Thais de Assis Ribas

Evaluation of Reck tumor supressor gene`s role in neuronal differentiation

Dissertação apresentada ao Programa de Pós-Graduação Interunidades em Biotecnologia do Instituto de Ciências Biomédicas da Universidade de São Paulo, para obtenção do Título de Mestre em Biotecnologia.

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Orientadora: Profa. Dra. Mari Cleide Sogayar

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Orientador: Profa. Dra. Mari Cleide Sogayar

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São Paulo, 22 de março de 2019.

Ilustríssima Senhora
Profa. Dra. Mari Cleide Sogayar
Departamento de Bioquímica do Instituto de Química
UNIVERSIDADE DE SÃO PAULO

Prezada Professora

O Comitê de Ética em Pesquisa do Hospital Universitário da Universidade de São Paulo analisou o Projeto de Pesquisa de mestrado da aluna, **Thaís Assis Ribas**, intitulado: ***"Análise do papel do gene supressor de tumor RECK durante a diferenciação neurogênica"***, e considerou tratar-se de pesquisa que não envolve seres humanos, não necessitando, portanto, de avaliação e acompanhamento dos aspectos éticos do Comitê de Ética em Pesquisa em Seres Humanos.

Atenciosamente,

Dr. Mauricio Seckler
Coordenador do Comitê de Ética em Pesquisa
Hospital Universitário da USP

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que sempre acreditaram em mim e me ensinaram que

eu posso ser e fazer o que quiser.

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“Dificuldades preparam pessoas comuns para destinos extraordinários”

C.S Lewis

Resumo

Assis-Ribas T. ***Análise do papel do gene supressor de tumor RECK durante a diferenciação neurogênica****. [dissertação].* São Paulo: “Instituto de Ciências Biomédicas, Universidade de São Paulo”; 2019.

O gene supressor de tumor Reck (*REversion-inducing Cysteine-rich protein with Kazal motifs)* codifica uma glicoproteína multifuncional que inibe a atividade de diversas metaloproteinases de matriz (MMPs), como também modula a atividade de Notch e vias de Wnt canônico. Células neuroprogenitoras com *Reck* deficiente sofrem uma diferenciação precoce, entretanto, a modulação da expressão de *Reck* durante a progressão da diferenciação neuronal ainda precisa ser caracterizada. No presente estudo, nós verificamos a assinatura da expressão de *Reck* e caracterizamos a atividade do promotor de *Reck* murino durante o processo de diferenciação neural. Foi possível verificar um aumento na atividade e níveis de expressão do promotor de Reck em três modelos de diferenciação celular: PC12 feocromocitoma, P19 teratocarcinoma derivado de embrião e USP-4 célula tronco embrionária de murinos, que foram submetidas a indução da neurodiferenciação. Além disso, a superexpressão de Reck antes do início da diferenciação celular leva a uma diminuição na eficiência do processo de neurodiferenciação. Levando em conta os dois dados obtidos, eles sugerem que em oposição ao aumento gradual de *Reck* durante a diferenciação neuronal, a superexpressão nos estágios mais precoces de diferenciação dificulta as células progenitoras a se comprometerem com o destino para células neuronais. Nossos dados reforçam o potencial do uso da modulação da expressão de *Reck* para otimização dos protocolos de diferenciação *in vitro.*

Palavras-Chave: Diferenciação neuronal, teratocarcinoma, Reck, promotor, protocolos de diferenciação

Abstract

Assis-Ribas T. ***Evaluation of Reck tumor supressor gene`s role in neuronal differentiation****. [dissertassion].* São Paulo: “Instituto de Ciências Biomédicas, Universidade de São Paulo”; 2019.

Reck (*REversion-inducing Cysteine-rich protein with Kazal motifs)* tumor suppressor gene encodes a multifunctional glycoprotein that inhibits the activity of several matrix metalloproteinases (MMPs) and is also able to modulate the Notch and canonical Wnt pathways. *Reck*-deficient neuroprogenitor cells undergo precocious differentiation; however, modulation of *Reck* expression during progression of neuronal differentiation process is yet to be characterized. In the present study, we assessed the *Reck* expression signature and characterized the mouse *Reck* promoter activity during the *in vitro* neural differentiation process. We found increased *Reck* promoter activity and expression levels in three different cellular models, namely: PC12 pheochromocitoma, P19 embryo-derived teratocarcinoma and USP-4 murine embryonic stem cells, upon subjection to neurodifferentiation induction. Moreover, *Reck* overexpression prior to the beginning of the differentiation protocol leads to diminished efficiency of the neurodifferentiation process. Taken together, our findings suggest that in opposition to the gradual increase of *Reck* expression during the neuronal differentiation process, its overexpression at early stages of the process hinders the progenitor cells commitment to a neuronal fate. Our data reinforces the potential use of *Reck* expression modulation to optimize *in vitro* neurodifferentiation protocols.

Keywords: Neuronal differentiation, teratocarcinoma, Reck, promotor, differentiation protocols

1. Introduction

1.1. Stem cells

Stem cells are characterized by their potential for self-renewal and, for its ability to differentiate into other cell types (Caplan, 1991). Some types of stem cells have already been described and characterized for their potential for differentiation, such as: (i) totipotent stem cells, which are able to differentiate into embryonic and extraembryonic tissues; (ii) pluripotent stem cells, which give rise to all embryonic tissues, being represented by the embryonic stem cells; and (iii) multipotent stem cells, which give rise to a limited range of cells of a given tissue, being, for example, adult stem cells (Barry and Murphy, 2004; Bonfield et al., 2010; Caplan, 1991; De Los Angeles et al., 2015; Kobolak et al., 2016).

For many years, hematopoietic stem cells have been considered as the only stem cells that could be isolated from an adult organism (Caplan, 1991; Guida et al., 2016; Müller, Huppertz, & Henschler, 2016; Mayani, 2016). However, in the late 1970s, Friedenstein identified new stem cells, which were isolated from the bone marrow of adult mice and were initially called colony forming cells. These cells grew in monolayer, had fibroblastoid morphology and ability to differentiate into bone cells (Caplan, 1991). However, it was only in the year 1990 that Caplan and colleagues named these mesenchymal stem cells (MSCs) (Friedenstein, Chailakhyan, and Gerasimov 1987).

A valuable model for in vitro differentiation assays was described in 1982 by McBurney et al., Consisting of p19 cells derived from rat teratocarcinomas, which are considered pluripotent cells, i.e. having the potential for differentiation into several cell types (neuronal cells , adipocytes, chondrocytes, myocytes, among others), according to the stimulus provided (M. W. McBurney and Anderson, 1982; Jones-Villeneuve et al., 1982).

These cells are widely used for neuronal differentiation, because they are easy to obtain and have protocols optimized for differentiation in neuronal or glial cells,

already widely described in the literature, which makes the use of these cells very interesting (M. W. McBurney and Anderson, 1982; Zhang et al., 2012).

1.1.1. **Differentiation of Stem Cells**

One of the main characteristics of stem cells is their ability to differentiate in a range of specialized cells, such as: adipocytes, chondrocytes, myoblasts and neuronal cells (Caplan, 1991).

1.1.2. **Neuronal cells**

Neurons and glial cells are considered neuronal cells. Neurons are formed by the cell body, axon (which may be short or long) and dendrites, and may or may not be associated with oligodendrocytes, which form the myelin sheath and play an important role in the transmission of the nervous impulse (Neuro-histologia Martinez, 2014; Neuroscience, 2nd Edition, Purves, 2001 - book). Astrocytes are cells responsible for supporting the function of neurons and oligodendrocytes, as well as their metabolic function (Peferoen et al., 2014; Kriegstein and Alvarez-Buylla, 2009).

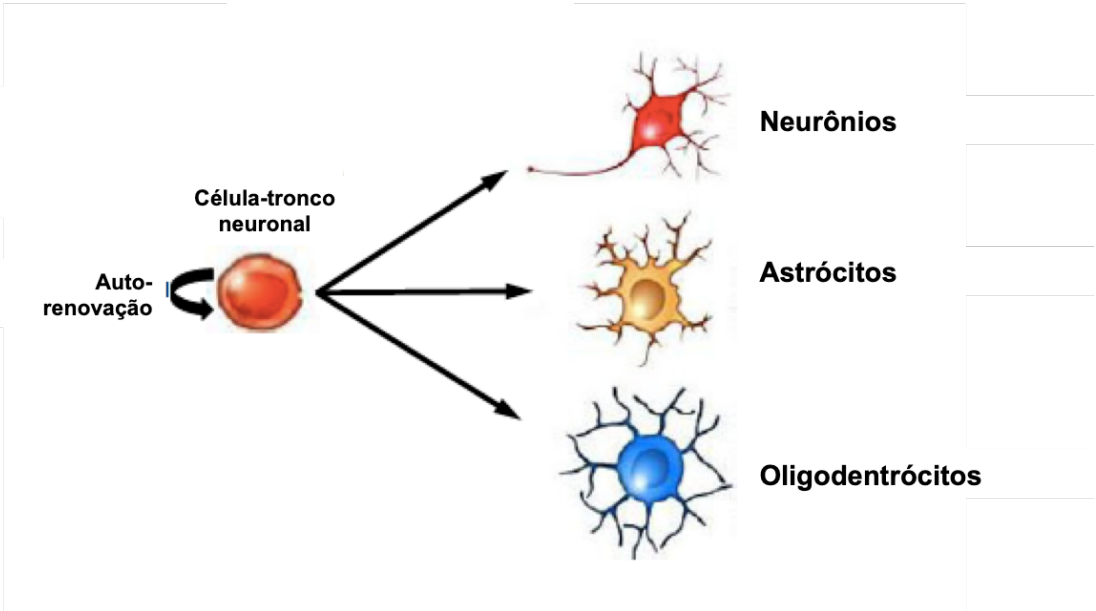

Figure 1. Differentiation of Stem Cells in nervous tissue cells. (Adapted from Killick*et al.,* 2011). Neuronal precursor cells are pluripotent cells that can self-renew

and differentiate into cells of nervous tissue, which can generate neurons, astrocytes, and oligodendrocytes.

Cells capable of differentiating into neuronal cells are also called neuronal precursor cells (NPCs). For in vitro Neuronal differentiation assays, NPCs can be used as experimental models of pluripotent cells, such as embryonic stem cells (ESCs), teratocarcinomas, neural precursors isolated from animal nervous tissue, and iPSCs (induced pluripotent stem cells), among others.

Neuronal differentiation can generate three cell types: neurons, astrocytes and oligodendrocytes. Neuronal differentiation in vitro attempts to reproduce the neurogenesis that occurs in vivo. The undifferentiated cells have the deactivated BMP pathway and the activated FGF pathway, activating this signaling pathway for the differentiation in neuronal cells, as it happens in vivo. After activation of the FGF pathway and inactivation of the BMP pathway, in order for the final phase of differentiation to occur, it has already been verified that an extracellular stimulus is required that will activate the final phase of differentiation in neuronal cells, which is given by Retinoic Acid (RA) in most protocols.

1.1.3. Neural precursors (NPC) and types of differentiation

In order for Neuronal differentiation to occur, different protocols have been described that involve: formation of embryoid bodies, co-culture with stromal cells and monolayer culture. Each of the protocols involves different signaling pathways triggered and different degrees of efficiency. However, a point common to all strategies is that the use of retinoic acid may lead to greater process efficiency (Azari and Reynolds, 2016).

Jones-Villeneuve, in 1982, described the importance of retinoic acid in the differentiation of neural precursors in the case of the use of p19 cells, noting that cellular cultures stimulated with Retinoic Acid presented neuronal cells, whereas cultures without treatment had cells similar to the extra-embryonic endoderm (M. W. McBurney and Anderson, 1982).

During development, retinoic acid has been described as being an important factor for the survival of dentate gill cells and its deficiency may cause decrease of neurogenesis in vivo (Jacobs et al., 2006).

Retinoic acid acts by stimulating retinoid receptors, which promote Neuronal differentiation through the activation of FGF pathways and inactivation of the BMP pathway (Jacobs, 2006). In addition, it has recently been described that retinoic acid is an important factor for the proliferation of neuronal cells in vivo (Mishra et al.,2018).

1.2. Microenvironment

The microenvironment is essential for the homeostasis of the organism, being crucial for processes like: differentiation, migration and cell growth. An important part of this microenvironment is the matrix extracellular (MEC), which is composed of macromolecules of various natures, such as: collagen fibers, proteoglycans and glycoproteins, acting as structural support for the cell and modulating its behavior (Oh et al., 2001; Trombetta-Lima et al., 2015).

1.2.1. Matrix Metalloproteinases and their Inhibitors

Embedded in the extracellular matrix are the Matrix Metalloproteinases, zinc-dependent proteolytic enzymes, which are also called MMPs. In humans, 24 members of this family can be found, which can be divided into different subfamilies: collagenases, matrilisins, stromelysins, gelatinases, membrane MMPs, transmembrane and secreted. MMPs are essential for the remodeling of ECM, degrading its components and being responsible for cleaving different cell surface molecules, thus helping processes of differentiation, angiogenesis, embryonic development, among others (Fabre, Ramos, and de Pascual-Teresa, 2014; Fanjul-Fernández et al., 2010).

Mmps can be divided into sub-families according to their structures. The basic

structure of an MMP is composed of propeptide, which contains a cysteine, a metalloproteinase catalytic domain, a binding peptide (which may have variable sizes) and a hemopexin domain (Figure 3), and can be subdivided into groups:

Mmps archetypes, which have the basic structure of an MMP. In this group are collagenases (capable of degrading collagen), among other enzymes, stromelysins (which in spite of degrading other components of the ECM, such as laminin and integrin, are not able to cleave collagen) and other Mmps are in the previous ones because they have different sequences).

I. Matrilisins, which lack the domain of hemopexin and cleave collagen IV, laminin and entactin, as well as non-ECM proteins.
II. Gelatinases, which has a fibronectin binding domain, which allows binding and denaturation of collagen or gelatin.
III. MMPs activated by furin, which in addition to the basic structure, containing a furin domain, which allows cleavage of the substrate. In this category are secreted Mmps (which are processed inside the cell), membrane (which has a membrane anchoring domain) and transmembranes (which do not have a hemopexin domain, but have a cysteine domain and immunoglobulin).

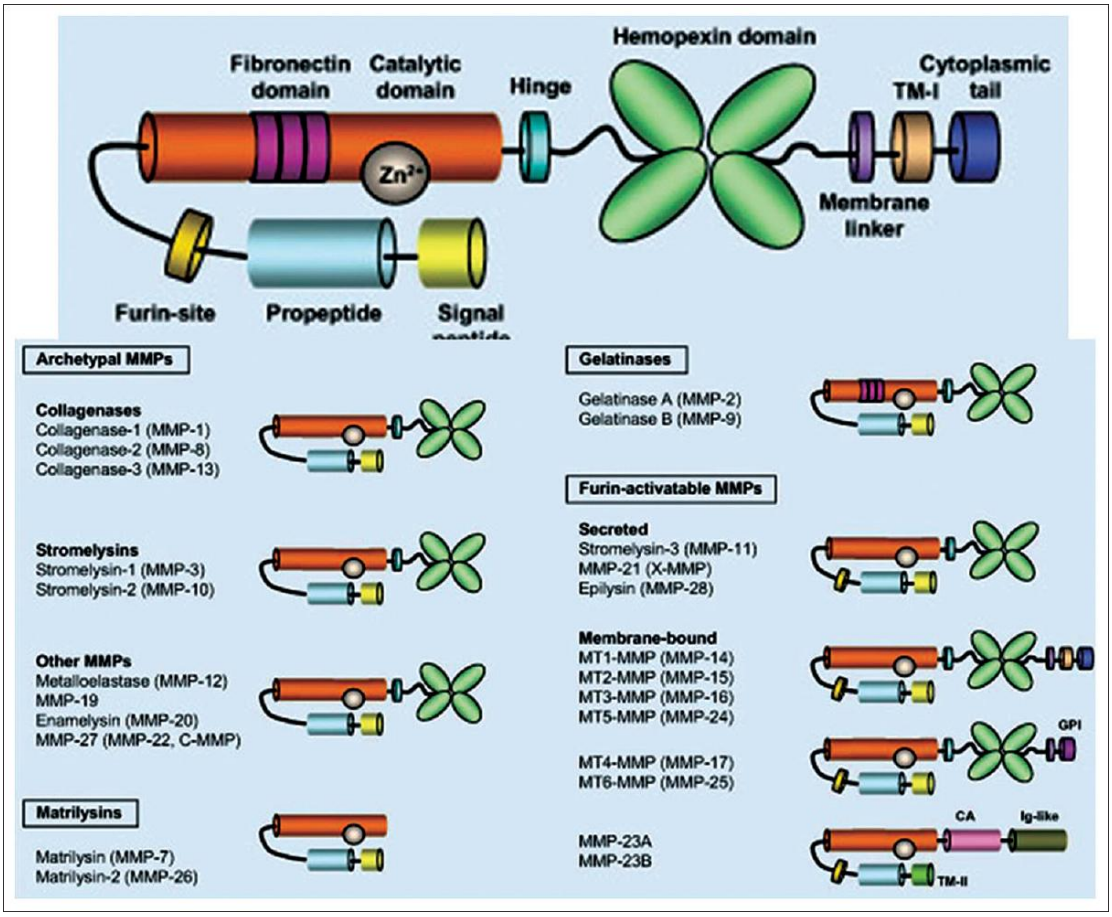

Figure 2. Metalloproteinases of matrix and their structures. The basic structure of a MMP is composed of propeptide, which contains a cysteine, a metalloproteinase catalytic domain, a binding peptide (which may have variable sizes) and a hemopexin domain, and changes in this structure classify them into different families (Kapoor et al., 2016).

However, for remodeling to occur so that the body's homeostasis is maintained, the expression and regulation of MMP inhibitors are also essential. Two types of inhibitors can be highlighted: TIMPs (Tissue Inhibitor of Metalloproteinases), a family composed of four proteins that are secreted into the extracellular medium, being reversible inhibitors of MMPs; and Reck (REversion-inducing-Cysteine-rich protein with Kazal motifs), the only described MMP inhibitor that is anchored to the cell membrane (Gupta et al., 2014; Takahashi et al., 1998).

1.3. **Reck gene**

The Reck gene was characterized by Takahashi et al in 1998, for leading, when overexpressed, to the reversal of the tumor phenotype of DT-transformed NIH-3T3 cells, transformed by v-K-ras. The Reck protein is 110 kDa, being anchored to the membrane through its carboxy-terminal portion by glycosylphosphatidylinositol (GPI). Reck regulates negatively at least four MMPs: MMP-2, MMP-7, MMP-9 and MMP-14 (the latter is also known as MT1-MMP) (Figure 4), and has low expression in tumor cells (Oh et al., 2001; Omura et al., 2009; Takahashi et al., 1998).

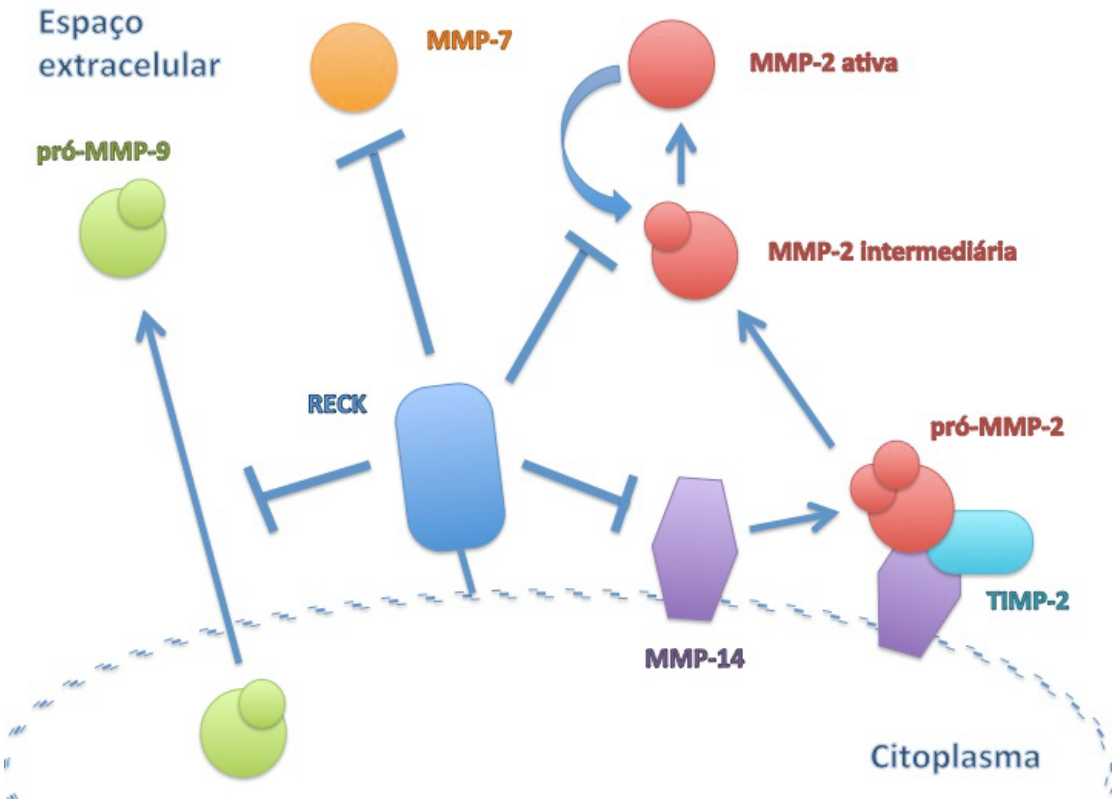

Figure 4. Regulation of MMPs by RECK. Schematic representation of the regulation of MMP-2, MMP-7, MMP-9 and MMP-14 by Reck. Reck inhibits the secretion of pro-MMP-9; the activation of pro-MMP-2 at two distinct points of its activation cascade: by inhibiting the formation of TIMP-2, MMP-14 and pro-MM-2 ternary complex and by inhibiting the last step of autocatalytic activation of the intermediate form of MMP-2; and the activities of MMP-7 and MMP-14 (Adapted from Noda & Takahashi, 2007; Trombetta-Lima et al., 2015).

1.4. Extracellular matrix and its role in Neuronal differentiation

The extracellular matrix is important for the physiology of nervous tissue and for the differentiation of Stem Cells into neuronal cells (Bikbaev, Frischknecht, and Heine, 2015; Vaillant et al., 1999), being essential, also, for the connection of the neural networks by facilitating the exchange of neural stimuli and to promote the nervous conduction (Bikbaev, Frischknecht and Heine, 2015).

It has been extensively reviewed in Barros (2011) that the extracellular matrix is important for the involvement of the neuronal precursor in its differentiation process for neuronal cell or glial cell, being possible to stimulate a NPC to differentiate into oligodendrocytes with the addition of laminin (Barros, Franco, and Mu, 2011; Li et al., 2014), highlighting the importance of Mmps and their inhibitors, such as Timps for tissue physiology (Kaczmarek, Lapinska-Dzwonek, and Szymczak, 2002; Dzwonek, Rylski, and Kaczmarek, 2004).

The expression profiles of Mmp-2 and Mmp-9 have already been described in vivo differentiation, since Mmp-9 plays an active role in neuronal cell migration, whereas Mmp-2 plays a role in both cell migration and proliferation (Vaillant et al.,1999). About the in vitro differentiation models using neuroblastoma cells, the constant expression of Mmp-2 in cells not induced to differentiate was verified, however, the expression of Mmp-9 is induced by Retinoic Acid (Chambaut-Guérin et al., 2000).

1.5. RECK and its role in cell differentiation

MMPs -2 and -9 play an important role during adipogenic differentiation, more specifically, in the transition phase from pre-adipocytes to mature adipocytes, in which the inhibition of these MMPs leads to a decrease in differentiation efficiency, inhibiting the accumulation of lipids and the morphological change of the cell, which occurs in both murine and human cells, since these MMPs present a greater expression in the later stages of differentiation (Yamamoto et al., 2007; Mohammadi et al., 2015; Campagnoli et al., 2001).

Recently, RECK expression has been reported to be lower in the initial stages of

the adipogenic differentiation protocol and its depletion favors adipogenesis, to the detriment of osteogenesis (Bouloumié et al., 2001). It was also described that murine cells (MCT-3T3-E1 pre-osteoblast lineage) and human MSCs undergoing osteogenic differentiation show a higher expression of Reck until the fourteenth day of the protocol, when there is a sudden drop in the expression of this gene , a level that is maintained during the later stages of the protocol (Zambuzzi et al., 2009; Mahl et al., 2016).

Reck plays an important role in embryo development since it makes knockout mice exhibit high activity of Mmps and abnormal organogenesis, resulting in death around E10.5 (Oh et al., 2001). In addition, Muraguchi et al.,Analyzed the Neuronal differentiation of mice with conditional Reck knockout in NPCs, observing an early Neuronal differentiation in these animals. However, NPCs isolated from these viable embryos did not have the ability to form neurospheres, which means, they were not able to proceed with differentiation (Muraguchi et al., 2007).

In the adult brain, Reck has already been analyzed in a model of ischemia, in which it was possible to verify a greater expression of Reck in the region of recovery of the neuronal cells after ischemia (Ortega et al., 2010). Thus, due to the importance of Reck as a modulator of the microenvironment and in view of the existing evidence that its expression changes the fate of differentiation in different models, this project aims to analyze the role of Reck during Neuronal differentiation *in vitro*.

2. Objectives

2.1. General Objective

The presente work has as objective to analyse the role of the tumor supressor gene *Reck* during the process of neuronal differentiation *in vitro*.

2.2. Specifics objectives

Objective 1. Analyse the expression profile of gene *Reck* during the neuronal differentiation *in vitro* by qRT-PCR.

Objective 2. Analyse the activity from promotor region from gene *Reck* during the neuronal diferentiation *in vitro*.

Objective 3. Evaluation of the effect of *Reck* superexpression on the efficience of the neuronal differntiation protocol *in vitro.*

Objective 4. Elaborate a citical revision aboyt the Mmps e their inhibitors (*Reck* e *Timps*) in the differentiaion process.

3. Materials and methods

3.1. Solutions and culture media

3.1.1. For mammalian cells:

- DMEM (Dulbecco´s Modified Eagle Medium) (Life Technologies, Carlsbad, CA, EUA).
- α-MEM (*Minimum Essential Media*, Life Technologies).
- PBSA (Phosphate Buffered Saline – without calcium or magnesium): buffered saline solution pH 7.2, composed of 140mM NaCl, 2,7mM KCl, 8mM Na_2HPO_4 and 1,5mM KH_2PO_4.
- Trypsin: 0.1% trypsin solution (Life Technologies) in PBSA containing 1 mM EDTA (pH 8,0).
- Fetal bovine serum: FBS (Atená Biotecnologia, Campinas, SP, Brasil).
- Ampicillin (Sigma-Aldrich, St. Louis, MO, USA), concentration used: 25mg/ml.
- Streptomycin (Sigma-Aldrich), concentration used: 100mg/mL.
- Versene: PBSA containing 30mM EDTA.

3.1.2. For bacterial cells:

- Liquid culture medium SOC (BD, NJ, USA): 2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl 2, 20mM MgSO 4, 20mM glucose.
- LB medium (Luria-Bertani, Gibco LB-Broth, Life technologies): 10g/L Tryptone; 5g/L yeast extract; 10g/L NaCl (pH 7.5).
- LB-agar: LB medium containing 1.5g/L of agar (Gibco).

3.2. Cell lines used

Cells P19 (ATCC® CRL-1825™): embryonic mouse teratocarcinoma line.

Cells PC-12 (ATCC® CRL-1721™): rat adrenal gland pheochromocytoma line.

Cells USP-4: embryonic stem cells derived from murine blastocyst (Sukoyan et

al.,2002).

3.3. Ethics Committee Approval

The nature of the project does not require approval of an Ethics Committee, as judged by the Research Ethics Committee of Universidade de São Paulo Hospital, and the committee's opinion is attached here (Attachment 1).

3.4. Cell culture conditions and maintenance

Cells were maintained in α-MEM medium (Minimum Essential Media, Life Technologies) supplemented with 10% FBS (fetal bovine serum). In addition, 25mg/ml ampicillin (Sigma-Aldrich) and 100mg/ml streptomycin (Sigma-Aldrich) were added to the culture medium. Cells were maintained under stable conditions at 37°C, 2% CO2 and controlled humidity.

The culture medium of the adherent cells was changed every 2 or 3 days. When the culture reached a confluence of approximately 80%, the cells were subcultured, in which the cell layer was washed with PBSA and the cells were removed from the plastic surface by digestion with 0.1% trypsin in the presence of 1mM EDTA (pH 8.0) (Life Technologies) in PBSA.

Cell stocks were maintained in liquid nitrogen and the cell suspension (in the approximate concentration of 1x106 cells/mL), was frozen in solution containing 80% or 75% of DME, 10% or 15% of FBS and 10% of DMSO (dimethylsulfoxide). The cell suspension, in freezing medium, was placed in freezing ampoules and held for 15min on ice and then stored, first at -80°C and subsequently in liquid nitrogen, at least 190°C.

For thawing of the cells, one of these ampoules was withdrawn from the storage tank, -80°C or -190°C, thawed and the cell suspension suspended in a tube containing 5 ml of culture medium and centrifuged in a bench centrifuge (Baby®I, Model 206 BL, FANEM, Guarulhos, Brazil) at 70g for 5min. Then the supernatant containing DMSO was removed and discarded and the pellet containing the cells was

resuspended in culture medium suitable for that cell type and then incubated in the presence of 2% CO2 at 37°C.

To test the presence of Mycoplasma spp., all cell cultures were tested by Nested-PCR reactions using a method developed in our laboratory by Dr. Ana Cláudia Oliveira Carreira and Ms. Marluce Mantovani, based on Uemori et al. (Harasawa et al., 1992).

3.5. Bacterial transformation with plasmid DNA

3.5.1. Obtaining electrocompetent bacteria

E. coli bacteria from strain XL1-blue were inoculated into 5mL of LB medium containing tetracycline (1:1000) and incubated at 37°C for 16-18h under stirring (10g). After incubation, the bacteria were diluted 1:100 in 20 ml of LB medium and then incubated at 37°C until the absorbance of 0.5-0.8 at 600ηm was reached (exponential growth phase), the culture was centrifuged for 5min at 1700g, the supernatant discarded and the pellet resuspended in 20mL of ice-cold Milli-Q water. The final step was repeated 3 times to remove excess salt from the solution, the pellet was resuspended in 400µL of LB solution containing 10% glycerol and the cells were then stored at -80ºC in 50µL aliquots.

3.5.2. Bacterial transformation by electroporation, storage and amplification of plasmid DNA

For the storage and amplification of the vectors used in this work, we added 2-4µL of the vectors, with a minimum of 10ng, to 50µL of an electrocompetent bacteria suspension, which were then electroporated in a 2,800V cuvette. After electroporation of the bacteria, 300 µL of SOC culture medium was added to the cuvette and the bacterial suspension was transferred to a 1.5 mL microtube, which was incubated at 37°C in a dry bath for 1 hour for recovery of the cells. These bacteria were then seeded in LB-agar plate containing 50µg/mL ampicillin.

The bacterial clones obtained were expanded and incubated in LB medium at 37°C for 16-18h under stirring (10g). Cell stocks were prepared by adding to the cell

suspension a 30% glycerol solution in the ratio 1:1 (v:v) and stored at -80°C.

For plasmid DNA purification, it was used the GeneJETPlasmidMIniprep kit (ThermoScientific, Waltham, Massachusetts, USA), following the manufacturer's guidelines. After termination of the protocol, plasmid DNA concentration was determined by absorbance at 260 ηm on the ND-1000 Spectrophotometer (NanoDrop Technologies, ThermoScientific) spectrophotometer.

3.5.3. Expression vectors

The expression vector pCXN2 containing the coding sequence for murine Reck was kindly provided by Professor Makoto Noda, Kyoto University, Japan, to Dr. Regina Maki Sasahara who developed her PhD project in our group in collaboration with the group of Prof. Noda.

- pCXN2-Reck: murine Reck expression vector.
- PCXN2: empty vector used as control in assays.

3.6. Cell differentiation

Cells PC12 were differentiated by addition of 10nM basic fibroblast growth factor (bFGF) to the culture medium for two days and, after removal of bFGF, that culture was maintained for seven days and then collected for analysis. USP-4 cells were differentiated using the protocol that consists of plating the cells in Hanging drops containing 1,250 cells/25µL of 5% FBS in BMC (Basic Media Culture) dropwise in the lid of a Petri dish with 148cm², containing PBSA. After three days the embryoid bodies (EB) formed were transferred to a Petri dish of 9cm² in diameter and after 24 hours the culture medium was changed to neurobasal medium (NB medium) (GIBCO), supplemented with B27 and 0.1µM of retinoic acid. After four days, the retinoic acid was removed and the cells were maintained for seven days in NB medium supplemented with B27 (Hayashi et al., 2010)

The cell differentiation of p19 line was based on the protocol of Martins et al (2005)

and Santiago et al (2005). Cells maintained in adherent culture in the incubator at 37ºC with 5% CO2 and controlled humidity were washed with PBSA and released of the plastic surface by digestion with 1.0% Trypsin, 1 mM EDTA in PBSA (saline-phosphate without calcium or magnesium). These cells were then plated (5x105 cells) in Petri dishes for bacteriology 100x20mm (without cell adhesion treatment) in the presence of 1μM of retinoic acid and incubated for 3 days, after which the cells that were in suspension were collected and transferred for a cell wall plate 100x20mm adherent surface and maintained for 7 days, when they were collected for further analysis. In this stage, the spheroids were also collected for analysis.

3.7. Flow cytometry

The non-differentiated, spheroid and differentiated cells, collected at different time periods, were submitted to the flow cytometry protocol using the FACS ARIA II (BD) for analysis, using antibodies against the following proteins: Map2, nestin , s100, β-3-tubulin, and GFAP. For this analysis, cells were removed from the surface, washed and incubated with blocking buffer (4% SFB, 0.1% Triton-X-100 in PBSA) for 30min at 4°C. After incubation with the Anti-β-3-Tubulin antibody (1: 1,250, Cat # ab56676, Abcam) for 30min at 4°C, the cells were washed with PBSA, resuspended and incubated with the secondary antibody (1: 2,000; Cat # A-11001, Thermo Fisher Scientific) for 30min at 4ºC. The cells were then washed three times with PBSA and subjected to analysis on the FACS ARIA II.

3.8. Analysis of RECK gene promoter activity

Analysis of the RECK gene promoter activity was performed as previously described (Sasahara, Takahashi, and Noda, 1999) and luciferase activity was evaluated essentially as previously described by Guerreiro et al. (2005). Briefly, for the protocol of transfection, the cells were plated 24 hours earlier in 24-well plates containing 106 cells/ml in each well. Transfection of purified plasmid DNA into Qiagen columns (Qiagen, Inc., Hilden, Germany) was performed using calcium phosphate (Ausbel et al., 1995). To detect the activity of the promoter present in the 5 'region of the Reck gene in mice, 3mg of the luciferase plasmid and 0.25mg of pRL-TK (Promega,

Madison, WI, USA) were used. Plasmid pRL-TK, which carries the Renilla luciferase reporter gene, was added together with the promoter to be used as internal control for the transfections, which were performed in triplicates. 20 hours after transfection, these cultures were washed with PBSA and analyzed for expression of the reporter gene after 24h using the Dual-Luciferase Reporter Assay System (Promega) and a Microplate Luminometer (Dynex Technology, Chantilly, VA, USA), which expresses the luminescence in units of relative light.

3.9. RNA extraction

The cells total RNA used in this study (undifferentiated or submitted to neuronal differentiation protocols, described in item 3.9, was isolated using Trizol (Sigma-Aldrich) and the AllPrep DNA/RNA/Protein Mini Kit (Qiagen) For the purification of DNA, RNA and proteins, cell cultures were washed with PBSA two times prior to the addition of 250μL of Trizol, cell lysate was collected with the aid of a scrapper, transferred to a microtube of 30 μL of chloroform was added and the lysate was agitated again for 30 sec and the sample was then centrifuged for 5 min at 20,817g (Centrifuge 5417R, Eppendorf, Hamburg, Germany) for the phase separation. The aqueous phase, in which the total RNA is present, was withdrawn and transferred to a new tube. Then, 100 μl of ice-cold isopropanol was added and the samples were incubated at -20°C overnight. The next day, the samples were centrifuged for 15min at 20,817g in a benchtop centrifuge and the RNA was then sedimented, washed with 100μL of 70% ethanol and centrifuged again at 20,817g for 10min, the supernatant was discarded, and the RNA, which was found in the sediment, then resuspended in 30μL of RNase-free water.

The concentration of RNA obtained in each sample was determined from the spectrophotometer (ND-1000 Spectrophotometer, NanoDrop Technologies - Thermo Fisher Scientific) by measuring the absorbance of the samples at 260ηm, considering the correspondence between one unit of absorbance at this wavelength and the concentration of 40μg/mL of RNA. The degree of purity of the RNA was analyzed by the ratio Abs260ηm/Abs280ηm, with a satisfactory purity of about 2.0.

3.9.1. Synthesis of cDNA and quantitative RT-PCR (qRT-PCR)

The total RNA, extracted according to item 3.9, was used as a template for the synthesis of the cDNA in reverse transcription reaction. For this purpose, 1µg of total RNA in a total volume of 5.5µL was used as substrate, adding 2µL of 5x buffer for synthesis of the first SuperScript III enzyme (Life Technologies), 0.5µL of RNase OUTTM (40U/µL, Life Technologies) and 2µL of DNase I (10,000 U/ml) in a final volume of 10µL. These samples were then incubated at 37ºC for 10min and the enzyme was inactivated by heating at 75ºC for 10min. Subsequently, for each sample, 1µL dNTP (10mM, Life Techonologies) and 0.5µL OligodT (0.5µg/µL, Life Technologies) were added in a final volume of 12µL. The samples were then incubated at 75°C for 10 min for denaturation of the DNA molecules and immediately placed on ice. Subsequently, it was added 7µL of a mix composed of: 2µL SuperScript III first ribbon synthesis buffer, 2µL DTT (0.1M, Invitrogen), 0.5µL RNase OUTTM (40U/mL) and 2.5µL of H2O. These samples were then incubated for 10min at 25ºC to anneal the primers and then at 42ºC (optimal temperature for the SuperScript III enzyme) for 2min, and then 1µL SuperScript III enzyme (200U/µL, Invitrogen), and the samples were incubated at 50ºC for 2 hours, to allow the synthesis of the cDNAs and later at 72ºC for 10min to inactivate the reverse transcriptase. 1µL RNase H (5U/µL, Fermentas - ThermoScientific) was added for degradation of template RNA molecules that were still present in the sample, incubating at 37ºC for 30min and then at 72ºC for 10min. Finally, the cDNA samples were diluted 3x in Milli-Q® water for use.

3.9.2. *Primers* design

For the quantification of gene expression through qRT-PCR primers were designed having as substrate the target transcripts of this study. The primers were designed with the aid of the Primer-BLAST (NIH) program. The following parameters were adopted for the design of the primers: (i) product size between 70 and 200, (ii) primers between exon-exon junctions, (iii) specificity for the organism of interest. The quality of these primers was confirmed using the IDT software (Integrated DNA Technologies), ensuring that there was no hairpins

formation at the annealing temperature of these primers, also avoiding the formation of homodimers (formation of these should have larger ΔG than -9 kcal/mol) and heterodimers (the formation of these should have ΔG greater than -9 kcal/mol). After this first analysis, the sequence of the primers was analyzed in the NucleotideBlast program and at BLAT to ensure that they covered distinct exons and were specific to the gene being sought. The oligonucleotides/primers thus designed are listed in Table 1.

Table 1: Sequence of oligonucleotides used for quantification of gene expression by quantitative RT-PCR assays.

Target transcript	*Sequence of the Primers (5'®3')*
Reck	Forward –CCCAGATTATTGCCCAGAGACA
	Reverse – ACACCTGGCAAAGATGAGTTCA
β -Actina	Forward –CGAGTACAACCTTCTTGCAGC
	Reverse – ATACCCACCATCACACCCTGG
HPRT	Forward –CCCAGCGTCGTGATTAGTGA
	Reverse – TGGCCTCCCATCTCCTTCAT

3.9.3. Determination of the optimum final concentration of primers

Prior to the qRT-PCR assays, the optimal concentration of each of the primers was standardized. Thus, reactions containing the primers at final concentrations of 800 to 100nM were performed, using, as template, a mixture of the cDNAs. In this way, the lowest required concentration of primers was determined so that amplification of the product of interest occurred, without Ct having a variation in its value and, also, that

there was no variation in the profile of the gene amplification curve, besides the minimum or nonexistent formation of dimers.

3.9.4. Quantitative RT-PCR reaction (qRT-PCR)

For quantification of the product formed during the reaction of qRT-PCR, the fluorophore SYBR® Green Dye (Life Technologies) was used. When this reagent is not bound to the double strand of DNA, it exhibits fluorescence at a wavelength of 520ηM, but when it is intercalated in the DNA double-strand, because it has affinity with the minor DNA groove, there is an increase (of 100 times) in this fluorescence, allowing the qRT-PCR product to be detected.

The qRT-PCR reaction had, as a template, 1.5μL of the cDNA of interest diluted 20 times in Milli-Q water and 1.5μL of the primer mixture, forward and reverse, at the concentration determined in item 3.10.3, and 3μL of the Fast SYBR® Green Master reagent. PCR reactions were performed in triplicate on the ViiA 7 Real Time PCR System (AppliedBiosystems - Thermo Fisher Scientific). The reaction conditions were: 1 cycle of 95ºC for 20 seconds; 40 cycles of: 95ºC for 1 second, 60ºC for 20 seconds; after the PCR step, the dissociation curve of the molecules is determined, the temperature is raised to 95°C for 15 sec for denaturing the double strand of DNA; then the temperature is maintained at 60ºC for 1min, followed by a gradual rise in temperature to 95ºC. For the management of the thermal cycler and the data collection generated during the amplification, the QuantStudio Real-TimePCR Software (AppliedBiosystems) software was used.

3.9.5. Confirmation of differential expression

The reagent Fast SYBR® Green Master is inserted unspecifically in the double strand of DNA, thus, the presence of non-specific amplification and contamination, such as the formation of primer-dimers/initiators, could interfere with the intensity of fluorescence.

For this reason, the specificity of the fluorescence signal was confirmed by analyzing

the dissociation curves of the amplified product. When the sample temperature reaches the denaturation temperature (Tm) of the amplified product, the product is denatured and the Fast SYBR® Green Master is decoupled from the DNA, which causes the decrease of the fluorescence intensity detected by the apparatus. This way, when the reaction of qRT-PCR is terminated, the temperature is raised gradually and the fluorescence intensity is measured. With the derivative of the previous curve, a product dissociation curve was generated. As the products have different sizes and have different Tms, the curve allows the distinction between the different products that amplified during the reaction, besides the possible presence of amplification in the negative control and formation of primers-dimers.

For the QuantStudio Real-TimePCR Software program, the initial data analysis was done, defining a threshold in the exponential phase of amplification of the gene. From the intersection of the threshold with the amplification curve, it was possible to obtain the Ct of the sample (Threshold cycle, the cycle in which the fluorescence is above the background). To determine this Ct, each reaction had a cut-off point of 0.1, which was determined manually.

In the qRT-PCR experiments, there may be a variation of the initial cDNA concentration in the reaction mixture, so that for the data to be compared, they were normalized. Normalization was done through qRT-PCR reactions using primers for constitutive expression genes, which served as internal controls for the amount of cDNA used in the reactions. Thus, the expression of the target gene was determined from the expression levels of the control genes.

The average Cts was calculated from the Cts of the samples, in triplicate. As the expression of the gene is analyzed in relation to a reference sample, the difference between the means of the Cts of the reference sample and the mean of the Cts of the sample studied was calculated. This difference was defined as ΔCp. For the analysis of the primers, it was used the method of Liavk, 2001, in which the 2-ΔΔCT in which the normalization of the primers is considered the maximum, would be equal to 1 (Livak and Schmittgen 2001). The endogenous expression genes used were: β-actin and HPRT.

3.10. Statistical analysis

Differential expression of the target genes was determined by qRT-PCR, Western Blot, Luciferase Assay, and Flow Cytometry. The method of qRT-PCR analysis chosen was that described in Livak and Schmittgen (2001), which takes into account the efficiency of each pair of primers used. The results were submitted to the one way-ANOVA test, followed by the Tukey post-hoc test or Student's T test for comparison between the experimental groups. All comparisons and differences were considered significant when $p < 0.05$

4. Results

Chapter 1

Impact of *Reck* expression and promoter activity in neuronal *in vitro* differentiation

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Abstract

The *Reck* (*REversion-inducing Cysteine-rich protein with Kazal motifs)* tumor suppressor gene encodes a multifunctional glycoprotein, which inhibits the activity of several matrix metalloproteinases (MMPs) and modulates the Notch and canonical Wnt pathways. *Reck*-deficient neuro-progenitor cells undergo precocious differentiation; however, modulation of *Reck* expression during progression of the neuronal differentiation process is yet to be elucidated. In the present study, we assessed *Reck* expression and characterized mouse *Reck* promoter activity during the *in vitro* neuronal differentiation process. Increased *Reck* promoter activity and mRNA expression levels were investigated in PC12 mouse pheochromocytoma, P19 mouse embryonal carcinoma and USP-4 mouse embryonic stem cells, upon induction of neuronal differentiation. While *Reck* expression increased throughout neuronal differentiation in P19 and USP4 cell lines, the expression levels remained unchanged in PC12 cells. Interestingly, *Reck* overexpression in embryonic P19 cells led to a diminished efficiency of the neuronal differentiation process. Taken together, our findings suggest that in contrast to the gradual increase of *Reck* expression during late stages of the neuronal differentiation process in mice, increased *Reck* expression at early stages of this *in vitro* differentiation diminished the number of β-3 tubulin neuron-like cells. Our data reinforce the importance of *Reck* expression in regulating *in vitro* neuronal differentiation.

Introduction

The tissue microenvironment is a key driver of cellular differentiation [1], [2], [3]. In particular, the extracellular matrix (ECM) plays crucial roles by allowing flexibility for cell movement, and by modulating accessibility to the non-matrix components, such as peptide growth factors and cell-adhesion/cell-cell interaction molecules [4], [5]. Therefore, it is not surprising that molecules which modulate the

ECM composition are potential targets for intervention, aiming at optimization of *in vitro* neuronal differentiation protocols [6], [7], [8], [9], [10].

The *REversion-inducing Cysteine-rich protein with Kazal motifs (Reck)* gene encodes a multifunctional glycoprotein of 110 kDa, which modulates the microenvironment by acting in different pathways. This glycosylphosphatidyl inositol membrane-anchored glycoprotein inhibits the activity of several members of the Matrix Metalloproteinase (MMP) family, which is composed by key enzymes responsible for the ECM turnover, including: MMP-2, MMP-7, MMP-9, and MT1-MMP [11], [12], [13]. Other Reck targets have also been identified, as the extracellular ADAM10 metalloproteinase and CD13/aminopeptidase N, implicating Reck in Notch pathway modulation through ADM10 and Wnt7 pathway activation in a GRP124-dependent manner [14], [15], [16], [17], [18].

Mice lacking functional *Reck* die around embryonic day 10.5, displaying abnormal organogenesis and alterations in the basal membrane, with deprivation of fibrillar collagen and elevated MMP activity [12]. The *Reck* promoter region is susceptible to at least two myogenic regulatory factors, namely MyoD and MRF4, suggesting a possible role for *Reck* in myogenesis [19]. Furthermore, *Reck* expression has been demonstrated to be modulated during chondrogenesis and downregulated at late stages of *in vitro* osteoblast differentiation [20], [21]. In addition, *Reck* impairment in human mesenchymal stem cells was described to favor adipogenesis over osteogenesis [22]. On the other hand, *Reck*-deficient neural progenitor cells undergo precocious differentiation [15] and modulation of *Reck* expression during progression of the neuronal differentiation process is yet to be characterized.

In view of the importance of *Reck* as a microenvironment modulator and the evidence that *Reck* expression modifies the differentiation outcome in different cellular models, we aimed to investigate *Reck* expression and promoter activity during neuronal differentiation and its possible influence in neuronal cell fate. Therefore, murine *Reck* promoter activity and expression pattern were investigated during the *in vitro* neuronal differentiation process, and the effects of *Reck* overexpression in the neuronal differentiation process was also assessed.

Materials and Methods

Cell Culture. Rat pheochromocytoma PC12 cells were kindly provided by Dr. Irene Yan (Department of Histology, University of São Paulo, SP, Brazil). The culture was maintained essentially as previously described [23], in Dulbecco's modified Eagle Medium (DMEM), supplemented with 10% (v/v) fetal bovine serum (FBS), 5% horse serum (HS), 2 mM L-glutamine, 100 mg/mL streptomycin sulphate, and 100 U/mL penicillin G. Mouse embryonic stem (ES) USP-4 cells were maintained over a murine embryonic fibroblast (MEF) feeder layer [24]. USP-4 [24] and murine embryo-derived teratocarcinoma P19 cells [25] were cultured with Minimum Essential Medium Eagle, α-modification, with nucleosides (α-MEM) supplemented with 10% FBS, 2 mM L-glutamine, 100 mg/mL streptomycin sulphate, and 100 U/mL penicillin G, and maintained at 37°C, in a 5% CO_2 atmosphere.

Neurodifferentiation protocol. For differentiation of PC12 cells, 10nM of basic fibroblast growth factor (*b*FGF) was added to the cell culture medium for 48h. The USP-4 ES neuronal differentiation protocol consisted of plating the cells as hanging drops onto the cover of 148 cm^2 Petri dishes (Nunc-Sigma, MO, USA), with 1,250 ES cells/ 25 µL drop in 5% FBS basic culture medium (BCM) and filling the plate with calcium- and magnesium-free Dulbecco's phosphate solution (PBSA) to avoid evaporation of the hanging drops. After three days, the resulting Embryoid Bodies (EBs) were transferred to 9 cm^2 Petri adherent dishes (Nunc-Sigma). After 24h, the culture medium was replaced by neurobasal (NB) medium (former Gibco, Life Technologies Corp., Carlsbad, CA, USA) supplemented with B27 and 0.1 µM of retinoic acid (RA). After four days, RA was removed from the culture medium and the cell culture was maintained for additional seven days in NB medium supplemented with B27 [26]. For P19 cells differentiation, spheroid formation was induced by transferring the cells to plastic Petri dishes at a density of 5 x 10^5 cells/mL in medium supplemented with 1 mM RA. After four days, spheroids were transferred to adherent tissue culture dishes and maintained for additional seven days [27].

Reck promoter activity assessed by transient transfection and luciferase activity. Murine *Reck* promoter plasmid constructs are the same as those previously described in [28], and were kindly provided by Dr. Makoto Noda (Kyoto University, Kyoto, Japan). Luciferase activity was essentially determined as previously described [29]. In summary, 24 h before transfection, cells were plated onto 24-wells plates (10^5 cells/mL/well). Plasmid DNA, purified using Qiagen columns (Qiagen Inc., Hilden,

Germany), was then transfected using the calcium phosphate method [30]. To detect the promoter activity of the 5'-flanking region of the mouse *Reck* gene, 3 mg of the luciferase reporter plasmid and 0.25 mg of pRL-TK plasmid (Promega, Madison, WI) were used for each transfection assay. The pRL-TK plasmid, which carries the Renilla luciferase gene, driven by a constitutive (HSV-*tk*) promoter, was used as an internal control to normalize the transfection efficiency. All transfection assays were carried out in triplicate. Twenty-four hours after transfection, the cultures were washed with PBSA [137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4] and fresh medium was added. After additional 24 h, expression of the reporter genes was analyzed using the Dual-Luciferase Reporter Assay System (Promega) and a Microplate Luminometer (Dynex Technology, Chantilly, VA), expressing luminescence in terms of relative light units.

Identification of putative transcription factors binding sites within the (-817/-52) *Reck* promoter region. The murine *Reck* promoter fragment (-817/-52 bp) was analyzed using the PROMO tool and the TRANSFAC database version 8.3 for prediction of potential regulatory sequences [31], [32].

Real time qRT-PCR. Total RNA was extracted and purified using the Illustra RNAspin mini-isolation kit (GE Healthcare, Chicago, IL). The RNA pellet obtained was solubilized in Milli-Q water and then stored at -70°C. RNA was quantified using the Nanovue spectrophotometer (GE Healthcare, Piscataway, NJ) and the RNA quality was evaluated by electrophoresis in agarose gel. Subsequently, 1µg of RNA was used as the substrate for cDNA synthesis using the qPCR BIO kit (PCR Biosystems, London, UK). The primer set for *Reck* and endogenous control genes *Hprt* and *β-actin* were designed using the Primer Software (Applied Biosystems, Foster City, CA, USA): (*Reck-sense*) 5' CCC AGA TTA TTG CCC AGA GAC A 3', (*Reck-antisense*) 5' ACA CCT GGC AAA GAT GAG TTC A 3', (*Hprt-sense*) 5' CCC AGC GTC GTG ATT AGT GA 3' and (*Hprt-antisense*) 5' TGG CCT CCC ATC TCC TTC AT 3', (*β-actin-sense*) 5'-CGA GTA CAA CCT TCT TGC AGC -3' and (*β-actin-antisense*) 5' ATAC CCA CCA TCA CAC CCT GG 3'. Real-time PCR was carried out using the Fast SYBR® Green master mix (Applied Biosystems) for detection and relative quantification of the target sequences in a ViiA7 Real Time PCR System (Applied Biosystems). A dissociation cycle was carried out after each run to evaluate the primers specificity and for quality control of the reaction. The expression ratios

were calculated according to [33], and endogenous control normalization was achieved using geNorm [34].

Flow cytometry analysis. Cells were harvested, washed and incubated with blocking buffer (PBS, 4% FBS, 0.1% Triton-X-100) for 30 min at 4°C, followed by incubation with anti-β-3-tubulin antibody (dil. 1:1,250, Cat# ab56676, Abcam, Cambridge, MA, USA) or with anti-GFAP (dil. 1:1,000, Cat# Z0334, Dako, Glostrup, Denmark) for additional 30min at 4°C. Cells were washed three times with PBS, and then, ressuspended and incubated with the appropriate secondary antibody (1:2,000; Cat #A-11001, Cat # 31234, Thermo Fisher Scientific, Waltham, MA, USA), for 30 min at 4°C. Cells were washed three times in PBS and data were acquired using the FACS Aria IIu (BD Biosciences, San Jose, CA, USA).

Statistical Analysis. In the graphical representations, bars indicate the mean values ± standard deviation (S.D.) of at least three independent experiments. For the promoter activity assay, within each construct, the difference between the three time points was analyzed using two-way ANOVA followed by Bonferroni test. For the gene expression and flow cytometry assays, the statistical differences between groups were evaluated by Student's t-test or one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. Differences were considered to be statistically significant for values of $p < 0.05$.

Results

Reck gene promoter activity during the neuronal differentiation process

Using the previously described mouse *Reck* gene promoter constructs [28] the contribution of different *cis* regulatory elements to *Reck* transcriptional activity during neuronal differentiation was evaluated by sequential deletions of the promoter region and dual luciferase reporter assays. The PC12 cell line was employed as a model of neuronal cell differentiation [35]. As previously reported, three days after induction of neuronal differentiation, PC12 cells start to display neurites, and on day 7, these cells are considered to be fully differentiated, under the employed condition [29].

Undifferentiated PC12 cells were transiently transfected with the construction containing the longest 5' upstream sequence (–4110/+82) of the *Reck* gene promoter, and the promoter activity of this fragment was evaluated at days 0, 3 and 7 after the induction of neuronal differentiation with *b*FGF. Murine *Reck* promoter

activity increased along progression of the neuronal differentiation protocol, with the highest promoter activity being observed at day 7 ($p < 0.001$), when PC12 cells were fully differentiated (Figure 1).

Shorter constructs of the 5' upstream sequence of the *Reck* gene (-2204/+82, -1679/+82 and -817/+82, relative to its ATG start codon), produced by deletions with restriction enzymes (as previously described in [28]), displayed promoter activity similar to the full-length sequence activity ($p < 0.01$), with the characteristic higher activity at more advanced stages of the differentiation process, namely day 7 of differentiation, when cells were considered to be fully differentiated (Figure 1). Therefore, gradual deletion of the upstream fragments did not influence the characteristic of higher expression on day 7 of differentiation, since the total promoter activity was not significantly affected. In line with this result, the promoter activity at day 0 and 3 was also exactly the same among the full-length promoter and 5' upstream sequence deleted fragments (-2204/+82, -1679/+82 and -817/+82) of the *Reck* promoter region (Figure 1).

On the other hand, the activity of the shortest construct, comprising the -52/+82 *Reck* gene 5' upstream sequence, did not display this neuronal differentiation-dependent modulation. The activity of this shortest fragment at day 0 or 3 was similar to that observed for the full-length and also for the 5' upstream sequence deleted fragments of the *Reck* gene (namely -2204/+82, -1679/+82 and -817/+82), but not for day 7, in which significant differences in promoter activity were detected among all fragments.

Finally, in line with the previously described importance of the Sp1 site for *Reck* promoter activity [28], mutation of both Sp1 sites in the shortest evaluated fragment (-52/+82), which was described as fundamental for the minimal promoter activity, led to a strong suppression of the *Reck* promoter activity (Figure 2).

Putative transcription factors binding sites within the *Reck* promoter region

Aiming to identify the key regulatory regions which are present within the *Reck* promoter region, the (-817/-51) fragment sequence, whose deletion led to the suppression of the modulation of *Reck* promoter activity along the differentiation process, was analyzed using the PROMO tool [31], [32] for prediction of putative transcription factor binding sites (Figure 2, Table 1).

We detected putative binding sites for 20 transcription factors, which had previously been described to be involved in neuronal differentiation (Table 1), including Hes1, Ap-1 and JunD.

Table 1: Transcription factors with potential action upon Reck promoter region -52/-817

Transcription factor	Role in neuronal differentiation	References
c-Fos	during development, the neocortex of knockout mice displays less differentiaded cells and higher apoptotic rate when compared to the wild type; its expression increases during neuronal differentiation in pheocrhomocytoma cells; promotes neuronal differentiation in PC12 and its inhibition through siRNA impairs neurite outgrowth	[36], [37], [38]
HES-1	lower expression in embryonic stem cells favors neuronal differentiation, null mice embryos display at the same time defects in neural tube formation and precocious neurogenesis, its expression decreases during neuronal differentiation and its overexpression impairs the neuronal differentiation process	[39], [40], [41]
C/EBPbeta	activated by RA in neuronal differentiation, knockout mice display reduced neuronal differentiation rates while its overexpression leads to precocious neuronal differentiation	[42], [43], [44]
JunD	its expression increases during the neuronal differentiation process induced by DMSO in neuroblastoma cells	[45]
c-Jun	its activation is enough to induce the differentiation process and its overexpression leads to precocious neuronal differentiation, highly expressed during neuronal differentiation induced by VPA differentiation	[46], [47], [48]

AP-1	AP-1 ligand expression increases during neuronal differentiation induced by DMSO, its epression is inhibited by RA in Hela cells, it is a downstream effector in the neuronal differentiation induced by bFGF	[45], [49], [50]
HOXA5	it is expressed by motor neurons and interneurons during development, its ectoppical expression in the developing cervical and brachial dorsal spinal cord results in loss of dorsal horn neurons	[51], [52]
NF1	depleation in neuroprogenitor cells leads to increased glial proliferation and neuronal differentiation abnormalities, supression through siRNA leads to impaired neuronal function	[53], [54], [55]
GR	its inhibition reduces NPCs proliferation and promotes differentiation, its activation suppresses neuronal differentiation and neurite outgrowth	[56], [57], [58]
MyoD	its ectopic expression inhibits neuronal differentiation in chick neural tube	[59], [60], [61]
GATA-2	it is expression increases in the early stages of neuronal differentiation, its depletion leads to severe impairment of neurogenesis	[62], [63]
YY1	it represses neuroprogenitor proliferation, its depletion impairs oligodendrocyte differentiation	[64], [65]
C/EBPα	its expression is linked to neuronal survival	[66]
AhR	its deplation leads to cerebellar granule neuron precursor development disruption, with increased neurodifferentiation and neurite outgrowth	[67], [68], [69]
POU2F2	its expression regulates the diversification and distribution of interneurons in the developing spinal cord	[70]

NF-kappaB	its expression promotes developing sensory neurons neurite growth and enhances the size and complexity of pyramidal neuron dendritic arbors in the developing cerebral cortex, it is activated by the glutamate cascate	[71], [72], [73]
HNF-3	it is expressed in early stages of neuronal differentiation and its expression is induced by retinoic acid in P19 cells	[74], [75], [76]
E2F-1	it is expressed in proliferating neural precursor cells whereas its expression is not detected in differentiated cells	[77]
POU2F1	its interaction with SOX2 is important for Nestin expression during the neural differentiation of P19 cells	[78]
RXR-alpha	its expression is important for the terminal neural differentiation phenotype induced by retinoic acid and in the neurite outgrowth promoted by Docosahexaenoic Acid treatment	[79], [80]

***Reck* expression during neuronal differentiation**

In order to evaluate *Reck* expression during progression of the *in vitro* neuronal differentiation, three different cell models were employed, namely: USP-4 murine embryonic stem cells, P19 murine embryonal carcinoma and PC12 rat pheochromocytoma cells (Figure 3).

An increase of about 4-fold in *Reck* mRNA expression was observed upon induction of USP-4 embryoid bodies (EB) to neuronal differentiation ($p < 0.05$, Figure 2A). Curiously, *Reck* expression was decreased in the EBs by approximately 25-fold compared to the adherent USP-4 embryonic stem cells ($p < 0.01$, Figure 3A). In agreement with this result, murine P19 cells also displayed a sharp increase of about 20-fold in *Reck* mRNA expression along progression of the differentiation process ($p < 0.001$, Figure 3B).

Interestingly, rat PC12 cells did not display any expression modulation throughout the differentiation process (Supplementary Figure 1).

Effects of *Reck* expression upon the neuronal differentiation process

Here, we demonstrate that *Reck* expression is upregulated upon induction of neuronal differentiation. Nonetheless, since it was described that *Reck* depletion in neural progenitor cells leads to precocious differentiation [15], aiming to evaluate the influence of *Reck* expression upon the neuronal differentiation process, *Reck* was overexpressed in murine P19 cells prior to the onset of differentiation.

Although *Reck* expression was increased by only 4-fold in the undifferentiated transfected murine P19 cells ($p < 0.05$, Figure 4A), induction of neuronal differentiation led to an even greater increase, of more than 20-fold, in *Reck* mRNA expression at sphere or differentiated stages, for wild-type P19 cells, empty vector control or *Reck* overexpressing cells ($p < 0.01$, Figure 4A).

The differentiation outcome was assessed by flow cytometry, through evaluation of the cell subpopulation expressing β-3-tubulin, which is a classical marker of mature neurons [81]. Cells overexpressing Reck at the beginning of the differentiation induction displayed a decrease of about 14% in the β-3-tubulin expressing cell population ($p < 0.05$), indicating significantly diminished neuronal differentiation efficiency. Furthermore, there was no significant difference in the cell subpopulation expressing GFAP, which is a classical astrocyte marker, under the different conditions employed [82] (Supplementary Figure 2).

Discussion

Reck is a multifunctional protein which determines the tissue microenvironment, not only by directly inhibiting MMPs and ADAMs activity, but also, by modulating the Notch and Wnt canonical pathways [11], [12], [14], [15], [13], [16], [17], [18]. *Reck* expression was observed to be gradually upregulated during chondrogenesis and osteogenesis, while being downregulated during adipogenesis [20], [21], [22]. While *Reck* impairment in human mesenchymal stem cells was described to favor adipogenesis over osteogenesis, in neural progenitor cells, it leads to precocious differentiation during cortical development [15], [22]. Here, we characterized the mouse *Reck* transcriptional activity during *in vitro* neuronal differentiation aiming to assess the possible influence of this gene in this differentiation process. We demonstrated, for the first time that *Reck* promoter activity and mRNA expression are substantially upregulated during the neuronal

differentiation progression in different cell lines. The *Reck* promoter region determined to be crucial to this modulation during neuronal differentiation was localized between -817 to -51 bp, in which at least 20 distinct putative binding sites for transcription factors involved in this process were identified.

Taken together, these data suggest that *Reck* may play a potential role in cell commitment to specific differentiation fates, including neurons. In order to conciliate the upregulated *Reck* transcriptional feature, as observed in P19 and USP-4 cells submitted to neuronal differentiation, with the precocious neuronal differentiation reported by Muraguchi and collaborators in *Reck* deficient cortical development [15] *Reck* overexpressing P19 cells were submitted to neuronal differentiation induction. In accordance with Muraguchi's findings, *Reck* overexpression led to a diminished efficiency of the differentiation protocol.

Muraguchi and collaborators described that *Reck* deficient neuronal progenitor cells displayed impairment in Notch signaling in an ADAM10-dependent mechanism [15]. Notch signaling is known to upregulate the expression of the Hes1 transcription factor [83], [84], whose impairment is known to foster neuronal differentiation [41]. As Hes1 potentially acts upon the *Reck* (-817/-51) promoter region, the Reck-Notch-Hes1 axis might constitute a feedback loop that influences the neuronal differentiation outcome. Therefore, taken together, our data indicate that modulation of *Reck* expression may be used as a tool to optimize *in vitro* neuronal differentiation protocols.

Conflict of interest

The authors declare no conflict of interest.

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Figures:

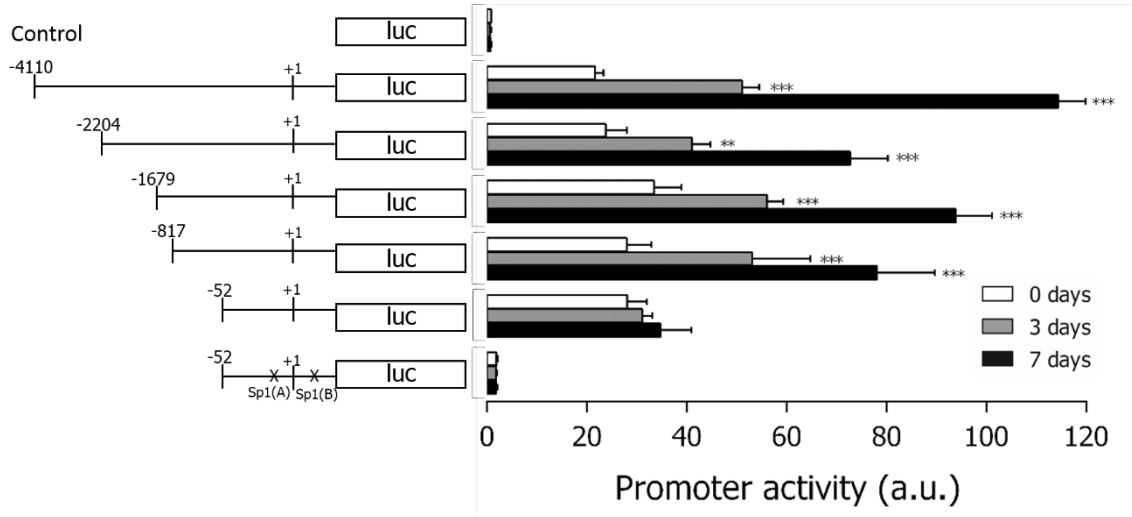


Figure 1. Effect of *Reck promoter activity in neuritogenesis during PC12 cell differentiation.* Schematic representation of a luciferase reporter gene containing the longest versus truncated 5´-flanking regions of the mouse *Reck* gene. Each of the luciferase constructs was assessed in the undifferentiated state (day 0, white), under differentiation with *b*FGF (day 3, gray) and in fully differentiated PC12 cells (day 7, black). The promoter constructs are represented by the size of their 5´-end relative to the transcription initiation site (+1). Data represent means ±SD of three independent experiments. Days 3 and 7 of the protocol are compared to day 0 for each construct. ** represents $p < 0.01$, and *** represents $p < 0.001$, in which the indicated group is compared to its corresponding undifferentiated one.

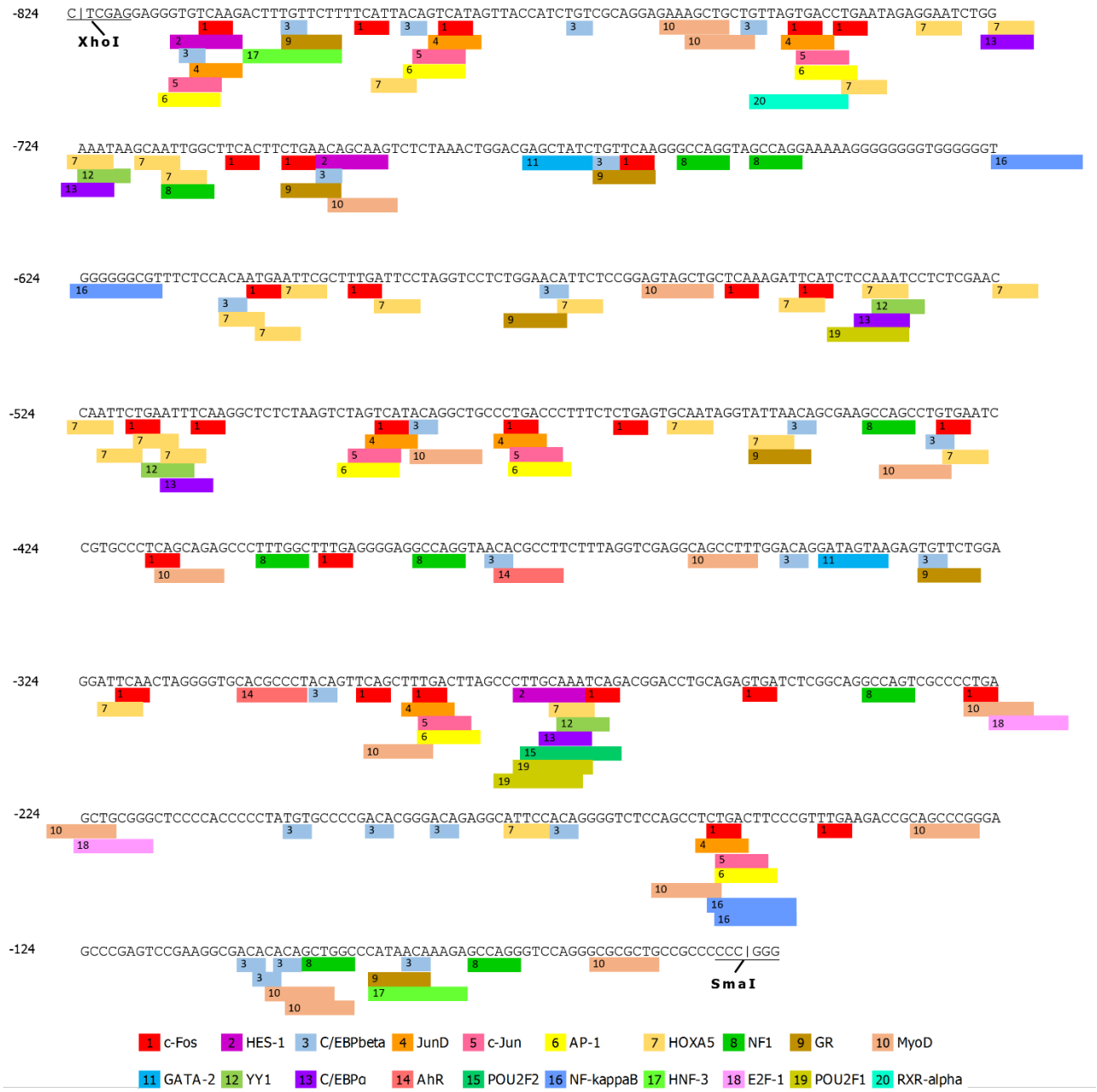


Figure 2. *Schematic representation of the putative transcription factor binding sites present in the -817 to -51 Reck promoter region*. Prediction using the PROMO toll (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3).

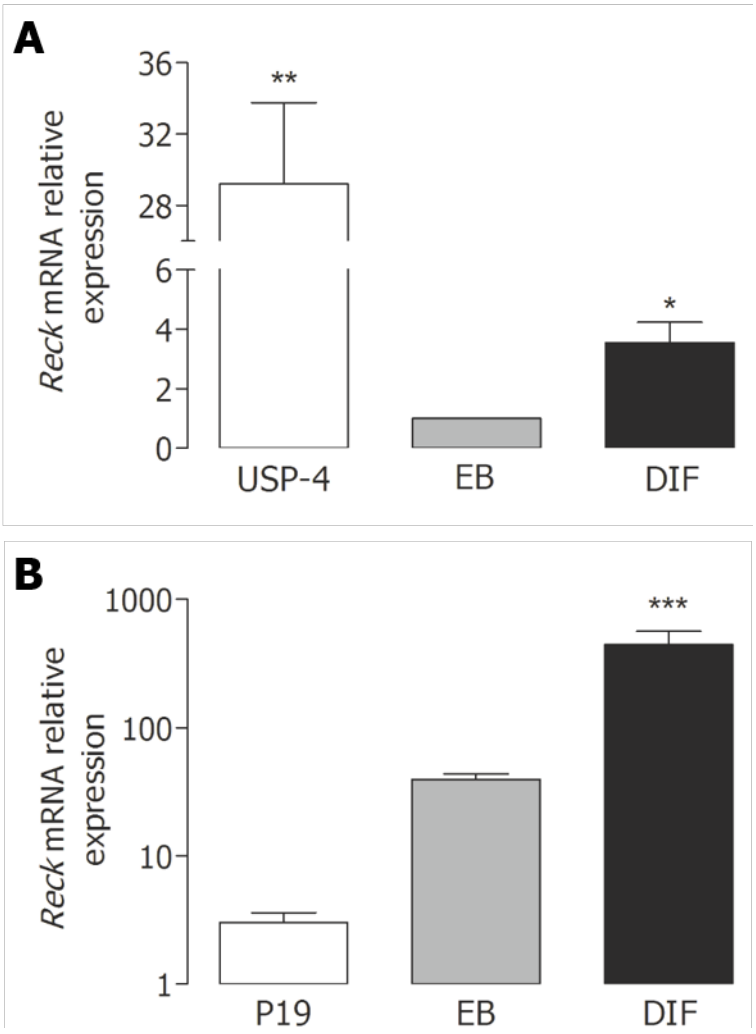


Figure 3. *Reck Expression During Neuronal Differentiation. Reck* mRNA relative expression in (A) USP-4 murine embryonic stem cells, (B) P19 murine embryo-derived teratocarcinoma cells, and (C) PC12 rat pheochromocytoma cells during the different stages of the neuronal differentiation protocols, from left to right: undifferentiated, under differentiation, and differentiated. EB stands for embryonic bodies, and DIF for the final differentiation stage. Data represent means ± SD of three independent experiments. * represents $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

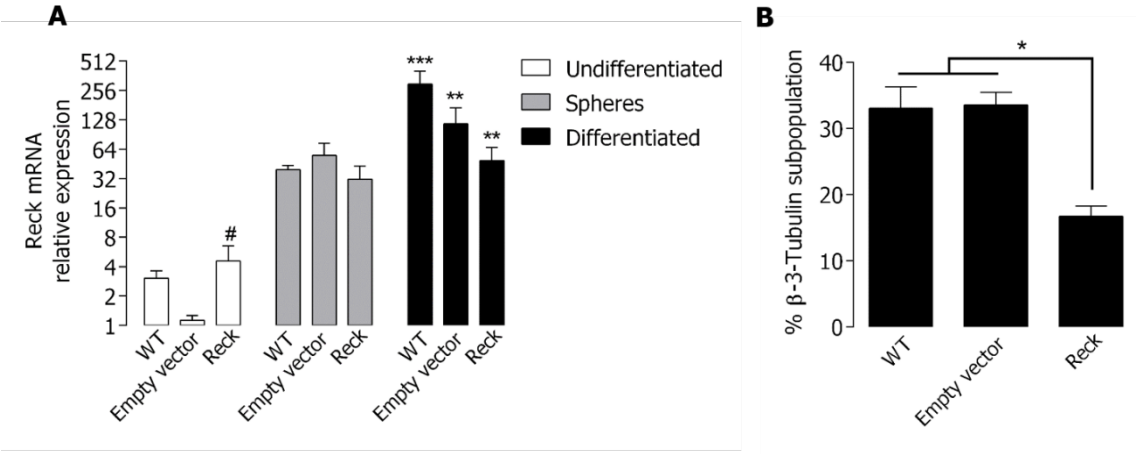
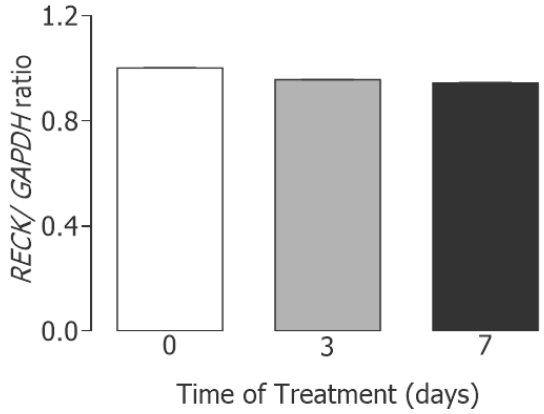
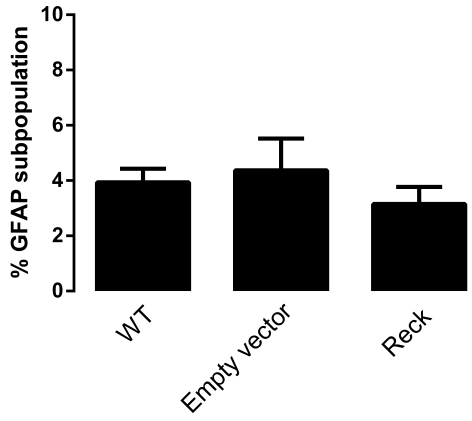


Figure 4. *Influence of Reck overexpression upon neuronal differentiation*. (A) *Reck* mRNA relative expression in wild type P19 murine embryo-derived teratocarcinoma cells (WT), transfected with the empty PCXN2 vector (empty vector) or with the PCXN2-Reck expression vector (Reck) during the different stages of the neuronal differentiation protocol: undifferentiated (white), under differentiation (Spheres, gray), and differentiated (black). (B) **β**-3-Tubulin expressing sub-population of P19 cells transfected with the empty PCXN2 vector or with the PCXN2-Reck expression vector at the end of the neuronal differentiation protocol. *represents $p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, in which the indicated group is compared to its undifferentiated counterpart. # represents $p < 0.05$ in the comparison between undifferentiated empty vector and *Reck* overexpression.

Supplementary Figures



Supplementary Figure 1. *Reck* mRNA relative expression in PC12 rat pheochromocytoma cells during the different stages of the neuronal differentiation protocols, from left to right: undifferentiated, under differentiation, and differentiated. Data represent means ± SD of three independent experiments.



Supplementary Figure 2. GFAP expressing sub-population of P19 cells transfected with the empty PCXN2 vector or with the PCXN2-Reck expression vector at the end of the neuronal differentiation protocol.

Chapter 2

Extracellular Matrix dynamics during Mesenchymal Stem Cells Differentiation

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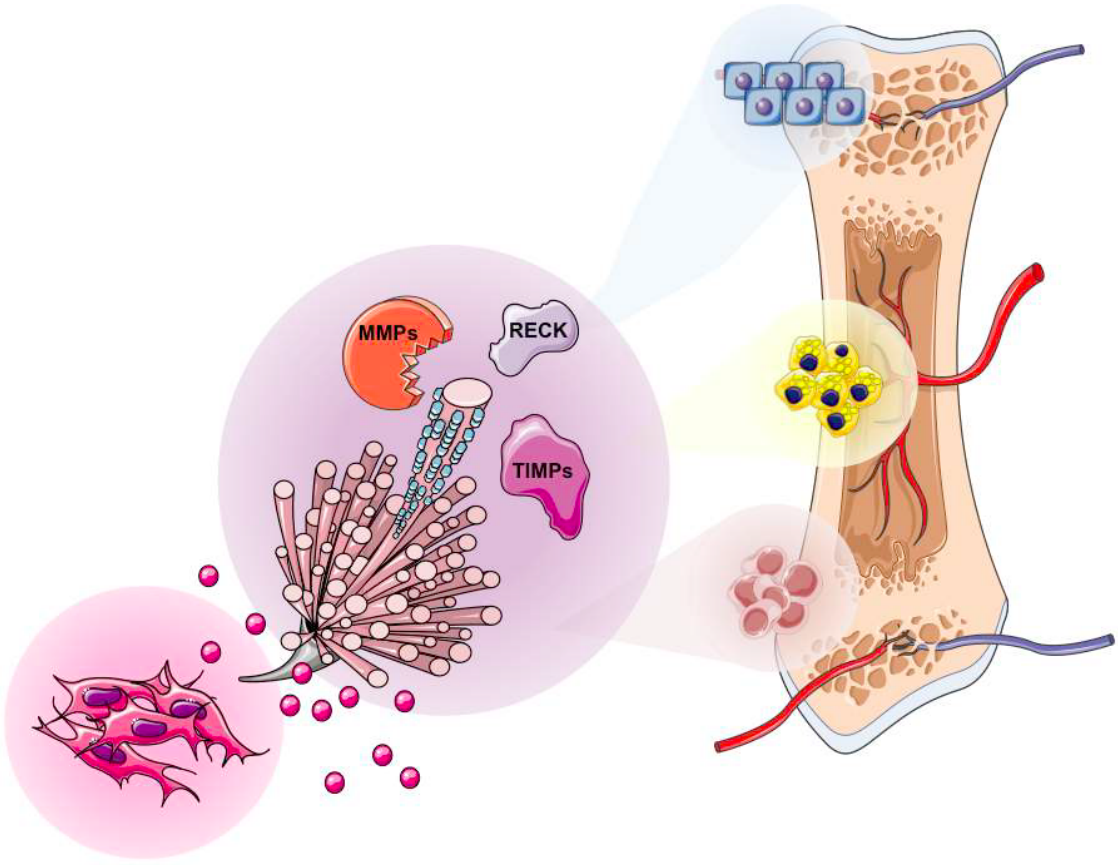
Abstract

Mesenchymal stem cells (MSCs) are stromal cells that display self-renewal and multipotent differentiation capacity. The repertoire of mature cells generated ranges but is not restricted to: fat, bone and cartilage. Their potential importance for both cell therapy and maintenance of *in vivo* homeostasis is indisputable. Nonetheless, both their *in vivo* identity and use in cell therapy remain elusive. A drawback generated by this fact is that little is known about the MSC niche and how it impacts differentiation and homeostasis maintenance. Hence, the roles played by the extracellular matrix (ECM) and its main regulators namely: the Matrix

Metalloproteinases (MMPs) and their counteracting inhibitors (TIMPs and RECK) upon stem cells differentiation are only now beginning to be unveiled. Here, we will focus on mesenchymal stem cells and review the main mechanisms involved in adipo, chondro and osteogenesis, discussing how the extracellular matrix can impact not only lineage commitment, but, also, their survival and potentiality. This review critically analyzes recent work in the field in an effort towards a better understanding of the roles of Matrix Metalloproteinases and their inhibitors in the above-cited events.



1. Isolation, *in vitro* characterization and *in vivo* origin of Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) were first described by Friedenstein *et al.,*(Reviewed in (1)) as spindle-shaped, adherent, non-hematopoietic stem cells resident in the bone marrow. Although MSCs represent only a minor fraction of the overall cell population in bone marrow, the facility to grow and expand these cells *in*

vitro overcomes this limitation (2). They are typically isolated from whole bone marrow aspirates after removing non-adherent cells. The remaining adherent mononuclear layer is often cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (3). After expansion and serial passaging, the enriched MSCs are usually heterogeneous. Individual MSC clones can be obtained through seeding cells by limited dilution, known as the CFU or Colony Forming Unit isolation methodology, which generates a homogeneous population (4).

MSCs can also be easily obtained from several other tissues, such as umbilical cord wall, blood, placenta, fat, lung, liver, and skin (5). Since no specific MSCs markers have been identified, MSCs are characterized by a combination of positive and negative markers. The positive markers are comprised by: Sca-1, CD44, CD71, CD73, CD90, and CD105. The negative markers may be the hematopoietic and endothelial markers (CD45, CD34, CD19, CD11b, CD11c, CD79a, and CD31), co-stimulatory molecules (CD80, CD86, and CD40), and MHC molecules (negative for class II and low for class I) (6) (Figure 1).

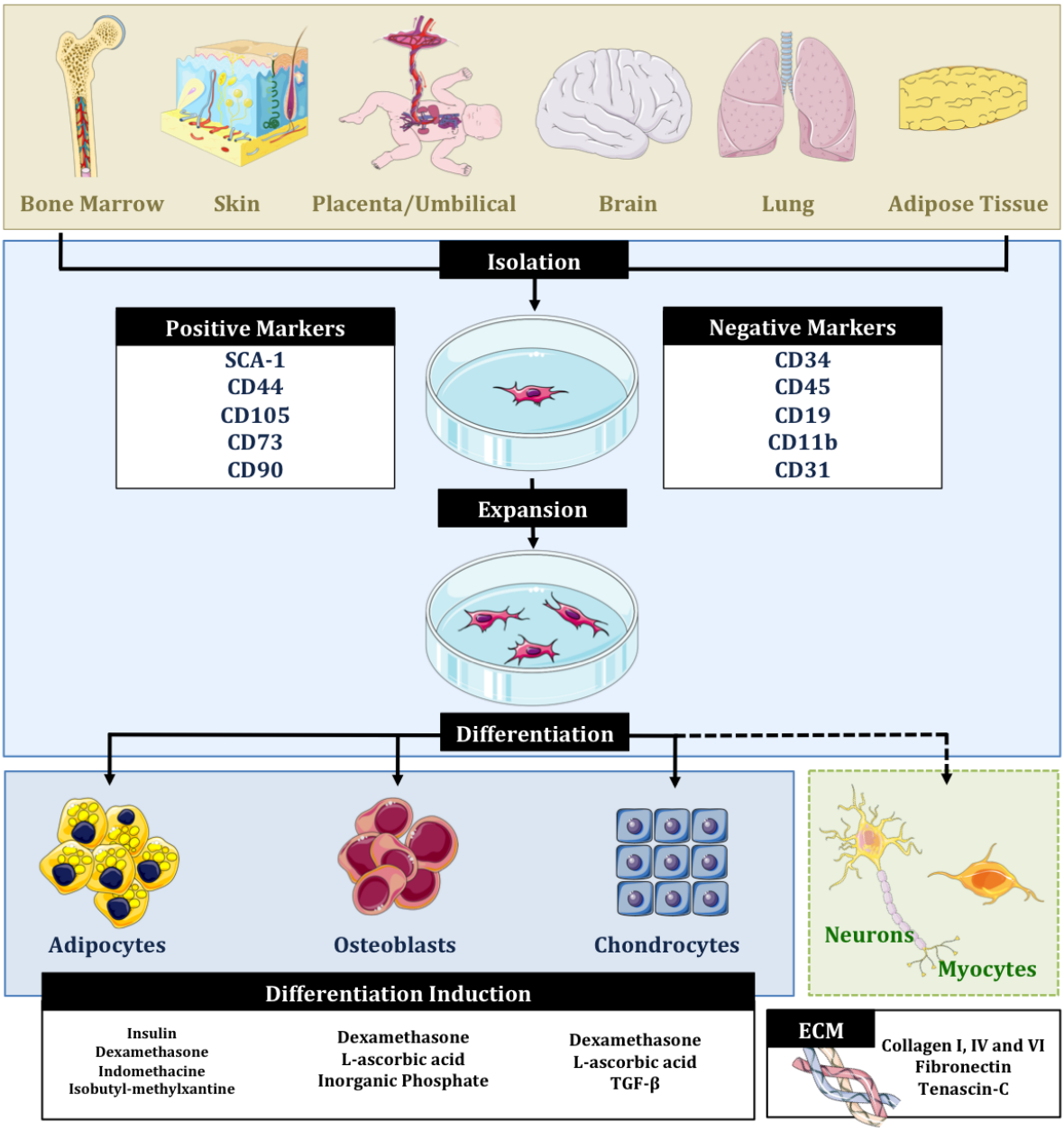


Figure 1

Figure 1 – Mesenchymal Stem Cells (MSCs) isolation, expansion, differentiation and niche. MSCs can be isolated from various tissues, such as the bone marrow, placenta and the skin. Upon isolation, MSCs may be, expanded and enriched by serial *in vitro* passaging. A combination of positive and negative markers can be used to determine the purity of MSCs. These cells can undergo differentiation *in vitro*, generating adipo, osteo, and chondrocytes (more uncertain fates, such as neurons and myocytes, have also been extensively reported). ECM plays an important role for MSCs, constituting the niche rich in collagen type I, fibronectin and

other compounds in the mesenchymal tissues. Servier Medical Art.

Despite the common properties of MSCs, listed in the International Society for Cellular Therapy (ISCT) guidelines, significant differences, such as propagation rates and differentiation spectrum were observed between mesenchymal stromal cells that were derived from different tissues (3).

The *in vivo* origin of MSCs is also a matter of debate. The perivascular niche was proposed as the source for MSCs in various tissues (7). Nonetheless, the pericyte nature of MSCs was recently questioned by reports showing that fat stromal cells do not localize in close proximity to blood vessels and, therefore, are, not of perivascular origin (8).

A molecular signature originated from the environmental niche most likely controls the tissue-specific phenotype of MSCs, which undergo a profound selection process during their cultivation. It is unclear whether this tissue-specific signature would be maintained under culture conditions (5). Although all MSCs are multipotent and adherent to plastic, accumulating data currently suggest that tissue-specific MSCs differ in basic and fundamental properties that may pose a critical effect on their differentiation capacity. The *in vivo* function of MSCs is largely unknown; nonetheless, they are believed to play an important role in tissue homeostasis and regeneration in mammals (8).

2. Mesenchymal Stem Cells Differentiation – Induction, Commitment and Pathways

Another important criterion for defining MSCs is their multipotency. MSCs have been shown to be capable of differentiating into mature cells of several lineages, especially of mesodermal- derived tissues. The International Society for Cellular Therapy (ISCT) establishes that to be recognized as an MSC, cells must differentiate into adipo, chondro and osteocytes (6) (Figure 1). Several studies have further reported mesenchymal stromal cell differentiation into other cell types of both mesodermal and non-mesodermal origin, including endothelial cells, cardiomyocytes, hepatocytes, and neural cells (9-12). Nevertheless, such multipotential capabilities are not universally accepted, mainly due to concerns generated by the lack of

globally standardized methods for their isolation, expansion and identification, as well as the range of assays used to define terminally differentiated populations (13).

MSC differentiation occurs in a two-step process. The first decision towards differentiation involves the generation of lineage-specific progenitors through a process called commitment. Once a cell is committed to differentiate, the process will resume unaltered and in the absence of any other input. The second phase, also referred to as maturation, involves further alterations, which will culminate in transformation of progenitor cells into terminally differentiated ones. The complete remodeling of transcription, translation and more recently described metabolism of the cell, in response to signaling transduction, sustains these processes (14, 15).

Let us go through some aspects of the cocktail induction and signaling transduction pathway involved with all three classical destinies followed by MSCs.

2.1 Adipogenesis

Adipogenesis, or the generation of lipid-droplet bearing adipocytes, is induced in the presence of isobutyl-methyl-xanthine (IBMX), dexamethasone, indomethacin and insulin.

The dual role of IBMX in adipogenesis involves phosphodiesterase inhibition, which culminates in intracellular cAMP levels elevation, followed by protein kinase A (PKA) activation, and, ultimately, leads to the activation of a set of adipogenic transcription factors. Simultaneously, IBMX may directly induce C/EBPβ expression. These elevated cAMP levels result in the phosphorylation of *cyclic AMP response element-binding protein* (CREB) (16). This mediator *per se* induces the expression of C/EBPβ. The two other members of C/EBP family, namely: C/EBPα and γ, have also been implicated in adipogenic differentiation. Binding of these three transcription factors to regulatory elements in the promoter region of PPARγ leads to its sustained expression, an event which is important not only for fate determination but, also, to cell identity, since its expression is sustained throughout the adipocyte lifetime (Nicely reviewed by (17, 18)).

Dexamethasone activates C/EBPγ through its binding to the intracellular glucocorticoid receptor, a process which is potentiated through PPARγ activation induced by indomethacin exposure. Another critically important chemical component

of adipogenic differentiation is insulin, which is long known to promotes glucose uptake for triglyceride synthesis, a hallmark of adipocytes (19).

2.2 Osteogenesis

In vertebrate embryogenesis, skeleton generation occurs through two different processes, namely: intramembranous or endochondral ossification. While the former is restricted to skull and clavicle bones, endochondral ossification is responsible for the rest of skeleton formation and is believed to be the process resembled *in vitro* by MSC specification. In this type of ossification, mesenchymal progenitors condense to form chondrocytes and perichondrocytes, generating a cartilage primordium. Chondrocytes will later undergo hypertrophy and cell cycle exit. The expression of Indian Hedgehog (IHH) by these cells is crucial to trigger the differentiation of perichondral cells into osteoblasts (20). Osteoblasts produce a unique combination of extracellular proteins, including osteocalcin and alkaline phosphatase, which are embedded in type I collagen. This ECM, also known as osteoid, is secreted and undergoes a process of calcium phosphate accumulation, called mineralization, generating hydroxyapatite. This process is the main generator of the composite found in bone, which is comprised of by both organic and inorganic material that can be stained by Alizarin Red (21).

Mineralized ECM deposition is the hallmark of osteogenesis being the final result of MSCs induction by the mixture of chemical factors, which include ascorbic acid, dexamethasone and β-glycerol-phosphate (BGP). Ascorbic acid is a well-known cofactor for collagen biosynthesis, constituting the basis for calcified extracellular matrix deposition. It also plays a major role in alkaline phosphatase (ALP) up-regulation, a process that is potentiated by dexamethasone. β-glycerol phosphate (BGP) acts as the substrate for ALP, generating high levels of phosphate ions for deposition of the mineralized ECM (20).

RUNX2 (Runt domain-containing transcription factor) and Osterix (OSX) are the main transcription factors regulating osteogenic MSCs differentiation. RUNX2 has been shown to induce the expression of almost all of the genes responsible for calcified and collagenic ECM production and deposition. Osterix is required later on, being regulated downstream of RUNX2, for proper osteoblast maturation, after

RUNX2 activity has already decreased, although it is never completely absent, since it is responsible for the synthesis of bone matrix in mature osteoblasts (22).

2.3 Chondrogenesis

Induction of chondrogenic differentiation greatly resembles osteogenesis, since these two processes share a SOX9+ progenitor, a transcription factor of the sex-determining region Y (SRY), related to the high mobility group box family of proteins, being indispensable for chondrogenesis and widely used as a marker of this process, along with collagen-2 expression (20).

The existence of a SOX9+ precursor shared by both osteo and chondrogenesis *in vivo* also explains the common presence of dexamethasone and ascorbic acid used in the induction cocktails used to induce these differentiation types. The main difference resides in the requirement for TGFβ to induce a signal transduction-mediated up-regulation of chondrogenesis-associated transcription factors. It is also important to stress that, since this process is inhibited by bFGF, generally, this induction is conducted in serum-free media, mostly due to the fact that many members of the FGF family can induce the expression of RUNX2 (23).

MSCs undergoing chondrogenesis present unique characteristics, expressing many biomolecules that are typically associated with hyaline cartilage, such as type II collagen and the proteoglycan aggrecan (24, 25). It is important to underscore that although these markers are associated with cartilage, the *in vitro* chondrocyte generation is, by far the most daunting of the three classical differentiations. This is mainly due not only to the fact that the proportion of chemical constituents in the induction cocktail tends to be completely abnormal, but, also, to the absence of the layered structure and spatial organization, which are normally found in native cartilage, resulting in poor mechanical properties, unlike the *in vivo* process (26).

One interesting fact is that few papers explore chondrogenesis differentiation *in vitro* in sufficient detail to establish whether one or more committed progenitors give rise to different subtypes of cartilage *in vitro*. The elastin-rich elastic cartilage is different from the hyaline one, which presents aggrecan, and is also different from fibrocartilage, which is rich in versican. Since type II collagen is a common feature in these three categories, most articles do not precisely describe what subtype of

chondrogenesis is occurring (27).

A significant number of critical signaling pathways are involved in regulating MSCs lineage commitment, including, but not limited to: Transforming Growth Factor-beta (TGFβ)/Bone Morphogenic Protein (BMP) signaling, wingless-type MMTV integration site (Wnt) signaling, Hedgehogs (Hh), Notch, and fibroblast growth factors (FGFs). Since these pathways are well established, we only briefly review their roles in MSC differentiation.

2.4. Signaling Pathways associated with MSCs Differentiation

TGFβ/BMPs family: The TGFβ superfamily, comprising more than 30 members, is involved in the control of cell proliferation, differentiation, and embryonic development. Different members exert various functions, being dose-dependent due to their differential role in selectively binding to their transmembrane serine-threonine kinase receptors. BMP4 can promote adipogenic differentiation while BMP2 needs to act synergistically with rosiglitazone for the same MSC fate induction. Furthermore, low doses of BMP2 promote adipogenesis, while high doses accelerate osteogenic and chondrogenic differentiation of C3H10T1/2 cells (28).

Wnt: The Wnt family consists of a vast number of secreted glycoproteins, which function both in a paracrine and an autocrine manner. As a highly conserved signaling pathway, Wnt signaling is involved in many critical biological processes, such as development, metabolism, and stem cell maintenance. By binding to its receptor Frizzled (FZD) and LRP5/6 co-receptors, Wnt ligands stabilize β-catenin, preventing its phosphorylation. Unphosphorylated β-catenin translocates into the nucleus and regulates target genes with a significant role in regulation of MSC differentiation. Activation of the Wnt signaling has been related to enhancement of osteogenesis in detriment of adipogenesis, in a plethora of cell models (Reviewed in (20)).

Notch: The Notch signaling pathway comprises two single transmembrane proteins, namely: Notch and Notch ligand (Delta/Serrate/LAG-2, DSL protein), which exerts its roles as signaling molecules through cell-cell tethering. Studies in 3T3-L1 pre-

adipocytes show that the adipogenic expression of PPARγ and C/EBPα was blocked by exposure to the jagged1 Notch ligand or by overexpression of the Hes-1 Notch target gene in 3T3-L1 cells (29). Moreover, it has recently been demonstrated that blocking Notch signaling promotes MSC adipogenic differentiation via the PTEN-PI3K/AKT/mTOR pathway (30). Notch signaling has also been shown to suppress osteogenic differentiation via inhibition of the Wnt/β-catenin signaling. In a contradictory manner, this pathway has also been implicated in osteogenic differentiation promotion, through crosstalk with BMP2 signaling (31). Therefore, Notch regulates both adipogenesis and osteogenesis in a complex manner, through direct targeting of pro-differentiation genes or in an indirect manner, by modulating other signaling pathways.

Hedgehogs: Hedgehogs are secreted proteins (Sonic Hedgehog [Shh], Indian Hedgehog [Ihh], and Desert Hedgehog [Dhh]), which can be cleaved to produce an active N-terminal fragment, which binds to two membrane proteins, namely: Patched (Ptc) and Smoothened (Smo) (Reviewed in (32)). This leads to Smo release, activating the Gli1, Gli2, and Gli3 transcription factors, culminating in differential regulation of the Hedgehog targeted genes. All the components of Hedgehog signaling pathway, such as Shh, IHh, and DHh, as well as Gli, are highly expressed in MSCs (33). During MSC adipogenic differentiation, Hedgehog signaling is down-regulated due to decreased expression of Gli. Moreover, activation of Hedgehog signaling blocked adipogenic differentiation. On the other hand, for osteogenic differentiation, the Hedgehog pathway has an active role, since its role reinforcing BMP signaling through Smad modulation has been shown to promote osteogenic differentiation (34). In conclusion, Hedgehog signaling pathway is pro-osteogenic and anti-adipogenic.

Other signaling molecules involved in MSCs differentiation: Several other signaling pathways, including FGFs, PDGF, EGF and IGF, have also been implicated in regulating adipo, chondro and osteogenic MSC differentiation. The roles of these peptide growth factors in MSC differentiation are mainly exerted through regulation of the previously discussed signaling pathways, such as Wnt and TGFβ/BMP (Comprehensively reviewed in (35)).

3. Mesenchymal Stem Cells and their Niches – A role for the Extracellular Matrix

Several studies have indicated that MSCs derived from different tissues differ in both their *in vivo* and *in vitro* phenotypes, but very little is known about the molecular events which control their tissue-specific nature. Due to the plethora of organs that harbor MSCs, tissue-specific characteristics of different MSCs populations could be caused by specific ECM composition in their resident niches. Stem cell niche has been defined as a specific microenvironment in the tissue where stem cells live in a quiescent stage, but can self-renew and differentiate in a controlled manner (36).

In vivo, MSCs interact with components of the microenvironment. Physical factors, including cell shape, external mechanical forces, ECM, among others, have been implicated in stem cell fate decision (37). It is essential to understand that the niche environmental signals do regulate stem cells behavior. One of the most important niche components is the extracellular matrix (ECM). A bulging amount of reports has offered insights into how stem cells sense signals from the ECM and how they respond to these signals at the molecular level, which ultimately regulates their fate. Interactions with the niche are reciprocal, since stem cells can remodel the niche in response to the signals they receive from it (38).

Virtually every cell in the body is exposed to ECM proteins. In epithelia, the ECM is organized into a basement membrane that confers polarity, i.e. cells contact the basement membrane environment. In the connective tissue, the MSCs residency site, these cells tend to be completely surrounded by the ECM. The ECM varies in composition and concentration, both within and between tissues (36). Basement membranes are typically rich in laminins and non-fibrillar type IV collagen, whereas in soft connective tissue fibrillar collagens, such as type I, predominate. Cells adhere to the ECM via several different cell surface receptors, of which integrins constitute the major class. This interaction allows cells to sense mechanical cues from the ECM, such as forces, and respond in an appropriate manner (for example: changes in cell shape and size and responses such as differentiation and proliferation) through a process known as mechano-transduction. Therefore, as a key component of the

stem cell niche, the ECM is not simply an inert scaffold, but rather can profoundly influence cell fate choices (39).

The importance of the ECM niche is highlighted by the fact that stem cell expansion or depletion phenotypes may be a direct result of disturbing stem cell–niche interactions or a secondary consequence of perturbing other aspects of tissue homeostasis (40). ECM turnover is mediated by several proteinases, among which the Matrix Metalloproteinases (MMPs) are prominent. MMPs comprise a family of more than 23 zinc-dependent enzymes that are crucial for ECM dynamics, therefore, changes in their activity are crucial for the organism homeostasis, development and, also, for the occurrence of several diseases (41).

4. Extracellular Matrix turnover during Adipogenesis

There are three types of adipose tissue, namely: white, brown and beige, also known as brite or brown-like. The white adipose tissue is mainly involved in fat storage, being characterized by adipocytes displaying a large unilocular lipid deposit surrounded by a thin layer of cytoplasm; their nucleus is flattened and located at the cell periphery. On the other hand, the brown adipose tissue is linked to thermogenic control through energy spending, with its adipocytes displaying smaller polilocular lipid droplets, a polygonal shape and being rich in mitochondria. When a white adipose tissue is submitted to a thermogenic stimulus, some cells acquire a brown-like phenotype, constituting the beige adipocyte cells (42, 43).

MSCs niche ECM has an important role during adipogenesis, being important not only for cell protection against mechanical stress, but, also, for the differentiation process *per se*, since ECM turnover is coordinated with changes undergone by the pre-adipocytes (44, 45). In pre-adipocytes, collagens types I, V, VI, and fibronectin are secreted in large quantities. Similarly, in the mature adipose tissue, ECM is mainly composed of collagen I, IV, V, VI, fibronectin and laminin complexes (46-49). In this tissue, all collagen types are highly represented with exception of type II collagen, which is weakly expressed (50) (Figure 2).

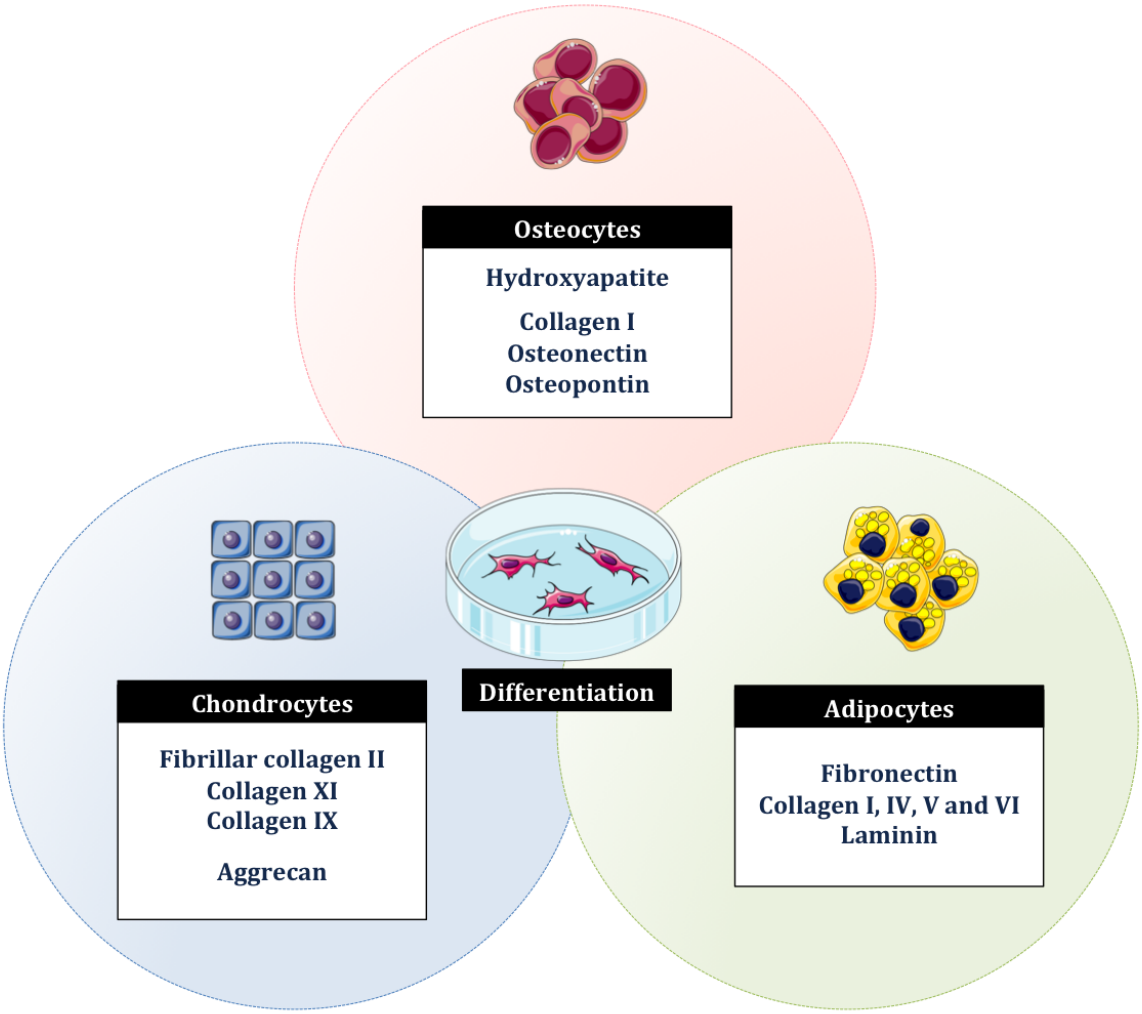


Figure 2

Figure 2 – Extracellular matrix (ECM) composition in bone, cartilage and fat tissue. The chemical nature of the ECM components will directly dictate tissues characteristics, such as softness, resistance, flexibility, permeability and hydration status. As MSCs commit to a certain differentiation fate, they are both influenced by the ECM composition, actively modifying this ECM. Matrix Metalloproteases (Mmps) and their inhibitors (Timps and Reck) play a crucial role in ECM turnover and cellular interaction with the microenvironment. Servier Medical Art.

The mRNA expression profiles of mature adipocytes from obese C57BL/6 mice and Wistar rats show that *Mmp-2*, *Mmp-3*, *Mmp-12*, *Mmp-14* and *Mmp-19*, as well as *Timp-1* are highly expressed (51, 52). Interestingly, even though *Mmp-12* is highly expressed in adipose tissue, *Mmp-12* depleted mice display no alterations in the tissue architecture (53). On the other hand, mice treated with the broad-spectrum MMP inhibitor galardin, which is known to inhibit at least MMP-1, MMP-2, MMP-3, MMP-8, and MMP-9, displayed a decrease in the fat pad mass when submitted to a high-fat diet (54). Nevertheless, male C57Bl/6J mice treated with another broad-spectrum MMP inhibitor, Ro 28-2653, known to inhibit MMP-2, MMP-9, and MMP-14, were shown to have a higher adipocyte count per tissue. However, these adipocytes were smaller than those of the untreated animals, an interesting alteration accompanied by a more intense collagen staining (55). The above-mentioned data suggest that MMPs, and, therefore, ECM remodeling, might be directly involved in the adipogenic process in adult depots.

The relevance of MMPs physiology to adipogenesis becomes clear during mammary gland involution, a process in which the death of secretory epithelium cells is synchronized with an increase in the number of adipocytes, which will repopulate the mammary fat pad. In accordance to the phenotype observed in the animals treated with broad-spectrum MMP inhibitors, the mammary gland involution of Mmp-3-deficient mice display a higher density of adipocytes/area, in comparison to wild type animals (56).

Complementary to *in vivo* models, several *in vitro* studies are available regarding the study of ECM changes during adipogenesis. In these studies, two important lineages are often used: 3T3-L1 and 3T3-F442A. These cells are derived from Swiss 3T3 cells, morphologically resembling fibroblasts, with a fusiform shape, but still retaining some undifferentiated phenotype. These precursor cells easily differentiate into adipocytes, showing a tendency to form lipid droplets, being, therefore, the most commonly used pre-adipocyte lineages (57-59).

In 3T3-F442A cells, *Mmp-2* knockdown by shRNA causes a reduction in the cell differentiation efficiency towards adipocytes. In agreement, *Mmp-2* overexpression in the same cell line leads to a higher differentiation efficiency, suggesting that *Mmp-2* is an important player during adipogenesis (60). In the same

model, it has been observed that the expression of *Mmp-2, Mmp-3*, *Mmp-7, Mmp-10, Mmp-11, Mmp-12* and *Mmp-13* mRNA is high in mature adipocytes. On the other hand, *Mmp-7*, *Mmp-16*, *Timp-3* and *Timp-4* seem to be down-regulated in these cells. During the differentiation protocol, the expression of *Mmp-9*, *Mmp-16* and *Timp-1* is down-regulated in the first stages of the differentiation process. Furthermore, *Mmp-14, Mmp-17, Mmp-19, Timp-2* and *Timp-4* display the opposite profile, showing a higher expression in the first stages of the differentiation process. However, it is important to keep in mind that there are significant differences between adipocytes differentiated *in vitro* and the isolated mature adipocytes. Remarkably, adipocytes isolated from C57/Bl6 male wild type mice do not display the same high mRNA expression of *Mmp-7*, *Mmp-9*, *Mmp-16* and *Timp-4* expressed by adipocytes which were maturated *in vitro* (52). Nonetheless, using microarray analysis, Alexander *et al.,*observed that, in 3T3-L1 cells induced to differentiate towards adipocytes, the expression of not only *Mmp-3* and *Mmp-13,* but, also, of *Mt1-Mmp*, as well as *Timp-1, Timp-2* and *Timp-3* were upregulated upon commitment towards adipocytes and increased as the differentiation program progressed (56). Interestingly, Bernot *et al.,*obtained opposite findings, since, upon adipogenesis induction, 3T3-L1 cells displayed a drastic decrease in *Timp-3* expression, which remained low during the whole process. Also, *Timp-3* overexpression in these cells led to an impairment in adipogenesis (61). Moreover, *Mmp-13* inhibition by siRNA compromised 3T3-L1 cells adipocyte differentiation cells *in vitro*, with the resulting differentiated cells displaying a lower lipid accumulation, visualized by Oil red staining (62).

MMP-2 and MMP-9 are already known to be expressed in human adult adipose tissue and to be modulated during adipogenic differentiation of 3T3F442A cells (63). Mmp-2 displays a steady increase in expression during the differentiation protocol, reaching a plateau at the 9th day of the process. On the other hand, Mmp-9 has a higher overall expression during the differentiation process, displaying a peak at the 7^{th} day, followed by a sharp reduction at the 9th day of the protocol (64). However, although *Mmp-9* silencing did not cause any alteration, *Mmp-2* inhibition led to impairment of the adipogenic differentiation efficiency (63).

Among MMP inhibitors, the membrane anchored protein RECK stands out by its unique localization at the cellular periphery (65). RECK inhibits the activity of at

least four members of the MMP family, namely: MMP-2, MMP-7, MMP-9 and MMP-14 (65, 66). Interestingly, RECK has also been implicated in influencing MSCs differentiation outcome. Its expression decreases at late stages of *in vitro* adipogenesis and its induced down-regulation by siRNA in human MSCs favors adipogenesis with consequent increase in cellular lipid droplets (67).

5. Extracellular Matrix turnover during Osteogenesis

Bone tissue has an important role not only for mechanical functions, such as locomotion and resistance, but also to its metabolism. This highly dynamic tissue is under constant ECM remodeling through the entire organism lifespan (68, 69).

The cellular compartment of this tissue is primarily composed by osteocytes, osteoblasts, and osteoclasts, whereas its MEC has an organic (mainly non-fibrillar collagen I and non-collageneic components, such as osteocalcin, osteonectin, and osteopontin) and an expressive inorganic composition, especially hydroxyapatite (70, 71) (Figure 2). Osteoblasts are the cells responsible for the mineralized matrix deposition; they can become bone-lining cells, present at non-remodeling ECM bone regions (72) or further differentiate to osteocytes. Osteocytes are embedded in this mineralized ECM, acting as sensors to modulate bone formation and resorption, which, in turn, is carried out by osteoclasts (69, 73, 74).

The roles played by MMPs in bone physiology and associated pathologies have been extensively characterized with clear examples in fractures, congenital diseases and development of the bone tissue (especially during endochondral bone formation), among others (75-79).

The importance of MMP-2 during bone development becomes evident through the analysis of the *Mmp-2* deficient mice. These animals display a variety of bone abnormalities, such as osteopenia, which derives from alterations in bone structure and ECM deposition. Remarkably, Mmp-2 -/- osteoclasts have impaired canalicular filopodia networks, known to be important for nutrients and metabolites flow (76). In humans, MMP-2 deficiency has been implicated in multicentric osteolysis and cranial mal formation (78-80).

Another MMP that is implicated in bone homeostasis is MMP-13. This collagenase is expressed by both osteoclasts and osteocytes. Therefore, it is not

surprising that Mmp-13$^{-/-}$ Wistar rats display bone mineralization alterations, more specifically, a heterogeneous hyper-mineralization, which results in loss of bone flexibility and susceptibility to stress fractures (81, 82).

MMP-14, also known as MT1-MMP, may have an important role in bone architecture and osteocyte function as well, since it is expressed in the first stages of endochondral bone formation at the bone canals (83-87). Remarkably, Mt1-mmp-deficient mice, even though viable, display severe collagen cleavage defects, arthritis, poor vascularization, and abnormalities in their skeletal structure, which includes craniofacial dimorphisms. These animals have impaired endochondral bone formation, cell proliferation abnormalities at the growth zone and, also, during the secondary ossification, which occurs after birth. The observed phenotypes may be partially explained by a deficient Mmp-2 activation cascade, and the presence of hyperactive osteoclasts and osteocytes that are more prone to undergo apoptosis (75, 84, 85, 87, 88).

Furthermore, MMP-3 is expressed at the chondro-osseous intersection and MMP-10 actively acts in the endochondral bone formation (89). MMP inactivation, either by treatment with a chemical inhibitor or through Timp-2 overexpression, leads to *in vitro* osteogenesis impairment of MC3T3-E1 pre-osteoblast mouse cells and calvaria cells isolated from postnatal day 1 mice (85). MC3T3-E1 shows an orchestrated MMP modulation upon osteogenesis induction, *Mmp-13* expression starts to increase after six days of the induction, whereas *Mmp-14* expression shows an increase in early phases of the process and starts to decrease at late stages of differentiation (90). In addition, MLO-A5 cells submitted to an osteogenic differentiation protocol display a peak of *Mmp-14* and *Mmp-19* expression, and a decrease in Mmp-2, Mmp-23 and Mmp-28 expression by the third day of the protocol. Also, expression of the Mmp inhibitor *Timp-1* is decreased during the differentiation protocol (91).

The Mmp inhibitor RECK is also modulated during osteogenesis. Its expression decreases after the 14th day of the *in vitro* protocol (92). In accordance with these findings, *Reck*, inhibition through shRNA, reduced the mice bone mineral density, inducing osteoporosis (93).

6. Extracellular Matrix turnover during Chondrogenesis

Elasticity and resistance are the main characteristics of the articular cartilage. These properties are a consequence of its unique ECM composition, since the cartilage framework consists mainly of fibrillar collagen II copolymerized with collagen XI and cross-linked to collagen IX at the surface. Moreover, it is a proteoglycan rich ECM, especially in aggrecan, which, in turn, is essential to the tissue resistance because it creates highly hydrated spaces (94) (Figure 2). In addition, the ECM plays a critical role not only in the tissue physical properties, but, also, modulating cell behavior. The chondrocyte pericellular-matrix is enriched with non-fibrillar collagen VI, which is believed to anchor the chondrocytes to the matrix and interact with cell surface integrins, thus acting as a mediator in cellular-matrix interactions (95).

In the light of ECM's complex role in cartilage characteristics and function, it is not surprising that a dynamic and multi-step ECM remodeling takes place during the course of chondrogenesis. In summary, a mesenchymal stem cell condensate is formed, accompanied by increased expression of collagen I and fibronectin with this cellular aggregate then commiting to the chondrocytic differentiation pathway (96). After the first step of differentiation, collagen type IIb, IX, and XI are expressed. It is important to highlight that articular cartilage chondrocytes display a low proliferative rate and a low collagen IIa expression. In the case of endochondral bone formation, in which the cartilage works as a template which is replaced by bone tissue, a second round of differentiation occurs leading to cellular hypertrophy and expression of alkaline phosphatase (Alp) and type X collagen, combined with decreased collagen IIb expression, forming the growth plate (which can be divided into: the resting, proliferative and hypertrophic zones). The surrounding ECM of these hypertrophic chondrocytes starts to mineralize and the cells become apoptotic. At this late step, ECM remodeling is intense and combined with VEGF expression, which contributes to the vascularization of the tissue ((97) extensively revised in (98, 99)).

There are several *in vitro* protocols for differentiating MSCs into chondrocytes (broadly revised in (100)). The majority of the procedures uses dexamethasone and/or TGF-beta treatment and has, as critical points, the cell number and cell-cell contact, many having as a first step the formation of MSCs condensates in suspension (97, 100-102). This is an important feature, since adipose MSCs cultured

as spheroids in ultra-low adhesive plates, were shown to display a better preservation of the ECM proteins fibronectin and laminin (103). This is an interesting optimization of the procedure, since suspension culture was shown to better preserve native ECM production in adipocytes (103). Furthermore, primary cultures of cartilage chondrocytes maintained in monolayer over an adherent substrate for more than four passages are known to acquire an amoeboid-like morphology and stop expressing collagen II, therefore, losing its specific cellular characteristics (104). Although several protocols are available in the literature, many suffer from one important critique, which is that the final stage usually displays the expression of late hypertrophic chondrocytes genes, such as collagen X and Mmp13, rendering it difficult to obtain cartilage chondrocytes from MSCs *in vitro* (105, 106).

Considering that the microenvironment is critical for differentiation and that we observe a clear change in ECM composition throughout the chondrogenic process, elucidating the role of proteases which are involved in ECM turnover should help to identify critical steps and allow manipulation and optimization of these protocols. Supporting this idea, Bertram *et al.,*performed a series of assays in which MSCs primary cultures from different origins were submitted to chondrogenic differentiation protocols in the presence of different protease inhibitors. In this analysis, a hydroxamate-based pan-MMP inhibitor stands out by completely suppressing chondrogenic differentiation through inhibition of proteoglycan deposition and of collagen II and X expression (106). This fact emphasizes the importance of proper MMP activity for chondrogenesis to occur.

As already mentioned, cartilage ECM is rich in fibrillar collagen, its main component, and proteoglycans. During endochondral bone formation in mice, Mmp13 is highly expressed in late hypertrophic chondrocytes, a step at which extensive ECM remodeling occurs; therefore, Mmp13 is believed to be one of the main collagenases acting at this stage (107). The aggrecan proteoglycan also acts by protecting collagen II from MMP cleavage, rendering its prior degradation necessary for collagen fibrils breakdown (108). It is interesting to notice that aggrecan is known to be cleaved by MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9 (with a low activity), and MMP-13 (109-111). Double knockout mice for Mmp13 and Mmp9 display impaired aggrecan degradation and, consequently, an accumulation of cartilage matrix that leads to an extended hypertrophic zone and increased

hypertrophic chondrocytes number (107). As a result, these animals present slower rate of endochondral ossification and vascularization (107). Interestingly Mmp13 -/-, Mmp9 -/- and the double knockout display normal chondrogenesis (107, 112). Moreover, treating MSCs with the specific MMP-13 inhibitor GG86/2 from the start of the differentiation protocol allows early chondrogenic differentiation, but partially reduces the expression of the late hypertrophic chondrocyte marker ALP (106). ALP, as already mentioned, is also an osteogenic marker whose presence in the microenvironment will foster osteoblast differentiation, playing an important role in endochondral bone formation. Taken together, these results indicate that MMP-13 and MMP-9 activity are promising intervention points for optimizing cartilage differentiation *in vitro*. The incorporation of chemical inhibitors, such as the MMP-13 inhibitor GG86/2, in the late stages of the chondrogenic protocols could contribute to arrest hypertrophic chondrocyte maturation and, therefore, could lead to cells that are closer to their *in vivo* cartilage counterparts.

Equally important to analyze MMP's roles during the chondrogenic differentiation process is to investigate the action of their inhibitors. Kondo *et al.,*reported that Reck expression is low at the early stages of ATDC5 mouse cells differentiation, in which cellular condensation, associated with MMP activity, is observed, and increases at later stages, co-localizing with collagen II deposition in the cartilaginous nodules (113). Impairment of Reck expression has dramatic effects in the differentiation efficiency, while *Reck* overexpression leads to impairment of the process by suppressing the initial cellular condensation and *Reck* knockdown does the same by suppressing ECM deposition on the cartilaginous nodules (113). Conversely, overexpression of Mmp9 and Mmp14 accelerated cellular condensation at the early stages of the protocol (113), whereas in the chicken model Mmp2 down-regulation led to promotion of cellular condensation, accompanied by a higher expression of fibronectin and integrins (114).

7. Future Perspectives

MSCs are multipotent stem cells, which are abundant and easily isolated from adult tissues of different organisms. Therefore, they currently represent a potentially viable and affordable alternative for autologous cellular therapy. An increasing

number of *in vitro* differentiation protocols targeting these cells have become available, but they need to be optimized in order to reach a higher efficiency. One practical approach for such optimization involves the modulation of the microenvironment in which these cells are cultured and to which they respond. Here we present integrated data that illustrate the orchestrated and fine-tuned role of MMPs and their inhibitors during MSCs differentiation, which may be fruitfully explored aiming at optimization of the efficiency and yield of the *in vitro* protocols.

8. Art work

Servier Medical Art (http://smart.servier.com, published by LES LABORATOIRES SERVIER, SAS) was used to generate the Figures**.**

9. Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

9. Acknowledgments

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5. Conclusion

Our data involving the expression and promotor activity of RECK during neuronal differentiation, as the disponible data in literature and discussed in our revision, they suggest that Reck expression can be used as a tool for the optimization of differentiation protocols.

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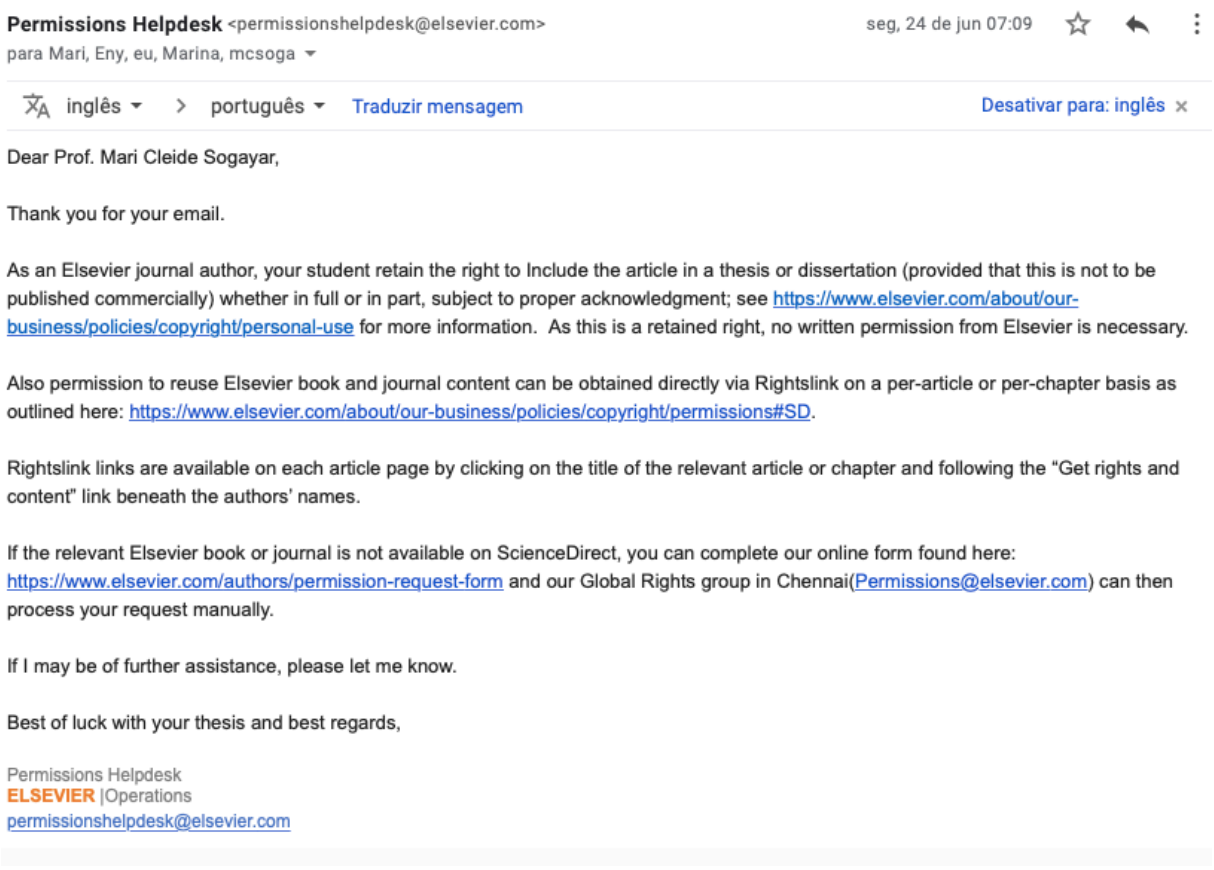
7. Appendix



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