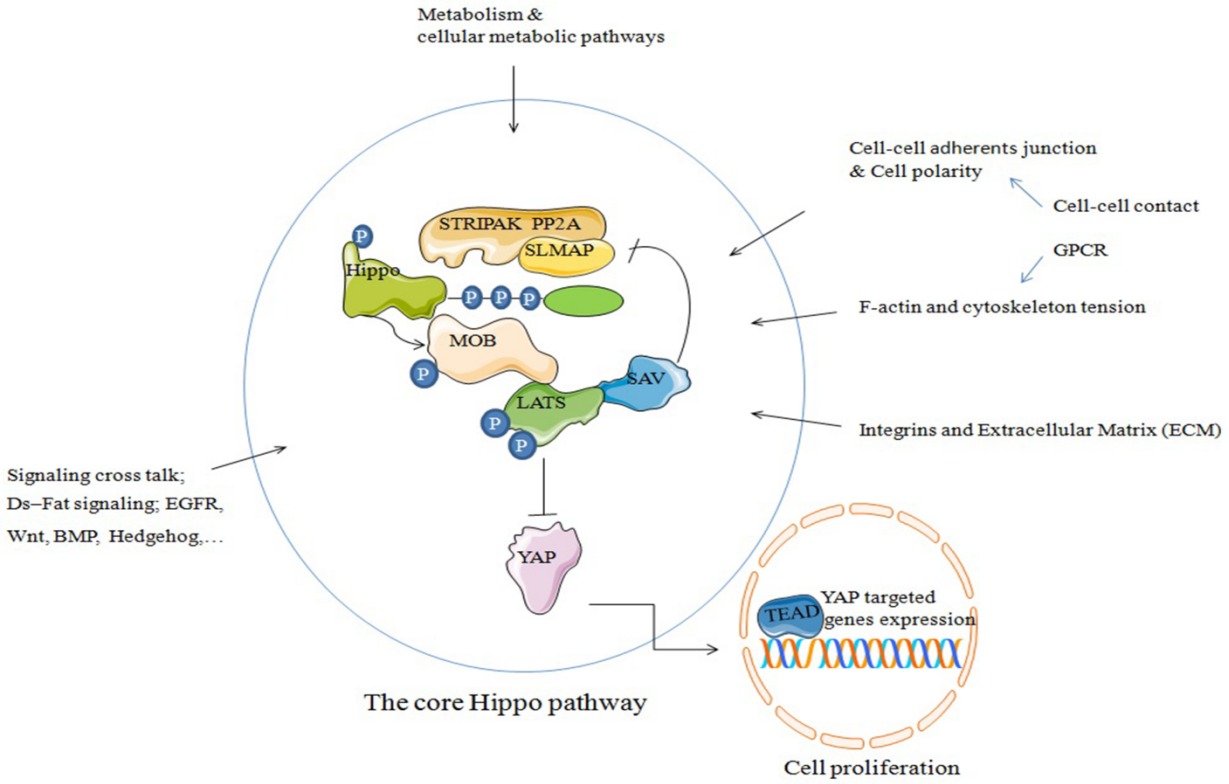

FIGURE 3| The activity of Hippo pathway heavily relies on cell junctions, cellular architecture, and the mechanical properties of microenvironment. Core Hippo pathway kinases phosphorylate the large tumor suppressor (LATS) family. Then, LATS family kinases inactivate YAP family proteins and this phosphorylation then inactivates YAP by inducing its retention in the cytoplasm by 14-3-3 proteins and thus preventing its association with its transcription factor partner (i.e., TEAD). Inactivation of YAP leads to the silencing of its downstream genes, which include anti-apoptotic factors such as cyclin E, DIAP1, and the microRNA. P: Phosphate; SARAH: Salvador/Rassf/Hippo domain; SAV: Salvador; STRIPAK: Striatin-interacting phosphatase and kinase; Ds: Dachsous; GPCR: G-protein-coupled receptor.

4.2.1 | Cell-cell Contact and Hippo Signaling

The proliferation inhibitory effect of cell-cell contact is essential for the development of embryo, and the maintenance of tissue architecture in adult organisms. The cell-cell contact strongly affects Hippo signaling, as well as the activation of LATS proteins. Thus, the isolated stem cells have typically high levels of YAP activity whereas cells cultured at high density or in EBs inner cell mass (ICM) have low levels. A chemical inhibitor of YAP acts as an inducer of differentiation and reducer of the proliferation of endocrine progenitor cells (Rosado-Olivieri, Anderson, Kenty, & Melton, 2019). In this respect, a study reported that

the Hippo pathway controls the proliferation and specification of pancreatic progenitors into the endocrine lineage (Rosado-Olivieri et al., 2019). The key contribution of cell-cell junctions is also reflected in the fact that the disruption of epithelial apical-basal polarity is associated with reduced Hippo signaling and the strong activation of YAP proteins (Misra & Irvine, 2018). The components of cell adherents junction complexes promote Hippo signaling; for example, the loss of Crb3, E-cad, α-Cat, or β-Cat can result in an increased YAP activity (Aragona et al., 2013; Pan, Heemskerk, Ibar, Shraiman, & Irvine, 2016). It is demonstrated that the high expression of LATS1/2 could activate the Hippo signaling pathway, promote mesenchymal stem cells (MSCs) differentiation into osteoblasts and adipocytes, and finally, inhibit MSCs proliferation (L. Li, Dong, Wang, Zhang, & Yan, 2018).

4.2.2 | Regulation of Hippo Signaling by Integrins and Extracellular Matrix (ECM) Attachment

The detachment of cultured cells from a substrate can cause cell death (anoikis) through the activation of Hippo signaling (Misra & Irvine, 2018; Zhao et al., 2012). Integrins play a crucial role in mediating the signaling from ECM, and multiple connections are identified between integrins and YAP activity. Integrin-linked kinase can inhibit the Merlin activation by inhibiting phosphatase MYPT1 or through activating RAC and PAK (Sabra et al., 2017; Serrano, McDonald, Lock, Muller, & Dedhar, 2013). Furthermore, it is found (Chakraborty et al., 2017) that the integrin-mediated regulation of Hippo signaling is linked to focal adhesion kinase (FAK). Integrins bound to fibronectin can stimulate FAK, which activates PI3K through Src. Moreover, PDK1, which is a downstream of PI3K, can then disrupt the core kinase cassette and inhibit Hippo signaling (Kim & Gumbiner, 2015). An alternative mechanism is also described for the FAK-linked regulation of YAP, involving the dephosphorylation of Ser397 by the protein phosphatase PP1A (Hu et al., 2017). Src can also promote YAP activity by its direct phosphorylation in addition to phosphorylating and inactivating the LATS (P. Li et al., 2016; Si et al., 2017; Taniguchi et al., 2015). The other downstream effectors of integrin signaling, including JNK, Rho, and Ras are also linked to the regulation of YAP proteins (Meng, Moroishi, & Guan, 2016). The related details are displayed in Figure 4.

4.2.3 | Cell Polarity and Hippo Signaling

Cell polarity is defined as spatial differences in the shape and structure inside a cell, which enables them to perform specialized functions. Furthermore, cell polarity is regarded important during various types of asymmetric cell divisions in order to set up functional asymmetries between the daughter cells. Previous research has demonstrated that apical-basal polarity, including tight junctions and the asymmetric distribution of organelles and cytoskeletal elements needs self-renewal and differentiation in the stem cells (Krtolica et al., 2007). Additionally, how ESCs become polarized and maintain this state is still unknown. The involved genes (i.e., ZO-1, E-cadherin, occludin, PKC ζ, PAR-3, along with PKC λ and laminin) are expressed in appropriate patterns and play important roles in cell polarization (Krtolica et al., 2007).

4.2.4 | Cell Metabolism and Hippo

There is a synergistic intertwining of metabolism, programming, differentiation, and fate. Cells rely on two pathways to make adenosine triphosphate (ATP), including glycolysis and oxidative phosphorylation (OXPHOS). In addition, cells have the flexibility to shift between metabolisms in order to match their requirements. Nonetheless, almost all differentiated cells rely on OXPHOS to complete their energetic requirements (Perestrelo et al., 2018). The skill to shift between metabolic states and to strongly regulate cellular mechanical properties is described as vital proceedings in achieving accurate embryonic development. These two modulators are usually studied separately, but they crosstalk in EB formation and differentiation. After EB formation, glycolysis as the main metabolic pathway inters the cell mass, where glucose is metabolized to lactate due to the hypoxic environment (Figure 2). Metabolism is a keystone in stem cell function and programming, chromatin remodeling, and gene expression regulation (Perestrelo et al., 2018).

4.3 | Downstream Factors Influencing the Activation of the Core Hippo Pathway

4.3.1 | YAP Proteins and Controlling Cell Fate Decisions

A wide variety of cell fate decisions depend on YAP proteins, including the earliest cell fate decision during mouse embryogenesis, the subdivision of the blastocyst into trophectoderm (TE), and the ICM (Nishioka et al., 2009). YAP is active in the outer blastocyst cells, which become TE whereas Hippo signaling in the inner cells suppresses YAP, and thus these cells form the ICM. Further, the regulation of Hippo signaling in the blastocyst is linked to differences in cell-cell contacts, cell polarity, and cytoskeletal tension between inner and outer cells (reviewed in (Sasaki, 2015)). For example, angiomotin is localized to the cell-cell junctions of the inner cells, where it promotes LATS activity, while it is localized to the apical membrane in the outer cells, where LATS is less active (Hirate et al., 2013). There is also evidence indicating that cell stretching contributes to YAP activation in TE cells (Maître et al., 2016). Many other cell fate decisions are influenced by YAP proteins as well. For instance, YAP directs the differentiation path of MSCs (Dupont et al., 2011). Furthermore, these proteins are linked to stem and progenitor cell fates in a wide range of tissues in mammals (reviewed in (Mo, Park, & Guan, 2014)). Similarly, YAP proteins suppress differentiation and promote the survival of the human ESCs (Lian et al., 2010). Meanwhile, the transient activation of YAP can convert differentiated cells into stem cells (Panciera et al., 2016). For instance, in response to TGFβ/BMP (i.e., transforming growth factor-beta/bone morphogenetic protein) signaling YAP and TAZ regulate ESCs self-renewal. In addition, YAP is inactivated during mouse ESC differentiation while it is activated in induced pluripotent stem cells. On the other hand, the knockdown of YAP in ESCs leads to the loss of pluripotency whereas the ectopic expression of YAP prevents the differentiation of ESCs. Further, the Hippo pathway regulates tissue-specific progenitor cells. Furthermore, the transgenic expression of YAP in mouse intestines causes a distinct expansion of the progenitor cell. Moreover, the physical

architecture of tissues can play a role in regulating the differentiation through Hippo signaling. For example, YAP is active in basal progenitor cells of the skin but inactive in most differentiating supra-basal cells due to the loss of contact with basement membrane and the acquisition of an apical membrane containing proteins that promote LATS activation (Elbediwy et al., 2016).

4.3. 2 | Regulation of Hippo Signaling by Crosstalk With Other Downstream Pathways

The integration of growth and cell fate decisions also occurs through the crosstalk of other signaling pathways with Hippo signaling, and so far, most major signaling pathways are found to regulate or crosstalk with Hippo signaling. For example, multiple G-protein–coupled receptor signaling pathways can regulate YAP through their influence on Rho activity (Yu et al., 2012). Several points of crosstalk with Wnt signaling are identified, including the interactions of YAP with Dsh and APC, as well as the coregulation of downstream genes by YAP and β-catenin (Hansen, Moroishi, & Guan, 2015). The interactions between YAP proteins and SMADs provide a point of crosstalk between Hippo and BMP pathways as does the regulation of Fat signaling by Dpp signaling (Varelas, 2014). EGFR and related pathways are also found to affect YAP activity through multiple mechanisms, including both Hippo pathway-dependent and -independent effects (Hong et al., 2014). The c-Jun N-terminal kinase signaling can exert multiple, even opposing effects on YAP activity as well (reviewed in (Irvine, 2012)).

5 | Mechanical Cues Interplay With Hippo/ Yes-associated Protein (YAP)

The mechanical properties of embryonic cells (ECs) are vital for their programmed development. The new knowledge of mechanical properties at different stages of development in embryo demonstrated the role of mechanosensing in cell fate decision. *In vitro* biophysical properties are the key modulators in ESCs fate and cellular programming. Mechanical properties have recently been documented as main regulators of cell fate. Changes in the mechanical properties of extracellular matrix (ECM), the stiffness of substrate, topography, and physical forces such as shear stress, tension, and compression are several factors of external mechanical cues that control cell fate through the mechanotransduction pathway. It is now confirmed that together with biochemical cues, these cues act in the regulation of embryogenesis (Perestrelo et al., 2018). Mechanical stimuli within the cells are converted to biochemical signals in a procedure called mechanotransduction. Meanwhile, Hippo signaling is sensitive to mechanical stress experienced by the cells. Therefore, the biomechanical regulation of Hippo signaling is revealed by the sensitivity of YAP localization to a variety of perturbations including the altered cell shape, ECM stiffness, cell stretching, cell density, and shear forces (Misra & Irvine, 2018).

Many of these effects depend on different factors such as the F-actin cytoskeleton, tension within the cytoskeleton, and the influence of this tension on cell-cell and cell-ECM attachments. Cytoskeletal tension is generated by myosin, and conditions associated with high levels of actomyosin contractility are generally

related to high YAP activity whereas conditions associated with low actomyosin contractility are due to low YAP activity (reviewed in(Misra & Irvine, 2018)). Moreover, in most cases, direct genetic or the pharmacological inhibition of actomyosin contractility decreases YAP activity while increasing actomyosin contractility leads to an increase in YAP activity. However, actomyosin contractility is reported, in some contexts, to inhibit rather than promoting YAP activity possibly because of the role of actomyosin activity in stabilizing cell-cell junctions (Furukawa, Yamashita, Sakurai, & Ohno, 2017; Hirata, Samsonov, & Sokabe, 2017). Hippo signaling can also feed back onto tissue mechanics and F-actin levels through YAP-dependent and-independent mechanisms. For example, YAP is essential for mechanical force production during lung branching morphogenesis.

Similarly, cells experience mechanical stress through their connections to the neighboring cells at adherens junctions, which are attached to the actin cytoskeleton. A related mechanism was recently identified in cultured mammalian cells by establishing that large tumor suppressor (LATS) proteins are recruited to adherens junctions and inhibited in a tension-dependent fashion (Ibar et al., 2018). Two different LIM domain-containing proteins are identified as required for this tension-dependent recruitment and the inhibition of LATS, namely, LIMD1 (Misra & Irvine, 2018).

6 | The interactions among Hippo signaling, mechanotransduction, and stem cells differentiation

In vivo gastrulation depends on the surrounding mechanical and biochemical cues linked to Hippo and mechanotransduction but the question is whether such cell signaling act in EBs or whether or not EBs pose the gastrulation process. Contrary to the previous view, EB formation and differentiation is not accidental, instead, they mimic the natural process of embryogenesis (Shkumatov et al., 2014). The expression of phenotypic markers of endoderm (e.g., Foxa2, Sox17, and GATA 4/6), mesoderm (e.g., Msp1/2, Isl-1, and Runx2), and ectoderm (e.g., Sox1 and Pax6) definitively reveal the skill of EBs to develop cells from all three germ layers (Itskovitz-Eldor et al., 2000).

Here, protein-protein interactions (PPIs) were analyzed using the STRING Database v. 10.5 (http://www.string-db.org), which includes direct and indirect protein associations with Hippo signaling, collected from different databases. Protein interaction networks were predicted using medium confidence scores (0.40) and clustered using MCL clustering algorithm (inflation parameter: 5). The proteins involved in mechanotransduction included Piezo1, Piezo2, TMC2, KCNK4, TRPA1, PKD2, and STOML3. The Hippo pathway encompassed proteins such as MST1/2, SAV1, LAST1/2, MOB1B, WWTR1, and YAP/TAZ, and differentiation proteins were involved in ectoderm, mesoderm, and endoderm formation (Figure 4).

There are several proteins that contribute to the main connection among the mentioned pathways. WW

domain-containing transcription regulator protein 1 (WWTR1) is a transcriptional coactivator which acts as a downstream regulatory target in the Hippo signaling pathway and plays a pivotal role in organ size. YAP is also a transcriptional regulator which can act both as a coactivator and a corepressor and is the critical downstream regulatory target in the Hippo signaling pathway which plays a pivotal role in organ size control. These two genes are major proteins in Hippo signaling in interaction with differentiation pathways and may in EB formation and size. Calpain small subunit 1 (CAPNS1) as a regulatory subunit of the calcium-regulated and PIEZO1 as mechanosensor channels are the main proteins of mechanotransduction that are in direct interaction with differentiation. Furthermore, the interaction between mechanotransduction and Hippo signaling is predicted, as Polycystin-2 (PKD2) that functions as a cation channel is involved in mechanosensation.

Experimental studies need to investigate the more vital role of these proteins and the interaction among three protein networks including mechanotransduction, hippo network, and three-layer cell type differentiation.

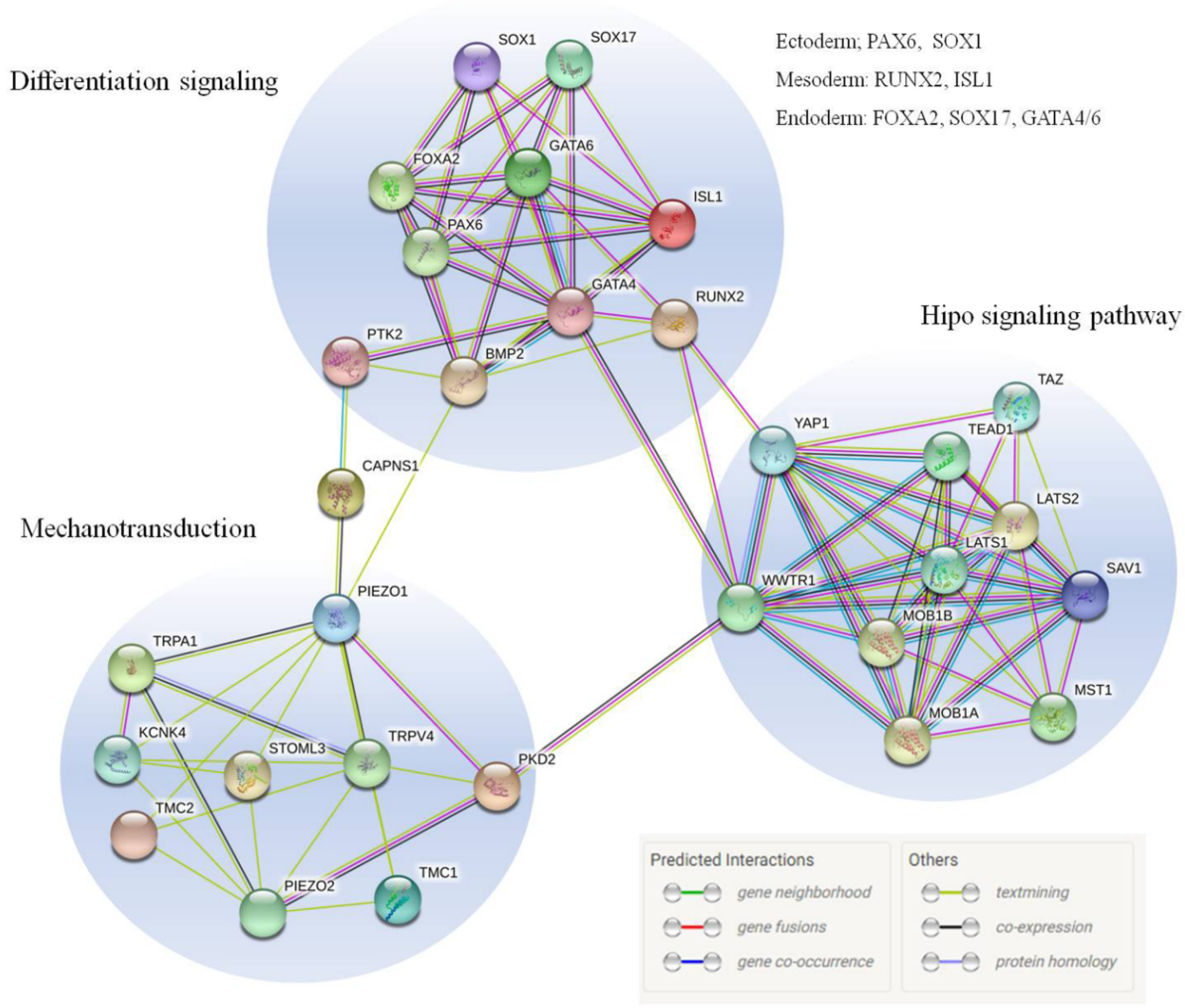

FIGURE 4| The cell fate stimulated by hippo signaling in coupling with direct and indirect protein associations in determination of stem cells proliferation and differentiation. The interaction network was prepared using the STRING Database v. 10.5 (http://www.string-db.org), collected from different databases. The medium confidence score was 0.40 and MCL clustering algorithm (inflation parameter: 5) was used. WW domain-containing transcription regulator protein 1 (WWTR1) is a transcriptional coactivator which acts as a downstream regulatory target in the Hippo signaling pathway and plays a pivotal role in organ size. YAP is also a transcriptional regulator which can act both as a coactivator and a corepressor and is the critical downstream regulatory target in the Hippo signaling pathway which plays a pivotal role in organ size control. These two genes are major proteins in Hippo signaling in interaction with differentiation pathways and may in EB formation and size. Calpain small subunit 1 (CAPNS1) as a regulatory subunit of the calcium-regulated and PIEZO1 as mechanosensor channels are the main proteins of mechanotransduction that are in a direct interaction with differentiation. Furthermore, the interaction between mechanotransduction and Hippo signaling is predicted, as Polycystin-2 (PKD2) that functions as a cation channel is involved in mechanosensation.

7 | CONCLUSION

The present study intended to answer this question that why the differentiation of embryoid bodies (EBs) in previous studies has had contradictory outcomes. The EB formation and differentiation are still not completely controlled, probably due to the insufficient knowledge of the biochemical and biophysical parameters of the EBs and its microenvironment. Although EBs have a spheroid shape, they are not the results of an accidental agglomeration of cells; they are deliberate aggregated cells with a potential smart programming for differentiation (Rungarunlert et al., 2009). The forced aggregation of the stem cells produces defective EBs with a changed cell-cell junction and polarity. Moreover, the size and uniformity of hydrodynamic conditions affect the EB formation. There are several methods for inducing the formation of EBs from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). The ideal system must avoid the formation of EBs with an optimal size, the agglomeration of cells, cell polarity defects, and past mechanical stresses. Additionally, forced aggregation disturbs normal metabolism and cell signaling and may influence the promising differentiation. Two major signaling pathways are reported in the stem cells and EB proliferation and differentiation (He et al., 2018; Park et al., 2018). By mechanotransduction cascade, cells translate mechanical cues into biochemical signals, thereby controlling several aspects of cell manners, including proliferation and differentiation. Piezo proteins, as the ion channels activated by membrane tension and mechanical signals, have an essential role in translating mechanical stress with the past memory, and the intra-EB mechano-information to the intracellular signaling pathways that are related to stem cell differentiation (He et al., 2018). If the aggregation of the stem cells to the spatial form of EBs are non-accidental, hence the Hippo signaling cascade controls the size through the regulation of cell proliferation and apoptosis (Park et al., 2018).

During the last decade, extensive studies have clarified the importance of Hippo pathway in the regulation of organ size, and several mechanisms have been proposed in this regard. Clearly, the cell polarity and adhesion complexes play a key role in Hippo pathway modulation. YAP/TAZ may promote organ size by inducing the progenitor cell proliferation. In addition, the inhibition of the core hippo may block the cell-cycle exit, leading to hyperplasia and differentiation defects. The Hippo pathway can also block proliferation and induce apoptosis in non-stem cells/non-progenitor cell types. Finally, an imbalance in Hippo signaling regulation in the neighboring cells may promote the cell competition through differential expressions. Moreover, the integration of growth and cell fate decisions occurs through the crosstalk of the other signaling pathways with Hippo signaling, and by now, most major signaling pathways have been found to regulate or crosstalk with Hippo signaling (Yu et al., 2012).

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General Conclusion and Future Directions

Conclusion Générale

Le tissu adipeux comme source de cellules souches mésenchymateuses.

Les capacités des CSMs à se renouveler et à se différencier en plusieurs types cellulaires ont fait de ces cellules une ressource thérapeutique majeure. (Lukomska *et al*., 2019). Les CSMs transplantées exercent des effets régénératifs dans les tissus lésés, les mécanismes proposés dans les processus de régénération comprennent la fusion cellulaire et les effets paracrines (immunosuppresseurs et anti-apoptotiques) et la différenciation ou la stimulation des cellules souches progénitrices locales (Teixeira & Salgado, 2020). Le tissu adipeux en tant que source de cellules souches présente plusieurs avantages en comparaison à d'autres sources. Il est facilement accessible en grandes quantités avec une procédure de récolte peu invasive et produit une grande quantité de cellules souches, point important pour la réussite les thérapies à base de cellules souches autologues et hétérologues permettant une injection directe et sans besoin de d'expansion. Dans notre étude, nous avons isolé les CSMs en raison de la potentialité thérapeutique. Pour cette étude, nous avons choisi les lambeaux adipeux comme source d'isolement des cellules souches et après caractérisation des marqueurs CD de surface cellulaire et différenciation cellulaire et moléculaire, nous avons selectionné les cellules exprimant les gènes de chemiotaxis et homing (CXCR4, CXCR7 et SDF1).

Les restrictions de la thérapie cellulaire aux CSMs

Dans la thérapie cellulaire, les CSMs sont isolées des tissus natifs (niche de cellules souches) puis, après expansion (ou sans expansion), les CSMs sont délivrés aux tissus lésés (niche dysfonctionnelle). Lorsque la fonctionnalité des niches dans les tissus lésés est déficiente cela peut entraîner la perte ou la dérégulation des cellules souches natives et/ou transplantées. Dans les tissus blessés il existe d'interactions entre les cellules, des signaux chimiques, la matrice extracellulaire et le système vasculaire. Les cellules sont maintenues par une signalisation concertée avec les cellules de la niche ; les signaux comprennent des signaux paracrines, autocrines et juxtacrines. Ainsi, les composants de la niche dans le site endommagé sont modifiés. Les cellules souches elles-mêmes ainsi que les cellules stromales, les facteurs solubles, la matrice extracellulaire, les connections neuronales, le réseau vasculaire et les composants de l'adhésion cellulaire subissent des perturbations et sont susceptibles de changer (Ushio-Fukai & Rehman, 2014). De cette manière, les signalisations moléculaires impliquées pourraient être affectées et de ce fait conduire à une programmation cellulaire indésirable.

Comme nous avons indiqué précédemment, un des principaux obstacles à la thérapie CSMs est le faible taux de survie après transplantation dû à la mort cellulaire par anoikis, au stress oxydatif et à la perturbation

du métabolisme énergétique (Chang, Song et Hwang, 2013). Des stratégies sont en cours d'élaboration pour améliorer les propriétés bio-fonctionnelles des CSMs explantées, telles que la pré-culture des cellules dans des conditions hypoxiques et le prétraitement avec des antioxydants. Ainsi, dans notre étude, nous avons utilisé une thérapie antioxydante pour évaluer l'adaptation des cellules souches en conditions d'hypoxie. Cependant il est clair que la relation entre taux d'hypoxie et concentration de ROS suit un modèle d'interaction très complexe dans la biologie des cellules souches.

Les ROS régulent la réponse des cellules souches à l'hypoxie.

Les ROS ont toujours été considérés comme des sous-produits métaboliques toxiques et des agents responsables d'une myriade de pathologies humaines. Des travaux plus récents, cependant, indiquent que les ROS sont des intermédiaires critiques des voies de signalisation cellulaire. Les producteurs de ROS cellulaires dédiés tels que les NADPH oxydases participent à la signalisation cellulaire (Hamanaka & Chandel, 2010). La production de ROS par les mitochondries est une réponse à l'état d'énergie cellulaire, aux concentrations de métabolites et à d'autres signaux en amont.

La réponse au stress mitochondrial induit par un stimulus potentiellement nuisible nécessite un dialogue coordonné entre la mitochondrie et le noyau cellulaire, connu sous le nom de communication mitonucléaire. Cette interaction est induite par la réponse hormétique dans les mitochondries et repose sur une variété de signaux parmi lesquels interviennent les ROS, les métabolites mitochondriaux, les signaux protéotoxiques, la réponse au stress mitochondrie-cytosol et la libération de mitokines (Bárcena, Mayoral et Quirós, 2018). Bien que plusieurs médiateurs et signaux de stress aient été proposés pour activer ce mécanisme de protection, les résultats bénéfiques de la mitohormèse sont probablement dus à une augmentation des ROS mitochondriaux. L'oxygène est l'un des composants essentiels de la niche (Mohyeldin, Garzón-Muvdi et Quiñones-Hinojosa, 2010), de faibles tensions en oxygène (hypoxie) maintiennent les phénotypes des CSMs dans des états indifférenciés et influencent également la prolifération et l'engagement du destin cellulaire (Eliasson & Jönsson, 2010).

Ainsi, le traitement des cellules souches avec des antioxydants qui est notre objectif de recherche est un paradigme. Plusieurs études ont montré que dans les tissus lésés, la tension d'oxygène est très faible par rapport aux microenvironnements de niche physiologiques des cellules souches. La figure 1 résume l'homéostasie redox dans l'auto-renouvèlement et la différenciation des cellules souches.

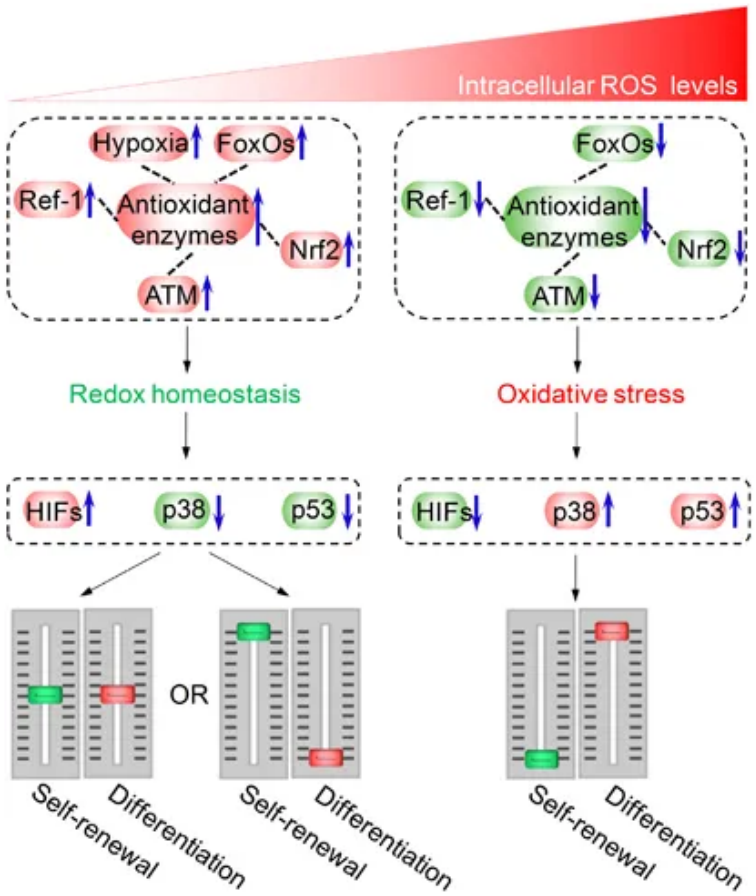

Figure 1. Homéostasie redox, auto-renouvèlement et différenciation des cellules souches. Les faibles niveaux de ROS maintiennent l'auto-renouvèlement des cellules souches et l'homéostasie redox, qui est bien régulée par les systèmes de défense enzymatique antioxydante et les niches hypoxiques ainsi que plusieurs régulateurs redox clés FoxO, Nrf2, l'endonucléase apurinique / apyrimidinique (AP) / facteur redox 1 (APE1 / Ref-1) et l'ataxie télangiectasie mutée (ATM); des niveaux élevés de ROS provoquent une différenciation anormale, une apoptose ou une sénescence des cellules souches par les molécules sensibles aux ROS, y compris les facteurs inductibles par l'hypoxie (HIF), p38 et p5 (Wang et al., 2013) Licence N4752901240168 @Elsevier ltd.

Les stratégies d'adaptation des cellules souches à l'hypoxie et au stress oxydatif.

Comme mentionné précédemment, les espèces oxygénées réactives sont des molécules de signalisation vitales dans la physiologie cellulaire. Cependant, en conditions d'hypoxie induite dans une zone lésé, les obstacles majeurs à la régénération du tissu, sont la très faible tension d'oxygène et l'inactivation des acteurs moléculaires spécialement HIF1α (Chang et al., 2013). Des stratégies sont en cours d'élaboration pour améliorer les propriétés bio-fonctionnelles des CSMs, telles que la pré-culture des cellules dans des conditions hypoxiques et le prétraitement avec des antioxydants. Il existe un certain nombre de stratégies qui ont été utilisées pour améliorer l'imbrication et la greffe de cellules. L'hypoxie relative est de loin la stratégie de

préconditionnement la plus étudiée pour modifier les cellules souches en culture afin de développer leur capacité de nidification (Theus et al., 2008).

Préconditionnement à l'hypoxie et adaptation à la niche dysfonctionnelle. La tension d'oxygène dans la plupart des conditions de culture cellulaire est généralement de 21%, mais la majorité des CSMs résident dans des niches par exemple dans la moelle osseuse dans lesquelles la tension d'oxygène est en moyenne de 5% et aussi faible que 1% dans le tissu ischémique (Miller, 2016). Réduire la tension d'oxygène à 1% en culture entraîne la perte d'un certain nombre de cellules, mais c'est le moyen le plus efficace de reproduire leur environnement natif dans le tissu cible où elles peuvent être placées et de définir les cellules les plus viables collectées (Miller, 2016). La signalisation moléculaire et, par conséquent la commutation métabolique pendant l'hypoxie ont pour objectif essentiel de réduire l'accumulation de ROS dérivées du métabolisme oxydatif, la prolifération cellulaire et la programmation de l'adaptation à la niche (C.C. Zhang et Sadek, 2014). Des études récentes indiquent que les différences dans les profils métaboliques des cellules souches pluripotentes sont étroitement liées à l'auto-renouvellement et à la décision initiale du destin cellulaire des cellules souches. Gu *et al* ont rapporté que les cellules souches pluripotentes naïves chez l'homme présentent un flux glycolytique élevé et une augmentation de l'activité de la voie du pentose phosphate (Gu *et al*., 2016). De plus, la commutation métabolique induite par l'hypoxie empêche la génération excessive de ROS et réduit la demande d'ATP, toutes deux étroitement associées au maintien des fonctions physiologiques et à la survie des cellules souches (Son et al., 2013). Afin de mimer l'hypoxie et le déficit respiratoire, dans cette étude nous avons utilisé l'antimycine A (AMA), un inhibiteur de la respiration cellulaire, en particulier la phosphorylation oxydative. L'AMA se lie à la cytochrome c réductase, provoque la perturbation de toute la chaîne de transport d'électrons et la formation du gradient de protons à travers la membrane interne des mitochondries. Cette inhibition entraîne également la formation de radicaux libres toxiques, les ions superoxyde ($O_2^{\cdot-}$) à des taux dépassant les mécanismes endogènes pour les neutraliser et entraînant mort cellulaire (Tzung et al., 2001). Dans cette recherche, nous n'avons pas utilisé de traitement au peroxyde d'hydrogène pour l'induction du stress oxydatif car le H_2O_2 lui-même agit directement comme messager du signal cellulaire et sans neutralisation mitochondriale, de faux positifs peuvent être obtenus grâce à la potentialité protectrice antioxydante. Les antioxydants peuvent neutraliser H_2O_2 par réaction chimique dans le cytoplasme, tandis que l'AMA induit la production de H_2O_2 par la cellule laissant aux cellules la décision et la possibilité de neutraliser les radicaux libres via des acteurs moléculaires ou par d'autres fonctions endogènes.

Prétraitements antioxydants. De nombreuses études ont rapporté que l'utilisation de molécules antioxydantes pour le prétraitement des cellules souches pourrait les protéger des conditions hypoxiques et pourrait

représenter une approche nouvelle et sûre pour améliorer les effets bénéfiques de la thérapie cellulaire (Mias, Trouche, Seguelas, Calcagno, Dignat - George et al., 2008). Ji Yong Kim *et al* ont signalé que le prétraitement des CSMs avec du lycopène atténuait le stress oxydatif et l'apoptose (Kim et al., 2015). Une autre étude a montré que la delphinidine empêche l'apoptose des cellules souches embryonnaires de souris induite par l'hypoxie par la réduction des ROS et l'activation de certaines voies moléculaires, dont JNK, NF-κB et Akt (Seo *et al*., 2013). Le prétraitement des CSMs avec de la mélatonine a amélioré la survie, l'activité proangiogénique et l'efficacité après injection dans le rein ischémique (Mias *et al*., 2008). De nombreux autres antioxydants, dont les caroténoïdes et les vitamines, ont été utilisés à cette fin. Dans cette étude, nous avons utilisé deux antioxydants, dont le mito-TEMPO et la N-acétylcystéine (NAC). Le NAC est le dérivé N-acétyle de l'acide aminé L-cystéine, et est un précurseur dans la formation du glutathion antioxydant dans les cellules. Le glutathion est largement connu pour minimiser la peroxydation lipidique des membranes cellulaires et bloquer le stress oxydatif mitochondrial. Un autre rôle important du glutathion concerne son impact sur la transduction du signal et l'expression des gènes à l'intérieur des cellules et également dans le métabolisme énergétique (Dringen et Hamprecht, 1998). Le NAC pourrait cibler les radicaux libres directement et indirectement et participer à l'homéostasie énergétique. Les CSMs ont été exposés à l'AMA comme inhibiteur de la respiration cellulaire. Sur la base de nos résultats, lors de l'exposition des CSMs aux conditions de déficience de la chaîne respiratoire, les cellules n'ont pas réussi à piéger les radicaux libres et le métabolisme énergétique a été perturbé. Le prétraitement avec le NAC a atténué les dommages à l'ADN, l'apoptose cellulaire et le stress oxydatif via les voies Nrf2 / Sirt3 et d'autres acteurs moléculaires, sans avoir d'effet sur le potentiel de la membrane mitochondriale ni la production d'ATP.
Le dysfonctionnement mitochondrial est probablement très important dans les CSMs dans un microenvironnement hypoxique. Pour cibler le stress oxydatif mitochondrial nous avons utilisé comme antioxydant le mito-TEMPO (mito-T). Les études antérieures ont rapporté que le mito-T pouvait affecter les tissus ischémiques liés au stress oxydatif induit par l'hypoxie (Du et al., 2019; Nautiyal, Shaltout, Chappell et Diz, 2019). Dans notre étude, le mito-T a considérablement réduit le stress oxydatif mitochondrial induit par l'AMA et a ainsi efficacement protégé les CSMs. L'analyse du cycle cellulaire a révélé que dans les cellules traitées par l'AMA, des cellules arrêtées en phase G0 / G1 étaient observées à environ 40%, tandis qu'un co-traitement avec mito-T arrêtait les cellules autour de 20% par rapport au contrôle. Ce résultat a montré que le mito-T n'agit pas seulement par piégeage des radicaux libres mais il joue un rôle dans les voies de signalisation.

Les limites de cette étude. Nos résultats montrent que la seule l'utilisation d'antioxydants dans le soutien des cellules souches en conditions hypoxiques ne suffit pas, car le métabolisme énergétique reste dysfonctionnel. De plus il apparaît clairement qu'une étude linéaire de l'effet des prétraitements sur des cellules

souches, y compris les prétraitements antioxydants et également le préconditionnement à l'hypoxie ne sont pas appropriés pour l'évaluation du devenir cellulaire. En effet, les signalisations cellule-cellule, l'adaptation des cellules souches par HIF1α / Sirtuins, les composants de la niche native et des systèmes de mécanotransduction pourraient chacun d'entre eux modifier les résultats linéaires obtenus avec des antioxydants dans le soutien des cellules souches en conditions d'hypoxie. De ce fait, il est nécessaire de compréndre de l'ensemble des facteurs intervenant et de les intégrer dans l'étude.

Par ailleurs, le terme antioxydants peut dans certains cas induire en confusion. Par exemple, les substances dont l'effet antioxydant a été démontré en laboratoire, les « antioxydants directs » n'ont parfois pas le même effet dans les cellules. Dans ce cas, le terme d'antioxydant pourrait être revu, en raison du mode d'action pléiotrope de ces substances bioactives. Par exemple, dans physiologie des cellules souches, l'acide ascorbique (vitamine C) agit non seulement comme un antioxydant, mais il pourrait induire les CSMs à se différentier en cellules ostéoblastiques (H. K. Choi et al., 2019; K.-M. Choi et al., 2008)

Orientation future et suggestions. Comme mentionné, dans la niche des cellules souches natives, les niveaux de ROS mitochondriaux sont vitaux pour la fonction biologique des cellules souches, y compris leur prolifération et leur maintien dans un stade indifférencié ou leur progression vers la différenciation. La niche est une structure complexe et dynamique qui transmet et reçoit des signaux par le biais de médiateurs cellulaires et acellulaires. Une niche est un réseau très compliqué, composé de protéines, de protéoglycanes, de signaux solubles (facteurs de croissance, cytokines, chimiokines), de signaux physiques (interactions avec des protéines telles que fibronectine, vitronectine, laminine, collagène, fibrilline) et de signaux provenant d'interactions cellule/cellule. Certains de ces composants peuvent être absents dans les tissus lésés. Après la transplantation de CSMs dans des lésions tissulaires, la mort des cellules souches que peut être détectée dès les premiers jours, n'est pas due ne totalité au stress oxydatif. Les anoikis, l'inflammation, le déficit de la chaîne respiratoire pourraient affecter le sort des cellules souches transplantées (Sart, Ma et Li, 2014). Ainsi, la manipulation des cellules souches et le préconditionnement des cellules souches pour former une mémoire et induire la signalisation de l'homéostasie de l'hypoxie sont nécessaires pour soutenir les cellules souches dans les tissus des lésions d'hypoxie, mais ce n'est pas suffisant. Il est nécessaire de prendre en compte les composants des niches natives afin de mimer par ingénierie tissulaire une nouvelle niche pour soutenir les cellules souches transplantées dans des conditions difficiles. Cependant, les composants précis peuvent être différents pour chaque type de cellules souches et au sein de chaque niche de cellules souches et source isolée, en particulier dans les tissus lésés.

Compte tenu de limites de notre étude, pour nos recherches futures nous suggérons :

• La répétition et la confirmation des résultats, avec une étude portant sur différents types de cellules souches.

• La prédiction du comportement des cellules en conditions hypoxiques avec une étude réalisé en parallèle sur plusieurs types de cellules normales et souches.

• La conception des systèmes de culture tridimensionnels prenant en compte les composants natifs de la niche avec l'ajout des molécules bioactives et des antioxydants.

General Conclusion and Future Directions

Adipose tissue, as the source of Mesenchymal stem cells. The abilities of MSCs to self-renew and differentiate into various cell types have made these cells a major resource for stem cell-based therapies*(Lukomska et al., 2019)*. The putative mechanisms by which transplanted MSCs exert regenerative effects in injured tissues include cell fusion, and paracrine effects, such as immunosuppressive and anti-apoptotic effects and differentiation or the stimulation of local progenitor stem cells(Teixeira & Salgado, 2020). Adipose tissue as a stem cell source has several advantages compared to other sources. It is easily accessible in large quantities with minimal invasive harvesting procedure, and yields a high amount of stem cells, which is essential for autologous and heterologous stem-cell-based therapies with direct injection and without need to more expansion. In addition, AD-MSCs possess the highest homing in the hypoxia/ischemic tissues due to the over expression of some molecular players including, the stromal cell-derived factor-1 (SDF-1) and chemokine receptor 4 (CXCR4) that play a major role in directing MSC homing to sites of injury(D. Zhang et al., 2008). For this reason, we choosen adipose tissues stem cell source and after characterization for cell surface CD markers and differentiation cellular and molecular certifying, we screened the cells under hypoxia for homing chemotaxis genes including, CXCR4, CXCR7 and SDF1. So, screened mesenchymal primary cells that we used in our studies was possed hypoxia resistancy behavior. Specillay these cells under hypoxia about %3 O_2 overexpressed the hypoxia master transcription factor that could upregulate the other signaling to nesting and adaptation at hypoxic harsh condition.

What is the MSCs therapy of injured tissues significant obstacles*?* In the cell therapy we isolate the MSCs from native tissues (niche of stem cells) and then, after expansion or without expansion, they are delivered to the injured tissues (dysfunction niche). Deficient niche function in the injury tissues may cause the loss or deregulation of native or transplanted stem cells. Injured tissues, involve a series of interactions between cells, chemical signals such as cytokines and growth factors, the extracellular matrix and the vasculature(Singer & Clark, 1999). The cells are maintained by signaling in concert with niche cells-signals include paracrine , autocrine and juxtacrine signals. So, the niche component in this damaged site will change. The stem cell itself, stromal cells, soluble factors, extracellular matrix, neural inputs, vascular network and cell adhesion components all will disrupt and change(Ushio-Fukai & Rehman, 2014). In this way, the molecular cell signaling involving in the inducing the fate will be affected or will be go to undesirable fate programming. The major obstacle in MSC therapy is the low survival rate after transplantation due to cell death via anoikis, oxidative stress and energy metabolism disruption(Chang, Song, & Hwang, 2013). Strategies are being developed to improve the bio-functional properties of MSCs such as

pre-culturing of the cells in hypoxic conditions and pretreatment with antioxidants. So, in our study we used antioxidant therapy for adaptation of stem cells in hypoxic condition. But there is one paradigm in the effect of hypoxia and free radical in biology of stem cells. And the search in this paradigm would be necessary to the clear of all players in stem cells therapy outcome.

ROS regulate the stem cells response to hypoxia. ROS have historically been viewed as toxic metabolic byproducts and causal agents in a myriad of human pathologies. More recent work, however, indicates that ROS are critical intermediates of cellular signaling pathways. Although it is clear that dedicated cellular ROS producers such as NADPH oxidases participate in signaling(Hamanaka & Chandel, 2010). Production of ROS at mitochondria thus integrates cellular energy state, metabolite concentrations, and other upstream signaling events and has important implications in cellular stress signaling, maintenance of stem cell populations, cellular survival(Hamanaka & Chandel, 2010).

The mitochondrial stress response activated by a potentially damaging stimulus requires a coordinated dialogue with the cellular nucleus, known as mitonuclear communication. This interplay induced by the hormetic response in mitochondria relies in a variety of signals among which the most relevant ones are reactive oxygen species (ROS), mitochondrial metabolites, proteotoxic signals, the mitochondria-cytosol stress response, and the release of mitokines(Bárcena, Mayoral, & Quirós, 2018). Although multiple mediators and stress signals have been proposed to activate this protective mechanism, beneficial outcomes of mitohormesis are most probably due to an increase in mitochondrial ROS. Activation of other protective stress mechanisms as mitochondrial unfolded protein response or the increase in the expression of mitokines are also associated with the positive benefits exerted by mitohormesis (Bárcena et al., 2018). As mentioned above, ROS cold be very important in stem cells behaviors as well. Oxygen is a one of the critical component of niche(Mohyeldin, Garzón-Muvdi, & Quiñones-Hinojosa, 2010). Low oxygen tensions (hypoxia) maintain undifferentiated states of MSCs phenotypes and also influence proliferation and cell-fate commitment(Eliasson & Jönsson, 2010). So the treatment of stem cells with antioxidants that is the our research aim is a paradigm. But several studies showed that in injury tissues the oxigene tension is very low than physiological stem cell niche microenvironments. The figure 1 summarized redox homeostasis in stem cell self-renewal and differentiation.

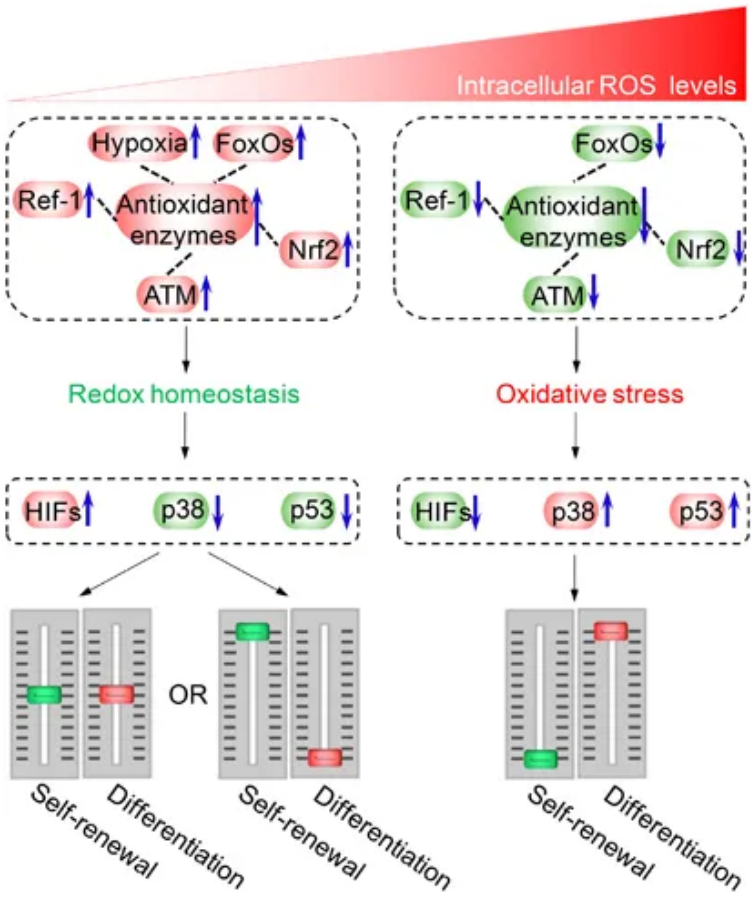

Figure1. *Redox homeostasis and stem cell's self-renewal and differentiation. Low ROS levels maintain the self-renewal of stem cells by monitoring the redox homeostasis, which is well regulated by the antioxidant enzymatic defense systems and hypoxic niches as well as several key redox regulators such as forkhead homeobox type O family (FoxOs), nuclear factor erythroid-2-related factor 2 (Nrf2), apurinic/apyrimidinic (AP) endonuclease1/redox factor-1 (APE1/Ref-1) and ataxia telangiectasia mutated (ATM); high ROS levels cause abnormal differentiation, apoptosis or senescence of stem cells by the ROS-sensitive molecules, including hypoxia-inducible factors (HIFs), p38 and p5(Wang et al., 2013) copy right @Elsevier ltd. 2010.*

The strategies for adaptation of stem cells in hypoxia and oxidative stress. As mentioned, although, ROS are a vital signal molecules in biology of cells and stem cells however in hypoxia conditions induced by injures, a major obstacle is oxygen tension in very low concentration and the inactivation of molecular players specially HIF1α (Chang et al., 2013). Strategies are being developed to improve the bio-functional properties of MSCs such as pre-culturing of the cells in hypoxic conditions and pretreatment with antioxi-dants. There are a number of strategies that have been used to improve cell nesting and engraftment. By far the most studied preconditioning strategy to modify stem cells in culture to develop their nesting capacity

is relative hypoxia(Theus et al., 2008).

Hypoxia preconditioning and adaptation to the injuries dysfunctional niche. The oxygen tension in most cell culture conditions is typically 21%, but the majority of MSCs reside in bone marrow niches in which the oxygen tension is on average 5%, and as low as 1% in ischemic tissue(Miller, 2016). Reducing the oxygen tension to 1% in culture causes the loss of a number of cells, but it is the most effective way to reproduce their native environment in the target tissue where they may be placed, and define the most viable cells collected(Miller, 2016). Molecular signaling and subsequently the metabolic switching during hypoxia is essential in decreasing the accumulation of oxidative metabolism-derived reactive oxygen species (ROS) and the proliferation and programming to the niche adaptation(C. C. Zhang & Sadek, 2014). Recent studies indicate that differences in metabolic profiles of pluripotent stem cells are closely related to self-renewal and initial cell fate decision of stem cells. Gu and colleagues reported that naive pluripotent stem cells in humans exhibit a high glycolytic flux and pentose phosphate pathway activity(Gu et al., 2016). Moreover, metabolic switching induced by hypoxia prevents the excessive generation of ROS and reduces the demand for ATP, both closely associated with maintaining the physiological functions and survival of stem cells(Son et al., 2013).For the mimic of hypoxia and respiration deficiency, in this study we used antimycin A, an inhibitor of cellular respiration, specifically oxidative phosphorylation . Antimycin A binds to the cytochrome c reductase, cause the disruption of the entire electron transport chain and the formation of the proton gradient across the inner membrane of the mitochondria. This inhibition also results in the formation of the toxic free radical superoxyde (O_2^{-}) The rate of superoxide production exceeds the cellular mechanisms to scavenge it, overwhelming the cell and leading to cell death(Tzung et al., 2001). In this research, we did not use hydrogen peroxide treatment to induction of oxidative stress in stem cells. Because, this H_2O_2, itself directly act as cell signal messenger and without mitochondrial neutralization, we may obtained a false results from antioxidant protective potentiality. Antioxidants can neutralize H_2O_2 through just one chemical reaction in the cytoplasm, whereas Antimycin a induce H_2O_2 in the bioactive and complex process. It let to the cells to decision and program to neutralize of free radical via molecular players or program to the other bio-functions.

Antioxidants pretreatments. Many studies reported that, the use of antioxidant molecules for pretreatment of stem cells could protect them from hypoxic condition and it may represent a novel and safe approach to improving the beneficial effects of cell therapy(Mias, Trouche, Seguelas, Calcagno, Dignat-George et al., 2008). Ji Yong Kim et al reported that, pretreatment of MSCs with lycopene attenuated the oxidative stress and the apoptosis(Kim et al., 2015). Another study showed that, delphinidin prevents apoptosis of mouse embryonic stem cell induced by hypoxia through reduction of ROS and activation of some molecular pathways including of JNK, NF-κB, and Akt(Seo et al., 2013). The pretreatment of MSCs with melatonin improved the survival, proangiogenic activity, and efficiency injected into ischemic kidney(Mias, Trouche,

Seguelas, Calcagno, Dignat-George et al., 2008).

In this study we used two antioxidants including mito-TEMPO and N-acetylcysteine (NAC). NAC is the N-acetyl derivative of the amino acid L-cysteine, and is a precursor in the formation of the antioxidant glutathione in the cells. Glutathione is largely known to minimize the lipid peroxidation of cellular membranes and mitochondrial oxidative stress. Another important role for glutathione relates to its impact over signal transduction of gene expression inside cells and also involved in energy metabolism(Dringen & Hamprecht, 1998). NAC is a precursor to the human endogenous antioxidant, glutathione that could target the free radicals directly and indirectly, and involve in the energy homeostasis. MSCs were exposed to AMA as an inhibitor of cellular respiration. Based on our findings, upon the exposure of the MSCs to the conditions of respiratory chain deficiency, the cells failed to scavenge the free radicals, and energy metabolism was disrupted. Pretreatment with NAC was found to alleviate the DNA damage, cell apoptosis, and oxidative stress via Nrf2/Sirt3 pathways and other molecular players, while not affecting the mitochondrial membrane potential and ATP production.

The mitochondria dysfunction is probably very important in the MSCs in a hypoxic microenvironment. And the evidence suggests that mitochondrial ROS is also a tightly controlled cellular oxidative homeostasis and propagation of cellular signaling pathways(Hamanaka & Chandel, 2010). So, in the other study we used mito-TEMPO (mito-T) for targeting of mitochondrial oxidative stresses. The prior studies reported that, mito-T could effect in ischemic tissues linked with hypoxia-induced oxidative stress(Du et al., 2019; Nautiyal, Shaltout, Chappell, & Diz, 2019).

Here, mito-T, dramatically reduced AMA-induced mitochondrial oxidative stress, and thus effectively protected MSCs. the analysis of cell cycle revealed that in the AMA-treated cells, G0/G1 phase arrested cells were observed approximately 40%, while co-treatment with mito-T arrested the cells around 19.84% compared to the control. This result showed that mito-T not only effect by free radical scavenging, it play in signaling pathway that in the future study we are going to focus it.

The restrictions of this study. Our results showed that for supporting the stem cells in hypoxia, the pretreatment of antioxidant is not enough, because the energy metabolism remains unresolved. In addition, the linear study in the effect of pretreatments including antioxidant and hypoxia preconditioning are paradigm according to the literature emphasize to the mitohormesis role and others report antioxidant properties. As well, the role of cell-cell junction, cell-ECM, the injured tissue's mechanoinformation, niche cells-signals include paracrine , autocrine and juxtacrine signals, and the molecular players especially HIF1α/Sirtuins must were considered. The antioxidants term could be confusing. The substances shown to have an antioxidative effect in the laboratory, the so-called 'direct antioxidants' will have not the same effect in the cellules. We could upgrade the term of antioxidants due to the pleiotropic mode of action to bioactives substances. For example in the stem cells field lens, ascorbic acid (vitamin C) acts not only as a antioxidant

but it could induce the MSCs to osteoblest cells(H. K. Choi et al., 2019; K.-M. Choi et al., 2008).

Future direction and suggestions. As mentioned, in the native stem cell niche mitochondrial ROS levels are vital for biological function of stem cells including the proliferation and stemness maintains or differentiation. The niche is a complex and dynamic structure that transmits and receives signals through cellular and acellular mediators. A niche is a highly complicated network, which is composed of proteins, proteoglycans, soluble signals (growth factors, cytokines, chemokines), physical signals (fibronectin, vitronectin, laminin, collagen, fibrillin) and signals arising from cell/cell interactions . Many of this conserved component may lost in the injury tissues. After transplantation of MSCs in tissue injuries, the death of stem cells within the first few days may be done. The all reason is not due to the oxidative stress. The anoikis, inflammation, respiration chain deficiency could effect to the transplanted stem cells fate(Sart, Ma, & Li, 2014). So, the manipulation of stem cells and preconditioning of stem cells to form a memory and induced the hypoxia homeostasis signaling is necessary to support the stem cells in hypoxia injury tissues but it is not enough. So, with considering of native niche component and with mimicking, the engineering of new niche to supporting transplanted stem cells in harsh condition is necessary. However, the precise components may be different for each type of stem cells and within each stem cell niche and isolated source, especially in the injured tissues.

With considering the limitations of this study, for the future research directions we suggest:

- *For the repeating and confirmation of the results, this study could perform to the other type of stem cells.*
- *To the comparison of results and prediction of cells behavior in hypoxic condition, the study could perform for several types of normal and stem cells.*
- *Nano-scaffold as 3D support could be designed with considering of native niche components and combined with different bioactive and antioxidants substances*

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