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DOTTORATO DI RICERCA IN AREA DEL FARMACO E TRATTAMENTI INNOVATIVI

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"From rat adipose stem cells to VEGF signaling: emerging role for neuropathic pain relief"

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List of abbreviations:

CTR	Control
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
ES	Embryonic Stem cells
HSCs	Hematopoietic Stem Cells
i.p.	intraperitoneal
i.t.	intrathecal
i.v.	intravenous
IL	Interleukin
MSCs	Mesenchymal Stem Cells
OXA	Oxaliplatin
PlGF	Placental Growth Factor
RASCs	Rat Adipose Stem Cells
RCEs	Rat Coronary Endothelial cells
sFlt-1	soluble Fms-like tyrosine kinase-1 (or soluble Vascular Endothelial Growth Factor- Receptor 1)
SVF	Stromal Vascular Fraction
TGF-β	Transforming Growth Factor-beta
TNF-α	Tumor Necrosis Factor- alfa
VEGF	Vascular Endothelial Growth Factor
VEGF-Rs	Vascular Endothelial Growth Factor-Receptors

Abstract

Adipose-derived stem cells are a class of mesenchymal stem cells mainly isolated from the stromal vascular fraction (SVF) of the adipose tissue. For many years the interest for MSCs of scientific community was concentrated on their ability to differentiate into mesodermal (chondrocytes, adipocytes, osteocytes and myocytes) and non-mesodermal (neurons, cardiomyocytes and endothelial cells) cells (Zuk et al., 2002; De Ugarte et al., 2003; Bunnel et al., 2008). By virtue of these features ASCs might be used in a cell-based therapy in which organ-specific tissue engineering and tissue reconstruction are the main purpose. Nevertheless, the recent scientific literature proposes an adjunctive mechanism of action for MSCs, based on their modulatory secretome.

Adult MSCs offer a pluripotent cellular source for replacing injured tissues and, at the same time, they represent a source of neuroprotective and anti-inflammatory mediators that counteract damage effects. Briefly, MSCs are able to modulate the inflammatory cascade associated to immune-related diseases, such as rheumatoid arthritis (Gonzalez-Rey et al., 2010), but also painful neuropathies consequent to nerve trauma (Sacerdote et al., 2013A; Watanabe et al., 2015) or to metabolic alterations (Shibata et al., 2008).

In this perspective, we investigated in a rat model of neuropathic pain induced by oxaliplatin, if intravenous and intrathecal administration of adult rat adipose stromal stem cells (RASCs) possess an anti-neuropathic effect (Di Cesare Mannelli, ..., Vona, et al. 2018).

Investigating a possible mechanism of action by which ASCs exert their effect, Pan-VEGF-A, EGF and TGF-β were assayed in plasma. EGF and TGF-β were not altered by oxaliplatin or ASCs treatments. On the contrary, Pan-VEGF-A concentration significantly increased in oxaliplatin-treated rats in comparison to the control group whereas ASCs were able to counteract this alteration, suggesting both a possible implication of VEGF modulation in the development of neuropathic pain and in ASCs pain relieving mechanism.

The study of the relevance of VEGF-A modulation in RASCs activity begun by the evaluation of the protein expression of the VEGF specific receptors, R1 and R2, on these cells; thus, the measurement of intracellular calcium dynamic induced by the VEGF receptors stimulation was performed.

RASCs express both receptors and the small increase in intracellular calcium induced by VEGF in RASCs suggests that VEGF receptors are actively linked to an intracellular signaling and calcium movement. In particular, calcium signaling was almost dependent on the stimulation of R2 receptor.

A mechanism for dumping VEGF activity is the autocrine production of sFlt-1. sFlt-1 is the extracellular fragment of R1 and an endogenous neutralizing ligand of the agonist. Therefore, we performed experiments aimed to investigate if VEGF activated RASCs can produce sFlt-1 "in vitro". In the same experiments the effect of VEGF stimulation on R1 expression was quantified. In control condition sFlt-1 was present in RASCs cytosol and also VEGF-R1 type was well expressed. One hour after VEGF165b administration, sFlt-1 and VEGF-R1 expression were decreased significantly in RASCs, while they were restored after 24h from VEGF administration. These experiments suggest that sFlt-1 can be released from cell after 1h and repristinated after 24h.

If similar mechanisms are active in the in vivo neuropathic model, the binding of sFlt-1 with VEGF or the internalization of VEGF could explain the anti-neuropathic effect of RASCs.

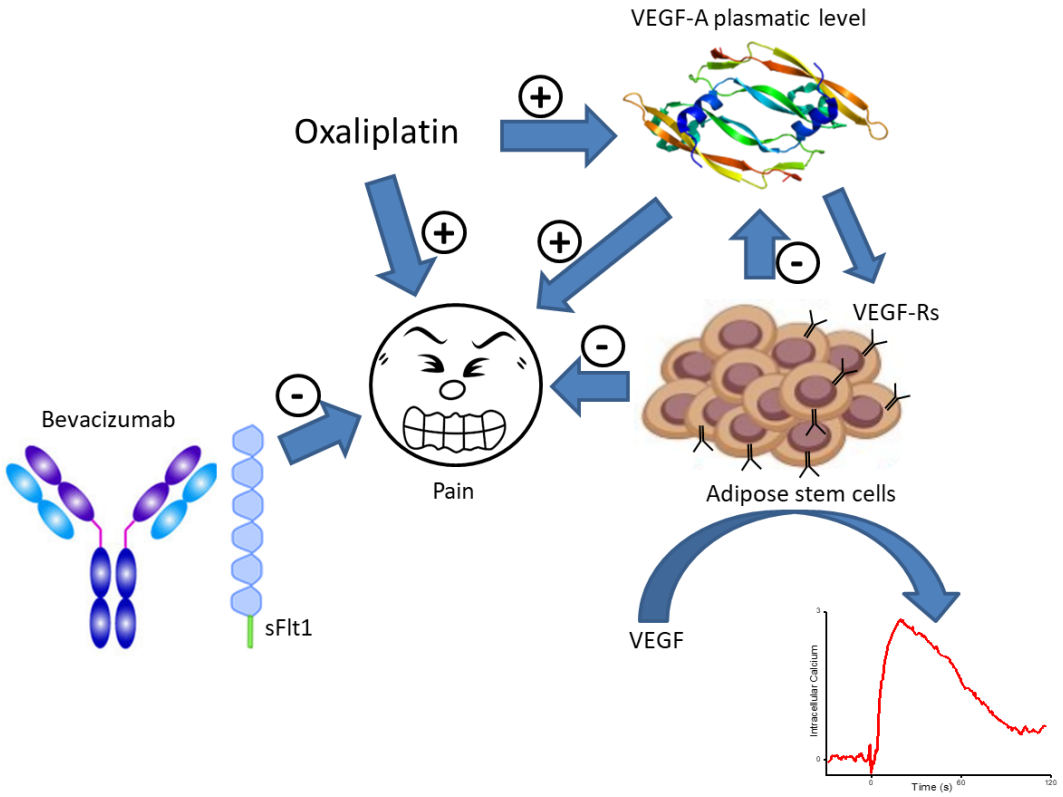
The modulation of VEGF-A is suggested as a key mechanism in the complex response orchestrated by stem cells against neuropathy and the role of ASCs "secretome" as effector of their efficacy is highlighted.

Supporting the hypothesis that VEGF increase plays a relevant role in the development of neuropathic pain, according to our experimental findings in rats, the involvement of VEGF in OXA-treated mice was evaluated by the efficacy of bevacizumab and sFlt-1. Indeed, intrathecally bevacizumab and sFlt-1 administration reduced oxaliplatin-dependent hypersensitivity induced by mechanical noxious stimulus, demonstrating that an increase in VEGF-A is involved in mice oxaliplatin-induced neuropathic pain and that the blockade of the growth factor effect can be mitigated by bevacizumab and sFlt-1.

The relevance of VEGF165b in pain perception was confirmed in mice since the directed administration VEGF165b by intrathecal injection rapidly induced a long-lasting mechanical hyperalgesia.

Moreover, the agonists for R1 and R2 receptor (VEGF165b, VEGF-E, PlGF-2) by intrathecal administration induced cold allodynia.

Oxaliplatin therapy of colorectal cancer induces a dose-dependent neuropathic syndrome in 50% of patients. So, the present data suggest an alternative approach in the treatment of oxaliplatin-induced neuropathic pain.



1. Stem cells

Stem cells are defined as non-specialized cells whose main features are the ability to self-renew completing an unlimited number of replication cycles and giving rise to differentiated cells. Both the ability of stem cells to replace mature cells at the end of their physiological lives and the ability to perpetuate themselves through self-renewal are crucial to maintaining tissue homeostasis and to ensure proper cell turnover of all body tissues (Ito et al., 2014).

An interesting, peculiar features of stem cells is their ability to do a symmetric mitosis that originates two daughter cells that maintain stemness, but also a mitosis defined as "asymmetric" or differentiating (Figure 1). This type of mitosis consists in giving birth to two daughters cells, of which only one proceeds to differentiation, while the other maintains the characteristic of stemness. With this replication strategy, the number of stem cells is maintained unchanged, while progenitor cells, further dividing, will give rise to mature cells that make up tissues (Cai et al., 2004). The choice between the two types of division is dictated by signals in the extracellular environment and hence the need for the body to restore the stem pool rather than forming new differentiated cells. In fact, when needed, progenitors produced by stem cells may face division cycles from which originates cells more and more "committed".

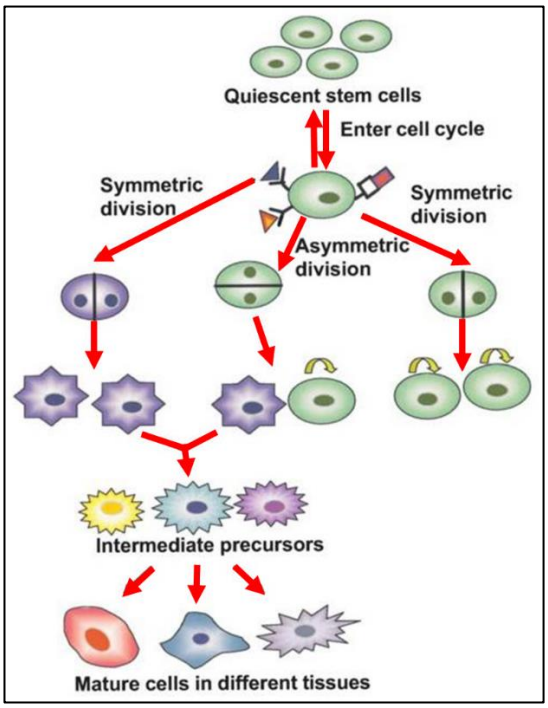


Figure 1. Possible stem cell division pathways. *Stem cells can remain quiescent or enter cell cycling in three ways: asymmetric division gives rise to a stem cell daughter and a precursor*

daughter; two kinds of symmetric division will generate twin daughter cells of either stem cell or progenitor properties (Cai et al., 2004).

1.1. Embryonic stem cells (ES)

Embryonic stem cells (ES) are derived from the pre-implantation blastocyst, a hollow sphere of cells containing an outer layer of trophoblast cell which give rise to the placenta and the inner cell mass (ICM), from which ES are derived. Cells of the ICM ultimately go on to form the embryo and therefore, under specific conditions, they are able to give rise to cell progenies representing all three embryonic germ layers and so to form all tissues in the body (Nichols et al., 2012; Reubinoff et al., 2000). The period of time during which ES are pluripotent is short; in fact, at the end of the second week of gestation begins a gastrulation process: within the embryo, the germ layers assume the proper layout for the beginning of organogenesis.

Pluripotent ES grow in round and compact colonies under the influence of leukaemia inhibitory factor (LIF), which contributes to maintaining stem cells in an undifferentiated and pluripotent state (Niwa et al, 1998). Moreover, they are characterized by the expression of embrionyc membrane antigens such as SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 and alkaline phosphatase (Henderson et al., 2002).

The remarkable duplicative properties of ES seem to be attributed to high levels of telomerase activity. Indeed, telomeres are non-coding regions that form the end of the eukaryotic chromosomes and are represented by a sequence repeated hundreds of times. The telomerase enzyme, a ribonucleoprotein involved in maintaining the length of the telomeres by added basal sequences at the extremity of the chromosomes, prevents their progressive shortening as a consequence of repeated mitosis, thus allowing the stem cell to perform countless cycles replicates (Thomson et al., 1998).

Because these types of stem cells are embryonic, their promising therapeutic applications are, first of all, hindered by ethical issues (Donovan et al., 2001), but also security concerns for the host organism may be associated to a therapeutic use. Indeed, the continued culture of ES cells in an undifferentiated state requires the presence of feeding layers and animal ingredients that could lead to a risk of transfer pathogens. Moreover, ES cells are characterized by a high level of genomic instability and their differentiation after long-term growth is not entirely predictable. It has been found the possibility of teratome formation following their transplantation, and the differentiated

ESs express molecules which could cause immune rejection by the host organism (Stojkovic et al., 2004).

1.2. Adult stem cells

Adult stem cells are multipotent or unipotent cells that are found in many tissues and organs. They first observed in the bone marrow (located in the bone marrow of long bones and in the spongy bony cavities) and unlike embryonic stem cells, adult stem cells have one ending replication potential beyond which they can no longer divide, thus achieving a stage known as replication senescence (Pittenger et al., 1999). Based on the presence of specific surface markers, two distinct populations have been identified: hematopoietic stem cells and mesenchymal stem cells.

1.2.1 Hematopoietic stem cells (HSCs)

Hematopoietic stem cells are roundish and multipotent stem cells deriving from mesoderma. HSCs are able to rebuild the entire hematopoietic system through the hemopoiesis process and they origin multipotent progenitors, oligopotent progenitors and progeny in the course of terminal differentiation. Oligopotent progenitors irreversibly directed differentiate into a precise cell line and have a progressive limitation of proliferative capacity. Instead, progenies in terminal differentiation are represented by all corpuscular blood elements: erythrocytes, platelets, granulocytes, monocytes and lymphocytes. With respect to immunophenotypic characteristics, HSCs express membrane antigens such as CD45, Thy1, c-kit and sca-1 but not Lineage antigens (Lin−), (Gallacher et al., 2000). The main HSC stem phenotype is associated with the CD34 antigen, that is, a transmembrane glycoprotein with a molecular weight of 120 kDa. Its functional characterization is not entirely clear, although a correlation between CD34 amino acid structure and adhesion and homing processes of stem cell was hypothesized (Krause et al., 1996). For many years these cells have been associated to the concept of plasticity, understood as the ability to respond to the need for blood cells maintaining a balance between extracellular stem cells and progenitor cells production. Instead, a concept of different plasticity has been introduced recently, based on the ability of stem cells, isolated from a specific tissue, to acquire the phenotype of another different tissue or organ. Stem cell plasticity could be associated to different processes (Figure 2). Cell trans differentiation is one mechanism by which

stem cells potentially could contribute to cell types of different lineages (Figure 2A). This lineage conversion was proposed to occur directly, by the activation of an otherwise dormant differentiation program to alter the lineage specificity of the cell dedifferentiation and cell fusion. Lineage conversion also could theoretically occur via dedifferentiation of a tissue-specific cell to a more primitive, multipotent cell and subsequent de-differentiation along a new lineage pathway (Figure 2B). A third explanation for observations of adult stem cell plasticity relates to the purity or homogeneity of the test population. In order to demonstrate definitively trans differentiation of a particular lineage-specific stem cell, it is essential to exclude the possibility that multiple, distinct stem cells could be contributing to the observed outcome, ideally by evaluating the potential of single isolated stem cells. Many purported demonstrations of stem cell plasticity inferred such activity following transplant of large numbers of heterogeneous populations of cells. Significantly, both unfractionated bone marrow (BM) and muscle side population (SP) cells likely contain multiple stem cell or progenitor cell populations, including HSC and nonhematopoietic mesenchymal stem cells, endothelial precursor and/or muscle progenitors, suggesting the possibility that distinct stem or progenitor cells could be contributing, consistent with their intrinsic lineage commitment and developmental potential, to each of the different lineage oucomes observed (Figure 2C). Contributions across multiple tissue types also could arise through the action of a single, rare pluripotent stem cell present in BM, and/or other tissues, which possibly copurifies in protocols designed to enrich for tissue specific stem cells such as BM HSC (Figure 2D). The final mechanism we consider here to explain observation of stem cell plasticity is cell-cell fusion, a process that occurs both in physiological and pathological conditions (Figure 2E) (Wagers et al., 2004).

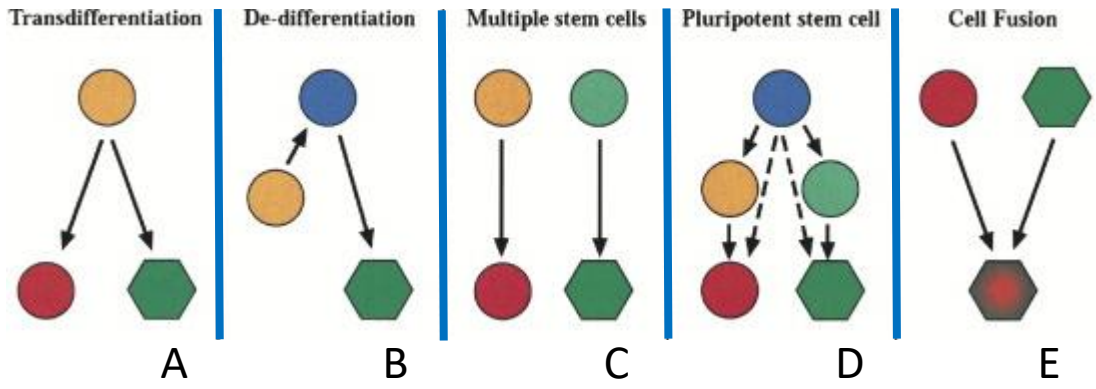


Figure 2. Schematic Diagram Depicting Potential Mechanisms and Explanations for Observations of Adult Stem Cell Plasticity. *Tissue-specific stem cells are represented by*

orange or green ovals, pluripotent stem cells by blue ovals, and differentiated cells of the "orange" lineage by red ovals and of the "green" lineage by green hexagons (Wagers et al., 2004).

Despite the potential of adult stem cells being more limited than embryonic stem cells, as they may only produce some types of specialized cells and to a lesser extent, they still have advantages; in fact, they can be extracted from an adult and re-planted in the same person, avoiding problems of incompatibility.
Unlike embryonic stem cells, adult stem cells do not pose ethical problems, since it is not necessary to destroy embryos to obtain them.

1.2.2. Mesenchymal stem cells (MSCs)

MSCs are non-hematopoietic and multipotent progenitor cells of star shape, which derive mainly from mesoderm, intermediate embryonic leaf from which originate all connective tissues of the organism. Indeed, it is generally agreed that in an embryo a mesenchymal stem cell is a pluripotent progenitor cell which divides many times and who progeny give rise to bone, cartilage, muscle and other mesenchymal tissues. But really, mesenchymal stem cells are very represented also in several adult tissues, where maintain the same ability to give rise to a spectrum of mesenchymal tissues differentiating along separate and distinct lineage pathways (Caplan, 1991).
Recently, MSCs have been shown their ability to differentiate into cell types other than those of mesodermic origin, and in a particular, under specific stimuli they can give rise to cell types of ectodermic and endodermic origin, including nerve cells, glial cells, hepatocytes and pneumocytes (Minguell et al., 2001).
For a long time, bone marrow was the main source of mesenchymal stem cells, but there are several limitations associated to bone marrow derived MSCs, such as the low rate of cells extracted, and the extremely invasive and painful withdrawal procedure for the donor. For these reasons today MSCs were recruited from other tissues including placenta, umbilical cord blood and adipose tissue. Adipose tissue is considered not only as an organ with the function of storing triglycerides, but also a structure that provides multiple functions. Recently, the importance of adipose tissue as an organ capable of regulating many physiological processes has been highlighted. In fact, it expresses and secrets a number of bioactive factors that may have autocrine, paracrine or endocrine function (Trayhum, 2004). Moreover, like bone marrow it is a mesodermally-derived and highly vascularized organ that give rise to a stromal

vascular cell fraction (SVF) characterized by a heterogeneous cell population including circulating blood cells, fibroblasts, pericytes, endothelial cells and mesenchymal stem cells. In recent years, indeed, adipose tissue has assumed a predominant role as a source of MSCs, since it can be withdrawn through less invasive procedures and a large amounts of mesenchymal stem cells were obtained (Kern et al., 2006).

1.2.3. Adipose stem cell identification: Cell surface characterization and evaluation of multilineage differentiation capacity

Stem cells obtained from adipose tissue (also defined adipose-derived stem cells – ADSCs or adipose stem cells - ASCs) are characterized by a cell surface phenotype similar to MSCs.

CD105, STRO-1 and CD166 are three common markers used to identify cells with multilineage differentiation potential and are consistently expressed on ASCs and MSCs (Barry et al., 1999; Gronthos et al., 2001; Majumdar et al., 1998; Pittenger et al., 1999; Dennis et al., 2002; Gronthos et al., 1994; Simmons et al., 1994). Also, the stem cell factor receptor CD117 has been shown to be expressed on an array of totipotent and pluripotent cells including MSCs and ASCs (Lemoli et al., 1993; Aye et al., 1992; Ogawa et al., 1993). In addition to these multipotent markers, ASCs display numerous other molecules including β-1 integrin CD29, hyaluronate receptor CD44 which plays a role in several pathologic and physiologic processes, α-5 integrin CD49e which mediate cell adhesion to fibronectin. ASCs also express high level of CD45 (ICAM-1) which is a member of the immunoglobulin supergene family and can be up-regulated in response several inflammatory mediators, and MHC Class I molecules which allows ASCs allogenic transplantation (Aust et al., 2004). On the contrary, ASCs do not express hematopoietic and endothelial markers including CD3, CD4, CD11c, CD14, CD15, CD16, CD31, CD33, CD38, CD56, CD62p, CD104 and CD144.

Adipose SCs possess the potential to differentiate toward a variety of cell lineages both *in vitro* and *in vivo*. Although ASCs are of mesodermal origin, they can differentiate into ectoderm and endoderm lineage cells as well as mesoderm (Figure 3) (Haddad et al., 2014). Regarding the differentiation into cells of the mesodermal lineages, ASCs,

under specific stimuli, may undergo along adipogenic, osteogenic, chondrogenic, myogenic, cardiomyogenic, angiogenic, tenogenic differentiation.

The differentiation of ASCs toward cells of the ectodermal lineage is induced culturing ASCs in specific conditions, based on this growing ASCs in monolayers with retinoic acid or on a fibrin matrix in the presence of EGF induces the expression of the epithelial markers, cytokeratin 18 or E-cadherin and cytokeratin 8, respectively (Brzoska et al., 2005; Long et al., 2010). As well as the exposure of ASCs to vasoactive intestinal peptide induces formation of retinal pigmented cells, which are of ectodermal origin as indicated by the expression of retinal pigment epithelium (RPE) markers, namely bestrophin, cytokeratins 8 and 18, and RPE 65 (Vossmerbaeumer et al., 2009).

It has also been demonstrated that ASCs can differentiate into neuronal or neuronal precursor cells (Safford et al., 2004).

Finally, it has been shown that ASCs can differentiate into endoderm lineage cells. Several reports have shown that ASCs have the potential to differentiate into hepatocytes under the stimulus HGF and FGF-1 and -4 (Aurich et al., 2009; Banas et al., 2009). In theory, ASCs could be used to reduce liver inflammation and treat liver fibrosis by differentiating directly into hepatocytes or by secreting factors such as angiogenic, antiapoptotic, anti-inflammatory, and antifibrotic factors. In addition to hepatic differentiation, the exposure of ASCs to nicotinamide, activin-A, exendin-4, HGF, and pentagastrin resulted in the production of pancreatic-like ASCs capable of insulin, glucagons, and somatostatin secretion (Chandra et al., 2009; Timper et al., 2006).

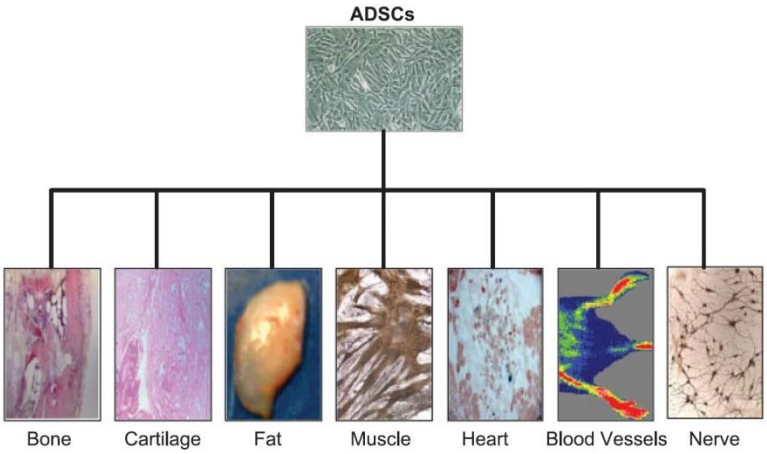


Figure 3. Tissues derived from adult ASCs. *ASCs can differentiate into bone, cartilage, adipose, muscle, stromal cells and tendon.*

1.2.4. Therapeutic application of ASCs

Originally, the application of ASCs in therapy was associated to their proliferation and differentiation potential, but in the last few years, the immunomodulatory and trophic activities of ASCs have been highlighted. Like MSCs (Bourin et al., 2013; Gimble te al., 2013; Nguyen et al., 2016; Bunnel et al., 2008), as a consequence of tissue damage ASCs are activated in "medicinal stem cells" able to modulate immune response acting on dendritic cells and B and T cells (Iyer and Rojas, 2008; Jones and McTaggart, 2008; Le Blanc et al., 2003). The immunoactivity of these cells has been shown to be mediated by both secreted bioactive molecules and by cell-cell contact. Moreover, ASCs-secreted molecules have also trophic effects that mediates inhibition of apoptosis and scar formation, angiogenesis stimulate tissue-intrinsic progenitors to divide and appropriately differentiate (Wagner et al., 2009; Rehman et al., 2004). For all these reasons therapeutic possibilities of ASCs are so many and include several clinical conditions, such as acute myocardial infarct, stroke, lung disease, diabetes, autoimmune and degenerative pathologies (osteoarthritis and rheumatoid arthritis, lupus, multiple sclerosis, amyotrophic lateral sclerosis, etc) but also painful neuropathies.

However, it is still only partially known if the reparative role of stem cells is manly dependent on the release of soluble factors or a direct commitment as cellular source. New correlated functionalities have been recently gaining ground: cell-delivery system and paracrine activity (Brini et al., 2017). Regarding the first function, MSC homing ability is exploited to modulate the release of growth factors and cytokines at the desired site and, moreover, MSCs can be bioengineered by insertion of nucleic acid sequences (e.g., messenger RNA and noncoding RNA), drugs or oncolytic viruses (e.g., myxoma virus to kill glioblastoma cells), to deliver their content to inflammatory areas or tumors, where they can produce or release therapeutic molecules (Sherman et al., 2015). Paracrine activity is linked to the cell-delivery system: the "paracrine hypothesis" states that MSCs are mostly effective through their secretome, which contains trophic, anti-inflammatory, immunomodulatory and antiapoptotic molecules, such as fibroblast growth factor, vascular endothelial growth factor (VEGF)-A, nerve growth factor, transforming growth factor-β and interleukin-10 (Murphy et al., 2013; Zhou et al., 2016; Brini et al., 2017).

Among all immunomodulatory and anti-inflammatory molecules secrete by mesenchymal stem cells, there are also neurotrophic factors which are growth factors able to promote the development and survival of neurons. They also maintain functional integrity, promote regeneration, regulate neuronal plasticity, and assist in repairing nerves damage. Neurotrophic factors include different types of molecules such as neurotrophins like NGF, BDNF, neurotrophin-3 (NT-3), and NT-4/5, but also insulin-like growth factor I and II (IGF-I and IGF-II), neurotrophic factor of the glial cell line (GDNF), ciliary neurotrophic factor (CNTF) (Apfel, 1999; Cova et al., 2010; Reid et al., 2011). The various neurotrophic factors affect different cell populations within the peripheral and central nervous system.

In addition to creating an environment rich in trophic factors, it has been shown that ASCs transplantation significantly reduces both mechanical allodynia and thermal hyperalgesia. Mechanisms considered responsible for this action are manifold, with the first of all decreasing proinflammatory interleukin IL-1β and IL-17 and increasing expression of anti-inflammatory protein IL-10 (Brini et al., 2017).

Although the mechanism of action of stem cells is not fully known, but data presents in literature show that stem cells have an interesting therapeutic potential to stop degenerative processes, inhibit apoptotic pathways, increase survival and recovery of damaged and unhealthy nerves. By virtue of these qualities, MSCs have been investigated as a valid treatment against several pathologies, among which the socially relevant painful neuropathies: cellular therapy would stimulate tissue repair and act paracrinely on inflammation, immune system and nervous system signaling (Brini et al., 2017; Di Cesare Mannelli et al., 2018).

1.2.5. MSCs and pain

Chronic pain is one of the most common medical complaints worldwide and it have an enormous social and economic impact. Current pharmacological and surgical treatments aim to relief pain and restore function; however, unsatiscactory outcomes are commonly reported. Therefore, studies on animal models have been performed for the use of stem cells (particularly adipose stem cells that can be obtain from the patient itself) in pain reduction.

Sacerdote et al., (2013) demonstrated that hASC and their secretome can control diabetic complications such as neuropathic hypersensitivity, acting on several

peripheral and central mechanisms involved in the development and maintenance of this condition, such as neural and immune elements; Watanabe et al., (2015) demonstrated that bone marrow-derived mesenchymal stem cells (BMSC) modulate inflammatory/immune responses and promote motor functional recovery after spinal cord injury. BMSC reduce neuropathic pain through a variety of related mechanisms that include neuronal sparing and restoration of the disturbed blood-spinal cord barrier (BSCB), mediated through modulation of the activity of spinal-resident microglia and the activity and recruitment of hematogenous macrophages. Di Cesare Mannelli et al. (2018), demonstrated that rat adipose mesenchymal stem cells (RASCs) administration rapidly and long-lastingly decreases pain due to oxaliplatin neurotoxicity and that RASCs pain relieving efficacy involves the modulation of VEGF-A;

1.3. Stem cell niche

Physiologically, stem cells are confined in specific microenvironments defined "niches", here stem cells receive stimuli that determine their fate. Therefore, the niche should not be considered simply a physical location for stem cells, but the place where extrinsic signals interact and integrate with the component of the niche to influence stem cell behavior. These stimuli include cell-to-cell and cell matrix interactions and signals that activate and/or repress genes and transcription programs. As a direct consequence of this interaction, stem cells are maintained in a dormant state, induced to self-renewal or commit to a more differentiated state.

Each different type of stem cells has their own anatomical site, for example hematopoietic stem cells reside in localized niches in the trabecular bone where they are in contact with osteoblasts. Epidermal stem cells, capable of supplying keratinocytes to cutaneous annexes and sebaceous glands, are located within the swelling of the hair follicle. Moreover, neuronal stem cells reside in specific niches within the subventricular area of the brain and the sub granular area of the hypothalamus.

The niche is generally constituted by an extracellular matrix structure with its proteins: integrin, laminin, fibronectin, tenascine and proteoglycans. It is additionally characterized by adherent joints, made up of N-caderine, E-caderineand B-catenine, which allow stem cell anchoring to the niche and their connection with the

extracellular matrix (ECM) (Moore et al., 2006). The ECM acts as a scaffolding system, in which stem cells, stromal cells and molecular cues are embedded. Its role is to retain the stem cells in place, to localize signals and to create gradients that guide stem cells in their processes of self-renewal and differentiation. Examples of these key properties are represented by β-1 integrins that are expressed in various stem cells types, such as HSCs, skin and muscle stem cells and that mediates stem cells adhesion to matrix components, regulating stem cell maintenance. Tenascin-C is another ECM component expressed in the stromal compartment of the brain (Garcion et al., 2004) and bone marrow (Ohta et al., 1998). In the brain, it increases sensitivity of NSCs to fibroblast growth factor 2 (FGF-2) and bone morphogenetic protein-4 (BMP-4) (Garcion et al., 2004). In bone marrow, osteopontin (OPN) is another matrix glycoprotein that interacts with cell adhesion molecules expressed on HSCs, like CD44 and other integrins (Stier et al., 2005; Nilsson et al., 2005) to facilitate HSC retention in their niche.

1.3.1. Stem cell niche function

The operation of the niches is not entirely clear. Recent hypotheses suggest that niches may act in different ways. Inside the niches stem cell adheres to the stroma asymmetrically and orients its dividing plane to ensure that only one of the daughters' cell make contacts with the basal membrane remaining anchored to the niche where it will continue to maintain the characteristics of stemness. The other daughters' cell, instead, detaches from the niche and moves away from the stromal cells and their signals, in this way it become a progenitor cell, and then begin to differentiate (Figure 4). In other cases, both daughter cells may remain anchored to the stroma, or they may both differentiate (Spradling et al., 2001).

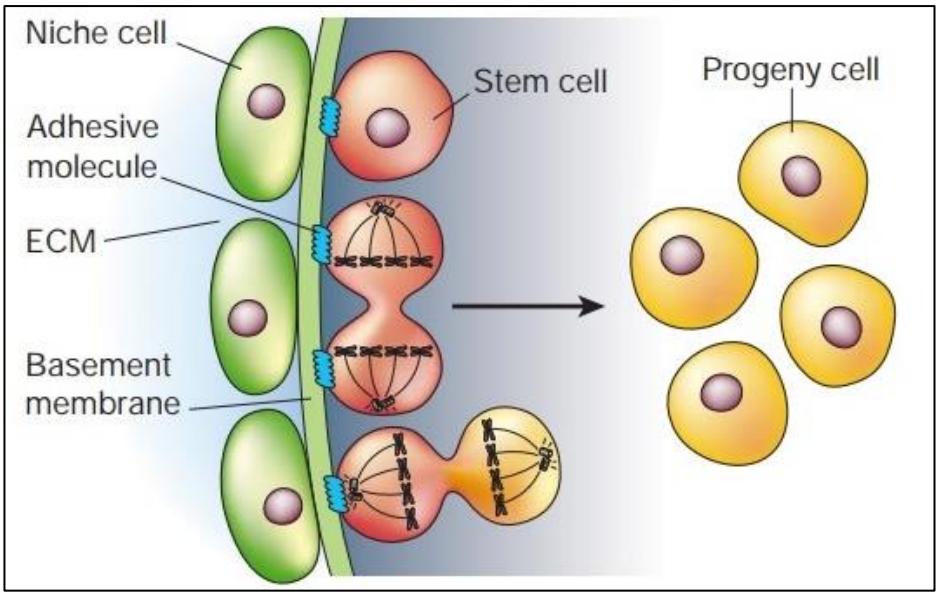


Figure 4. Niche structure. *Niche cells (green) underlying a basement membrane signal to stem cells (red) to block differentiation and regulate division. When a lineage mechanism prevails (lower mitotic cell), the stem cell divides such that one daughter retains its connections to the niche, while the other (yellow) becomes untethered and begins to differentiate. When a population mechanism prevails (upper mitotic cell), stem cell division may be either symmetric (shown) or asymmetric (not shown), as determined by local factors. ECM, extracellular matrix (Spradling et al., 2001).*

Regulating stem cell renewal is an essential feature of the niche, outside the niche, stem cells must possess sufficient intrinsic factors to overcome differentiation. Niches regulate the balance between the symmetrical and asymmetric division of stem cells, critical for maintaining the appropriate number of stem cells and for adjusting the demand for differentiated cells from the surrounding tissue.

Another stem cell niche-related phenomenon is the homing of stem cells to sites of injury and subsequent tissue healing. Although some tissue repair may be accomplished by the division of indigenous differentiated cells, such cells are most frequently post-mitotic. Thus, signaling to progenitor/stem cells to home to the site of injury and differentiate into the specific cell type is required. Damaged tissue expresses and releases a variety of chemokines, cytokines, adhesion molecules, proteolytic enzymes, growth factor receptors, and non-peptide mediators that recruit cell stem on the affected site to restore homeostasis tissue. MSCs are able to transmigrate through the endothelium, extravasate and reach the target site (Steingen et al., 2008). The mature cells that have undergone a damage are capable of secrete chemotactic signals but also differentiation signals, and these signals are a critical defining factor of stem cells identity (Gerstenfeld et al., 2003).

The homing phenomenon has also been observed in healthy animals: MSCs are able to circulate in tissues other than their home site, such as lung and muscle (Francois et al., 2006), although, in the absence of damage or inflammation MSCs are less able to migrate to specific organs than when inflammatory processes are present.

2. Pain

2.1. Epidemiology of pain

Pain represents a major unresolved health-care issue with widespread human and socio-economic impact. The American Academy of Pain Management reports that "uncontrolled pain can be considered as a silent epidemic, with 50 million Americans suffering from chronic pain and an additional 25 million people experiencing acute pain caused by accident or surgical procedures" (Weiner et al., 2003). In 1986 Koch estimated that 70 million visits to healthcare providers were motivated by pain complaints (Koch et al., 1986) and it is reported that 36 million Americans affected by pain miss nearly 4 billion workdays resulting in an extensive loss of work and productivity, all at an estimated cost exceeding $100 billion annually. Untreated pain has significant impact on patients and their family. Pain diminishes their ability to concentrate, do their job, exercise, socialize, perform daily tasks and sleep. All resulting in an unrelenting downward spiral of depression, isolation and loss of self-esteem. Clinical studies concluded that depression is the most frequent psychological reaction to chronic pain and that anxiety is the most frequent psychological reaction to acute pain (Sternbach et al., 1977).

2.2. Neurobiological basis and types of pain

According to the definition of IASP (International Association for the Study of Pain - 1986) and according to the association of O.M.S, *"Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage".*

Pain consists of several components: the physiological-discriminative, the emotional-affective and the cognitive-evaluative. Although the emotional and cognitive mechanisms influence the pain perception, the physiological-discriminative component is the most important especially for the pharmacological therapy.

The nociceptive stimuli are collected in the periphery from nociceptors, specialized primary sensory neurons that respond to mechanical, thermal and chemical noxious stimuli. The nociceptors represent the distal part of the nerve fibers that lead information from the periphery to the spinal cord. Nociceptive fibers have been

classified (Kumazawa et al., 1996) on the basis of their conduction velocity and sensitivity and threshold to noxious stimuli (Raja et al., 1988).
Based on axonal diameter and myelin thickness that confers them well-defined electrophysiological properties, the fibers are classified in:

- Aβ-fibers have a large diameter and a thick myelin sheath. These are very rapidly conducting fibers involved in detecting non-noxious mechanical stimuli (reviewed by Lynn 1994; Lewin and Moshourab 2004)
- Aδ-fibers have a somewhat smaller axon diameter and thinner myelin sheath. These axons are slowly conducting and are classified either as low-threshold, D-hair mechanoreceptors or mechanoreceptors activated by high-intensity, noxious stimuli:Aδ-mechanonociceptors (Koltzenburg et al. 1997; Lewin and Moshourab 2004)
- C-fibers are unmyelinated and slow-conducting (Van Hees et al., 1981)

Activation of nociceptors requires that adequate stimuli depolarize peripheral terminals, producing a receptor potential with sufficient amplitude and duration (Dubin et al., 2010). After excitation, the peripheral terminal of the receptors transduces the external stimuli and initiates an action potential conducted by the axon. Primary afferent nociceptors lead noxious information from dorsal root ganglion (DRG) to a second-order neuron located in the dorsal horn of the spinal cord. The afferent fibers of these neurons intersect and run along the entire spinal cord ascending through the contralateral portion of the medulla and leading the information to thalamus nuclei. Within the thalamus, nociceptive information is encoded regarding to the type, intensity and location of the pain. The second order neurons synapse with third order neurons which project to somatosensory and anterior cingulate cortices to drive sensory-discriminative and affective-cognitive aspects of pain, respectively (Renn et al., 2005). The spinothalamic tract is defined as the ascending pathway of pain control (Figure 5A).

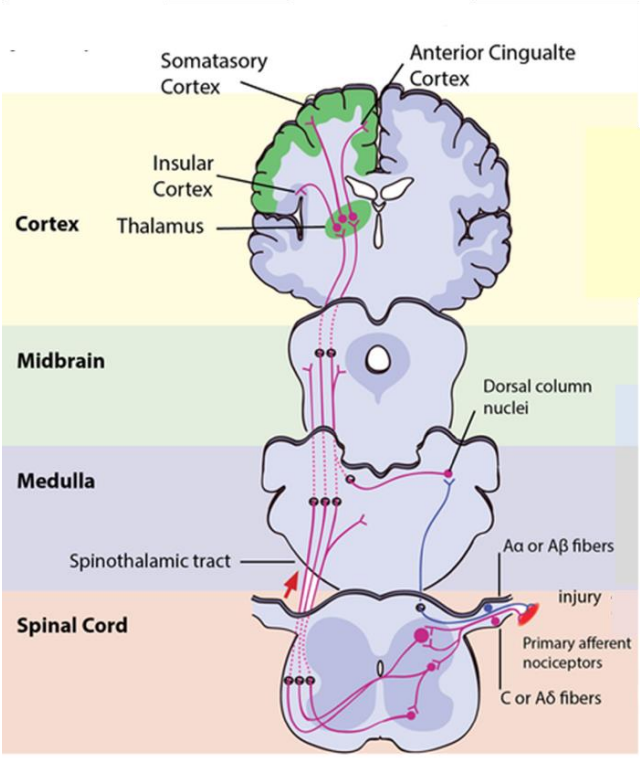


Figure 5A. *Schematic representation of the ascending pain pathway* (*Løseth et al., 2019)*

The functional activity of the ascending pathways above described is modulated by the descending control system (Figure 5B). Once the nociceptive information arrives to the higher-level centers, it is integrated to elicit a complex physiological response in front of the noxious stimuli, and modulated in order to reduce the intensity of the painful sensation. One of the most important regions involved in the descending pathway is the periaqueductal gray (PAG) in the midbrain, but there are other regions in the brainstem also involved in this process, such as parabrachial nucleus, medullary reticular formation, locus coeruleus (LC) and raphe nuclei. These centers use noradrenaline, serotonin, dopamine, histamine and acetylcholine to exert both excitatory and inhibitory effects on different sets of neurons in the dorsal horn.

Monoaminergic fibers originated from these nuclei run along the dorsal-lateral tracts of the spinal cord and terminate in the laminae of the spinal cord. These fibers control the first-order neuron activity, either directly or through the activation of inhibitory interneurons, and modulate the second order neurons activity through postsynaptic receptors activation by reducing the excitability. Overall, the pain control descending pathway is able to produce a reduction of the peripheral nociceptive input.

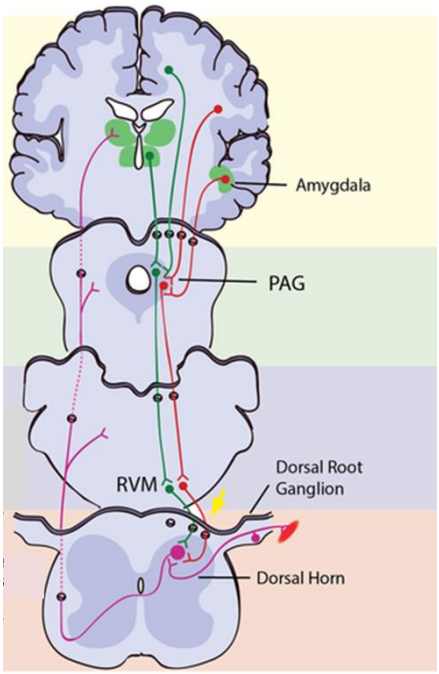


Figure 5B. *Schematic representation of the descending pathway of pain impulses transmission* (*Løseth et al., 2019)*

Concerning type of pain, there are four main categories of pain similar to each other:

- ***Nociceptive pain.*** To guard against tissue injury, it is imperative that the body is aware of potentially damaging stimuli. This awareness is achieved by a noxious stimulus-detecting sensory system (Figure 6A). Nociceptive pain is an alarm mediated by high-threshold unmyelinated C or thinly myelinated Aδ primary sensory neurons that feed into nociceptive pathways of the central nervous system (CNS) (Woolf & Ma 2007). These nociceptor neurons express specialized transducer ion channel receptors, mainly transient receptor potential (TRP) channels, tuned to respond to intense thermal or mechanical stimuli as well as exogenous and endogenous chemical mediators (Dhaka et al., 2006). Nociceptive pain occurs in response to noxious stimuli and continues only in the maintained presence of noxious stimuli.

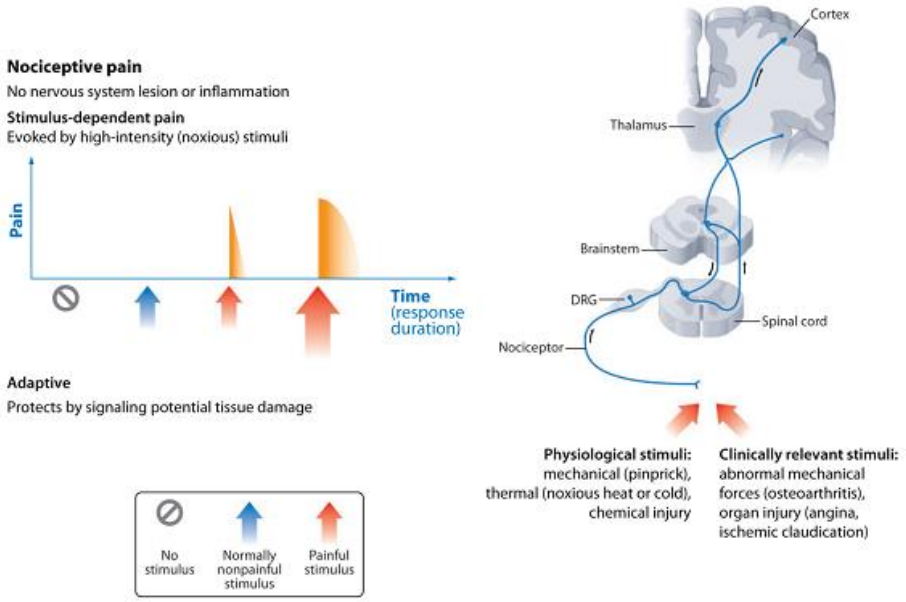


Figure 6A. *A summary of key features that distinguish and characterize the nociceptive pain (Costigan et al., 2009)*

- ***Inflammatory pain.*** This pain occurs in response to tissue injury and the subsequent inflammatory response. To aid healing and repair of the injured body part, the sensory nervous system undergoes a profound change in its responsiveness: stimuli that are normally innocuous now produce pain and responses to noxious stimuli become both exaggerated and prolonged (Juhl et al., 2008) (Figure 6B). Heightened sensitivity occurs within the inflamed area and in contiguous non inflamed areas as a result of plasticity in peripheral nociceptors and central nociceptive pathways (Huang et al., 2006, Hucho & Levine 2007, Woolf & Salter 2000). Because the pain system after inflammation is sensitized, it no longer acts just as a detector for noxious stimuli but can be activated also by low-threshold innocuous inputs. Typically, inflammatory pain disappears after resolution of the initial tissue injury. However, in chronic disorders such as rheumatoid arthritis the pain persists even if the damage is repaired (Michaud et al., 2007).

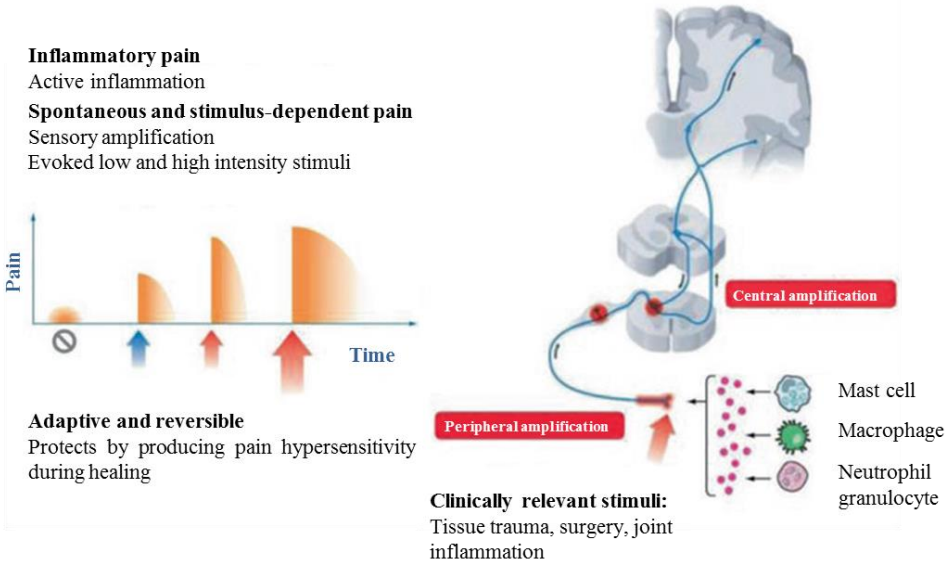


Figure 6B. *A summary of key features that distinguish and characterize the inflammatory pain (Costigan et al., 2009)*

- ***Dysfunctional pain.*** This pain is caused by a malfunction of the somatosensory apparatus. Dysfunctional pain occurs in situations in which there is no identifiable noxious stimulus nor any detectable inflammation or damage to the nervous system. It is unclear in most cases what causes the manifestation or persistence of dysfunctional pain. In conditions such as fibromyalgia, irritable bowel syndrome, and interstitial cystitis, the pain appears to result from an autonomous amplification of nociceptive signals inside the CNS (Nielsen et al., 2008, Staud & Rodriguez 2006) with a disturbed balance of excitation and inhibition in central circuits (Julien et al., 2005) and altered sensory processing that can be detected by functional imaging (Staud et al., 2008) (Figure 6C).

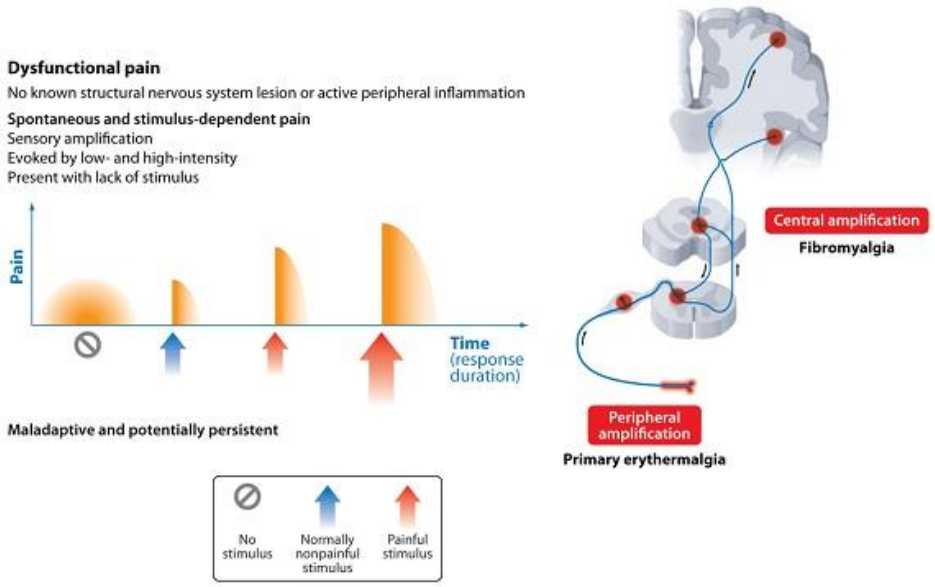


Figure 6C. *A summary of key features that distinguish and characterize the dysfunctional pain (Costigan et al., 2009)*

- ***Neuropathic pain.*** Neuropathic pain is defined as "Pain initiated or caused by a primary lesion or dysfunction in the peripheral or central nervous system" by International Association for the Study of Pain (www.iasp-pain.org). It can be divided into central or peripheral neuropathic pain based on the anatomical location of the injury or disease. Peripheral neuropathic pain results from lesions to the peripheral nervous system (PNS, eg. Peripheral nerves, DRG and dorsal root) caused by mechanical trauma, metabolic diseases, neurotoxic chemicals, infection, or tumor invasion and involves multiple pathophysiological changes both within the PNS and in the CNS (Dworkin *et al.*, 2003). Central neuropathic pain most commonly results from spinal cord injury, stroke, or multiple sclerosis (Ducreux *et al.*, 2006) (Figure 6D).

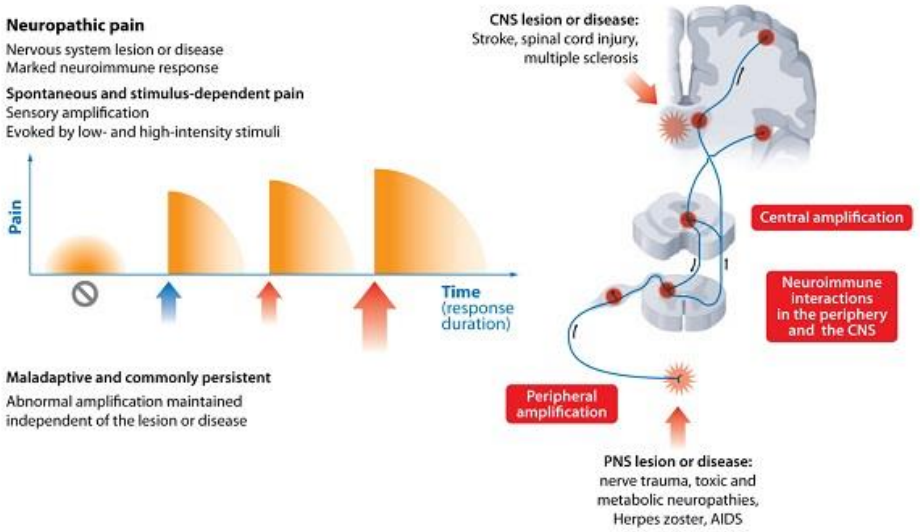


Figure 6D. *A summary of key features that distinguish and characterize the neuropathic pain (Costigan et al., 2009)*

2.3. Neuropathic pain

Pain and loss of function are intimately associated with the reaction of the nervous system to neural damage, and both provide important diagnostic clues that such damage has occurred. Peripheral neuropathic pain results from lesions to the peripheral nervous system (PNS) caused by mechanical trauma, metabolic diseases, neurotoxic chemicals, infection, or tumor invasion and involves multiple pathophysiological changes both within the PNS and in the CNS (Dworkin et al. 2003, Woolf & Mannion 2000). Central neuropathic pain most commonly results from spinal cord injury, stroke, or multiple sclerosis (Ducreux et al. 2006). The conventional approach to neuropathic pain has been to classify and treat it on the basis of the underlying disease (Dworkin et al. 2007). However, such an etiological approach does not capture the essential feature of neuropathic pain, which is the manifestation of maladaptive plasticity in the nervous system. The primary disease and the neural damage it causes are only the initiators of a cascade of changes that lead to and sustain neuropathic pain. Although treatment targeted at the primary pathology is obviously essential, understanding the mechanisms responsible for the maladaptive plasticity offers specific therapeutic opportunities to prevent the development of neuropathic hypersensitivity and normalize function in established neuropathic pain (Costigan et al., 2009).

Various factors such as trauma, infection, and tumor infiltration contributed to nerve injury which triggers in ectopic discharge or neurotransmitter release, leading to the generation of peripheral neuropathic pain. Although, neuropathic pain has different forms, the increased opening frequency of sodium channels in the axon membrane of injured nerve and maladaptive alterations caused by inflammatory mediators are the most common reasons for it.

2.3.1. Peripheral sensitization

Peripheral sensitization refers to that the threshold of a subset of nociceptors are decreased due to the accumulation of inflammatory mediators released from excitatory nociceptors or non-neural cells in the damaged tissue. These non-neural cells include mast cells, basophils, platelets, macrophages, neutrophils, endothelial cells, keratinocytes, and fibroblasts. They reside in or infiltrate into the inflamed tissue and release endogenous pro-inflammatory agents including neurotransmitters, peptides (SP, CGRP, bradykinin), eicosanoids, lipids (prostaglandins, thromboxanes, leukotrienes, endocannabinoids), neurotrophins, cytokines (IL-1β, IL-6, tumor necrosis factor-α), chemokines, and ATP, as well as proton and K+. Apart from inflammation, sympathetic excitation can also aid in release of these agents (Basbaum et al., 2009; Ballantyne, 2006). Most research on proinflammatory agents has focused on nerve growth factor (Chandran et al., 2016) and tumor necrosis factor-α (TNF-α). NGF directly interacts with peptidergic C fibers, co-expressing two structurally unrelated p75NTR and TrkA receptors, with high and low affinity to NGF respectively (Richner et al., 2014). NGF signaling via TrkA receptors is involved in the following three pathways: phospholipase C-γ(PLC-γ) pathway, mediating hypersensitivity; phosphatidylinositol 3-kinase (PI3K) pathway, inhibiting apoptosis of cells; mitogen-activated protein kinase (MAPK) pathway, promoting neuronal differentiation and axon growth (Ossipov, 2012). Additionally, NGF contributes to the initiation and development of pain by sensitizing the transient receptor potential cation channel subfamily V member 1 (TRPV1), activating mast cells to release pro-algesic agents (such as 5-HT, prostaglandins, histamine, ATP, etc.) and altering the gene expression of nociceptors (such as SP, Nav1.8 sodium channels, and brain-derived neurotrophic factor (BDNF)) (Khan et al., 2015). On the other hand, TNF-α mediated modulation is mainly due to the activation of two signaling pathways: IκB kinase activation of

nuclear transcription factor κB (NF-κB) and MAPK signaling pathway, which have been shown essential in the generation and sustenance of peripheral neuropathic pain (Shen et al., 2015). A nociceptor can express one or more cell-surface receptors to response to stimuli that give rise to inflammation or pain, hence, there is no broad-spectrum drug for neuropathic pain. Previous studies have shown that there are three classes of cell-surface receptors: ionic channel linked receptors, G protein coupled receptors (GPCRs), and enzyme linked receptors. In particular, receptors predominantly expressed in nociceptors are: P2 receptors (ATP receptors) (Kobayashi et al., 2013), TRPV1 receptors (Labuz et al., 2016), sensory neuron-specific receptors (SNSRs) (also known as Mas-related genes (Mrgs) receptors), acid-sensitive ion channels (ASICs) (Li et al., 2015), tyrosine receptor kinase (Trk) (Chang et al, 2016), and hyperpolarization-activated cyclic nucleotide-gated channels (HCN) (Jiang et al., 2008).

TRPV1, the unique capsaicin-gated receptor in the TRP family, not only can be served as the receptor recognizing and combining with certain inflammatory factors (such as lipid) directly, but also can be regulated by the downstream pathways signaled by the interaction between ATP, NGF or bradykinin and their specific receptors on the sensory neurons. ASICs, belongs to the DEG/ENaC (Degenerin/epithelial sodium channel) family, inducing the rapid depolarization of sodium current under acidic conditions. In damaged tissue, accumulation of prone and lactate due to anaerobic metabolism results in an increase in the extracellular proton concentration and consequent nociceptors activation. Besides, a host of receptors expressed on nociceptors belonging to GPCRs include B1, B2 receptors for bradykinin; EP1, EP4, EP3C receptors for prostaglandin E2; P2R2 receptors for ATP; metabotropic glutamate receptors; μ-, κ-, δ-opioid receptors; 5-HT receptors (Taguchi et al. 2015).

The expression and distribution of ion channels on the neuronal membrane are vital to the development and maintenance of membrane excitability. When nerve injuries, the function and density of voltage-gated sodium channels (VGSCs) and voltage-gated calcium channels (VGCCs) are changed, resulting in producing vast abnormal action potential and subsequent neuropathic pain. Previous studies suggested an important role of Nav1.3, Nav1.7, Nav1.8, Nav1.9 channel in sustaining neuropathic pain (Hains et al. 2003; Luiz et al., 2016; Siqueira et al., 2009). Apart from VGSCs, some types of calcium channels (e.g. N-, L-, Ttype VGCC) (Khomula et al., 2013; Leo et al., 2017; Orestes et al., 2013; Yamamoto et al., 2016), potassium channels (Busserolles et al.,

2016) and HCN also take part in the procession of neuropathic pain. Other related molecules and signaling pathways. Neural growth-related molecules, participated in several pathways including WNT, ephrinB-EphB, and Notch, are also involved in the initiation and development of neuropathic pain (Zhang et al., 2013). In addition, mTOR/VEGF signaling pathway has been revealed to regulate pain procession. When mTOR or VEGF receptors are inhibited, AMPA receptors, located in the downstream of the signaling pathway, are significantly downregulated, and produce an antalgic effect (Xie et al., 2017).

2.3.2. Central sensitization

Central sensitization refers to the process through which a state of hyperexcitability is established in the central nervous system, leading to enhanced reaction to nociceptive input. In the setting of nerve injury, persistent released neurotransmitters from nociceptors result in the depolarization of the postsynaptic neuron, thus, the N-methyl-D-aspartate receptor (NMDAR) on the postsynaptic neuron is activated and calcium influx induced by the depolarization strengthens the connection between nociceptors and neurons in spinal dorsal horns, which complicates the nociception. During this process, the long-term potentiation (LTP) produced by C fibers results in changes in synaptic plasticity of neurons (Amantea et al., 2000; Miletic et al., 2000). When metabotropic glutamate receptors (mGluRs) and substance P receptors are continuously activated, the upregulated calcium responses activate the downstream signaling and second messenger systems such as MAPKs, PKA, PKC, PI3K or Src, thus modulating the function of NMDARs. Intracellular mGluR5 were proposed to be a key receptor in neuropathic pain. After nociceptive hypersensitivity was induced, nuclear mGluR5 and receptor-mediated phosphorylated ERK1/2, Arc/Arg3.1 and c-fos have dramatically increased expression (Vincent et al., 2016). In addition to pain afferents from the injured area, central sensitization can also lead to pain surrounding the injured area, the generation of which is associated with the “sprout” of Aβ fibers. Normally, activation of Aβ fibers does not cause the excitability of pain sensory neurons as Aβ fibers terminated in the laminal I or laminal II in the dorsal horn. However, in the setting of nerve injury, the activity of nociceptors gets raised, and then C fibers disappear gradually thus deep Aβ fibers extending into the lamina I or lamina II, whereby establishing new synaptic connection with the existing neurons. This

phenomenon is called “sprout”. Under this condition, Aβ fibers, mediating innocuous stimuli previously, begin to mediate strong mechanic stimuli and thereby producing hyperalgesia.

When noxious stimuli transmit to the senior cortical central, GABA/glycine inhibitory neurons in the dorsal horn will be activated to reduce pain. Inhibitory dorsal horn interneurons synapse with the central terminals of primary sensory neurons and presynaptically modulate afferent input. Spinal interneurons also regulate activity in postsynaptic transmission neurons through GABAergic and glycinergic inhibition. In case of nerve injuries, excitotoxicity may cause these inhibitory neurons impaired or died. Because of the loss of inhibitory properties, transmission of nociceptive messages gets weakened and thus inducing hyperalgesia. A study suggested that injury in peripheral nerve downregulates the expression of potassium chloride transporter (KCC2) in the Laminae I, decaying $GABA_AR$-mediated postsynaptic currents producing hyperalgesia (Chen et al., 2014). Moreover, after nerve injury, tonic noradrenergic inhibition that acts on α2-adrenoceptors appears to be suspended (Rahman et al., 2008), and the net effect of descending serotoninergic input changes from inhibition to facilitation (Bee et al., 2008., Vera-Portocarrero et al., 2006). Amine uptake inhibitors like the tricyclic antidepressants or serotonin norepinephrine reuptake inhibitors (SNRIs) boost endogenous inhibition by increasing the levels of norepinephrine (Matsuzawa-Yanagida et al., 2008). Following nerve injury, primary afferents reduce their expression of μ opioid receptors, and dorsal horn neurons are less sensitive to inhibition by μ opioid agonists (Kohno et al. 2005).

In central nervous system, another important actor in the initiation and maintenance of neuropathic pain is represented by glial cells (Takeda et al., 2009; Tsuda et al., 2016). There are three main types of glial cells in CNS: microglia, astrocytes, and oligodendrocytes (Cao et al., 2008), wherein microglia and astrocytes are the most interesting cells. Microglia, as the inherent macrophages in the CNS, exist largely in the spinal gray matter. Astrocytes are the largest number of cells within the CNS. When nerve injury or inflammation is produced, astrocytes are activated later than microglia, but they remain functional for a long time (Thakur et al., 2017). Therefore, activation of microglia may be associated to the generation of neuropathic pain while activation of astrocytes may be related to the maintenance of neuropathic pain. Under common circumstances, glial cells are in static state. When nerve is injured, microglia and astrocytes are activated by cytokines secreted from injured peripheral nerve

terminals, and then large amounts of pro-algesic agents are released: neuropeptide (SP, CGRP (Calcitonin Gene Related Peptide), chemokines, neurotransmitter amino acids (for example glutamic acid), neurotrophic factors (NGF, BDNF), cytokines (TNF-α, IL-1β, IL-6), etc., further intensifying the central sensitization (Obata et al., 2008). Colony-stimulating factor 1 (CSF1), a cytokine involved in the differentiation and proliferation of macrophages and microglia, has been reported to serve as a new messenger for chronic pain, which could be a trigger for microglial activation (Thuault et al., 2016). Recently many studies confer attention to chemokines, including four subtypes: CC, CXC, XC, CX3C (Old et al., 2012), which function via regulation of GPCRs. Until now, there are over 20 types of chemokine receptors have been found. Most of chemokines can bind with several kinds of receptors, and one receptor can also recognize several kinds of chemokines. CCL3, CCL4, CCL5, CXCL2, and CX3CL1 in the PNS are identified as key mediators. In comparison, CCL1, CCL3, CCL7, CCL21, CXCL1, and CX3CL1 were found to be responsible for the central sensitization associated with neuropathic pain after the activation of glial cells (Kiguchi et al., 2012; Kiguchi et al., 2014). The downstream pathways including MAPK (Edelmayer et al., 2014), PLC, and PI3K (Jiang et al., 2016; Xu et al., 2007; Xu et al., 2011) are involved in the pain transmission. Toll-like receptors (TLRs) family, the membrane transporters expressed on peripheral immune cells and glial cells, are inherent component in the immune system. Microglia evoke immune response primarily via Toll-like-receptors (TLRs) such as TLR4 (Marinelli et al., 2015). In addition, blocking TLR4 and TLR2 can make pain relief and enhance the analgesia effects of buprenorphine in the CCI models (Jurga et al., 2016).

3. Vascular Endothelial Growth Factor (VEGF)

3.1. VEGF family of protein

The members of the VEGF family are dimeric glycoproteins with a molecular weight of between 34 to 45kDa. This family can be divided into five isoforms; VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF) in humans and other mammals. In addition, a protein related to the structure of VEGF was noted in the Orf virus, which came to be known as VEGF-E (Shibuya, 2003). Another group of proteins called VEGF-Fs were found in snake venom isolated from the Horned viper and Russell's viper (Suto et al., 2005; Yamazaki et al., 2009). Each isoform of VEGF forms dimers and bind to their receptor causing dimerization of the receptor which consists of any combination of three VEGF receptors (VEGF-Rs) as shown in Figure 7 (Cao et al., 2009). Although there are various combinations for each of the ligands and receptors, each combination would promote formation and development of vessels, lymph vessels and other vasculatures.

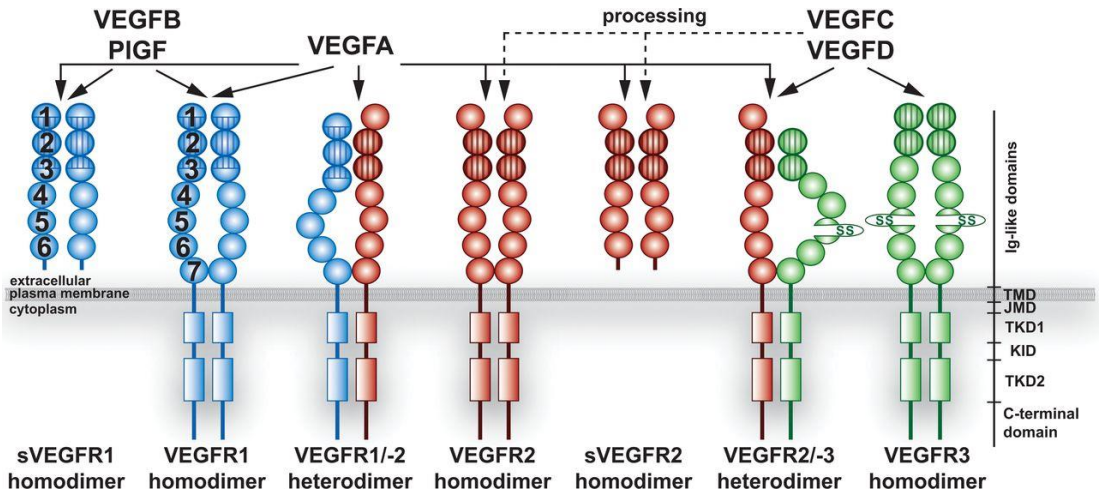


Figure 7. *The VEGF family and VEGF receptors (Koch et al., 2011)*

3.2. VEGF ligands and Functions

3.2.1. VEGF-A

VEGF-A (formerly known as vascular permeability factor/VPF) was identified separately by Senger et al. in 1983 and Ferrara et al. in 1989.

VEGF-A, usually called VEGF, is a highly specific vascular endothelial cell mitogen. Furthermore, it is also the strongest pro-angiogenic factor in VEGF family and it is expressed in every organ and tissue in the body (Breier et al., 1992; Ferrara et al., 1999). Hence, it is one of the most studied growth factors. The VEGF-A gene, containing eight exons and seven introns in the coding region, generates various isoforms after transcription, mRNA splicing variation and other steps that all have different biological properties. Among them, the isoforms of human VEGF-A are labeled as VEGF-A121, VEGF-A145, VEGF-A165, VEGF-A189, VEGF-A206 (Neufeld et al., 1999; Yla-Herttuala et al., 2000) (Figure 8).

Owing to various splice variants of the heparin-binding domain, different VEGF-A isoforms have different levels of solubility. For example, among three major isoforms, VEGF-A121 is completely soluble, VEGF165 is moderately soluble, but VEGF-A189 is almost insoluble (Ferrara et al., 2002). The most important isoform is the VEGF-A165, which is expressed in a variety of cells in the body. Isoform-specific mutant mice bearing either VEGF-A120 or VEGF-A188, but not VEGF-A164, died during embryonic life due to severe defective vessel development, indicating that the isoform VEGF-A164 is essential and sufficient for the normal development of the circulatory system (Maes et al., 2002; Carmeliet et al., 1999). Additionally, VEGF-A164 binds to NRP-1 that is a co-receptor for the collapsin/semaphorin family mediating neuronal cell guidance (Soker et al., 1996; Soker et al., 1998). VEGF-A is essential for embryonic and early postnatal development. The single allele knockout of Vegfa leads to embryonic lethality (Carmeliet et al., 1996; Ferrara et al., 1996).

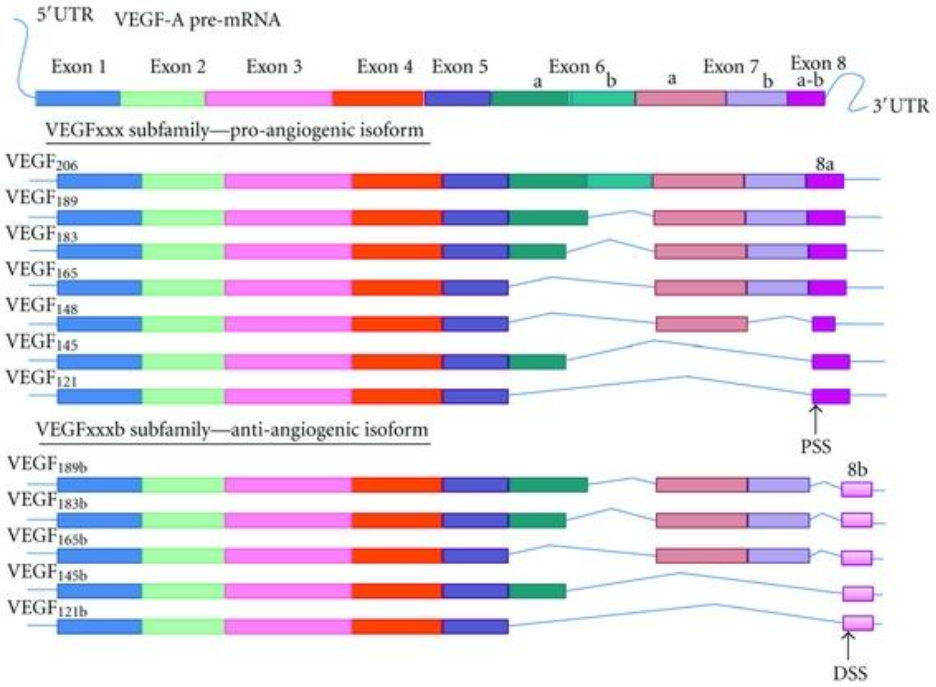


Figure 8. Alternative splicing of VEGF-A pre-mRNA. *The pre-mRNA of VEGF-A undergoes alternative splicing leading to pro-angiogenic isoforms notated with the number of amino acids and containing as last exon, the exon 8a stemming from the Proximal Splicing Site (PSS) located at the beginning of exon 8. The more recent subfamily of VEGF isoforms containing five members so far, are anti-angiogenic and contain as last exon, the exon 8b resulting of the splicing at the Distal Splicing Site (DSS) located after the exon 8a* (*Hilmi et al., 2012)*

3.2.2. VEGF-B

VEGF-B was first isolated in 1996, and two isoforms were identified, VEGF-B186 and VEGF-B167 (Paavonen et al., 1996). Both VEGF-B isoforms bind to VEGF-R1 and the co-receptor-NRP-1. VEGF-B186 is secreted from the cytoplasm into the extracellular matrix, while VEGF-B167 is secreted only under the stimulation by heparin. The isoform VEGF-B167 possesses the heparin-binding domain, whereas VEGF-B186 does not contain the heparin-binding domain (Olofsson et al., 1998; Makinen et al., 1999). Studies on the role of VEGF-B in angiogenesis mainly focus on heart and brain tissue revascularization.

3.2.3. VEGF-C

VEGF-C with the molecular weight of 46.9kDa was purified first by Joukov et al. in 1996. Lee et al. also isolated the corresponding gene, and the protein encoded by this gene was named VEGF-related protein (VRP) (Lee et al., 1996). VEGF-C is a mitogen

for lymphatic endothelial cells, and is involved in the development of lymphatic system (Kukk et al., 1996; Lymboussaki et al., 1999), and can promote lymphangiogenesis and induce lymphatic hyperplasia (Oh et al., 1997; Jeltsch et al., 1997). Its role in mediating lymphangiogenesis is caused by its ability to effectively regulate activation, proliferation and migration of lymphatic endothelial cells. As lymph node metastasis may occur by invasion of tumor cells into the lymphatic vessels, VEGF-C, is closely associated with the tumor metastasis (Joukov et al., 1996). Clinical data has demonstrated a correlation between VEGF- C levels in the tumor and metastasis process (Kitadai et al., 2001; Hashimoto et al., 2001). Additionally, VEGF-C may induce microvascular endothelial cell proliferation to some extent due to its ability to bind to VEGF-R2 (Cao et al., 1998).

3.2.4. VEGF-D

VEGF-D is similar to VEGF-C in structure. VEGF-D mRNA can be detected in the majority of human tissues, and is relatively abundant in the myocardium, lung, skeletal muscle, colon, and small intestine of adults (Achen et al., 1998). It not only induces lymphangiogenesis in tumors, but also causes diffusion of the tumor cells towards the regional lymph nodes through its expression in the tumor cells (Stacker et al., 2001).

3.2.5. VEGF-E

VEGF-E with the molecular weight of 20kDa is absent in humans and other mammals. It is, however, found to have the similar biological effects as VEGF-A165. At the protein level it demonstrates a ~25% sequence homology with VEGF-A165 at the amino acid level, though it lacks the basic domain and heparin binding domain (Veikkola & Alitalo, 1999).

3.2.6 PlGF

PlGF is a secreted glycoprotein, and is a dimer that first was isolated and purified from cDNA in human placenta by Iyer et al in 2001. There are four family members in this group, PlGF-1, PlGF-2, PlGF-3 and PlGF-4 based on the different mRNA splicing variants. PIGF is expressed in various kinds of tumors and in placenta in large amounts (DiPalma et al., 1996; Takahashi et al., 1994), which lasts the entire pregnancy, hence its name. P1GF can also bind to VEGF-A to form a heterodimer (De Falco et al., 2002).

3.3. VEGF Receptors and Signalling

The activation of the classical VEGF biological signal transduction pathways requires the specific binding of VEGF ligands and three types of transmembrane receptor tyrosine kinases (RTKs) in the cell membrane (VEGF-R1 (Flt-1), VEGF-R2 (KDR/FlK-1) and VEGF-R3 (Flt-4)) as. Among them VEGF-R2 is expressed in all endothelial cells and is the most important receptor mediating vasculogenesis. Human VEGF-R2 is called kinase inserted domain-containing receptor (KDR), while the murine receptor is called fetal liver kinase-1 (Flk-1).

3.3.1. Structure of VEGF Receptors

VEGF-Rs are members of the RTK superfamily and made up of one extracellular domain, one transmembrane domain and one intracellular domain (Manning et al., 2002; Krause & Van Etten, 2005). Among them, the extracellular domain is composed of seven immunoglobulins (Ig)-like folds (Shibuya et al., 2003). The intracellular domain contains two tyrosine kinase domains with a C-terminal tail at the end, and various cell signals are mediated from intracellular domain to downstream of signaling cascades (Olsson et al., 2006).

The activation of VEGF-Rs is controlled by its ligand. After the extracellular VEGFs dimer binds to the corresponding VEGF-Rs, the monomeric VEGF-Rs dimerize. According to the differences of VEGFs dimer structures and the specific binding capacity to VEGF-Rs, the dimerized VEGF-Rs will form into a homodimer or a heterodimer (Dixelius et al., 2003). Dimerized tyrosine kinases are activated and induce phosphorylation of its own tyrosine residues resulting in the phosphorylation and activation of other downstream proteins and pathways including the activation of a series of second messengers.

3.3.2. Membrane and soluble VEGF Receptor-1

Though VEGF-R1 was the first discovered RTK receptor of VEGF, its function is controversial due to its many roles. It regulates different signal pathways in various cell types and stages of the cell cycle. VEGF-A, VEGF-B and PlGF are all ligands of VEGF-R1. Park et al. thought that VEGF-R1 is a decoy receptor that creates no active signal transduction for cell mitosis working as negative regulator of VEGF (Park et al., 1994). The other possibility is that VEGF-R1 could block angiogenic cell signals

through competing with VEGF-R2 for binding of VEGF-A. It may then lead to less VEGF binding to VEGF-R2. VEGF-B and PlGF can only bind to VEGF-R1 and not to VEGF-R2 and VEGF-R3. Ligand binded-VEGF-R1 induces phosphorylation in the intracellular domain resulting in the recruitment of VEGF-R1 expressing cells such as monocytes as well as secretion of some factors, such as Hepatocyte growth factor (HGF) and Interleukin-6 (IL-6).

Due to various splicing variants, VEGF-R1 may also produce soluble VEGF-R1 (sVEGF-R1 or sFlt-1). VEGF-R1 and soluble VEGF-R1 can both bind to VEGF-A, with high binding affinity but VEGF-R1 has low intracellular response compared to VEGF-R2 (Seetharam et al., 1995; Waltenberger et al., 1994).

sFlt-1 comprises the extracellular domains of the vascular endothelial growth factor receptor-1 (VEGFR-1), and is soluble, being present in the circulation. It acts as an anti-angiogenic protein by antagonizing the actions of both vascular endothelial growth factor (VEGF) and placental growth factor (PlGF). Multiple splice variants of sFlt-1 have significantly different tissue distributions (Jebbink et al., 2011), raising the potential for different physiological and pathological roles. For example, in humans, the main sFlt-1 variant, known as sFlt-1 i13, is widely expressed in most tissues, whereas another variant known as sFlt-1 e15a appears to be almost exclusively expressed by the placenta (Jebbink et al., 2011).

sFlt-1 results either from alternative splicing of the VEGF-R1 pre-mRNA (He et al., 1999b) or through cleavage of the ectodomain of VEGF-R1 (Rahimi et al., 2009). Proteolytic cleavage of the extracellular region is thought to occur adjacent to the transmembrane domain (Raikwar et al., 2016) and is potentially due to the actions of proteolytic enzymes (Raikwar et al., 2014; Han et al., 2015). Proteolytic cleavage produces an sFlt-1 that is identical to the extracellular region of VEGF-R1, while the multiple variants of sFlt-1 that have been identified all have unique C-terminal sequences (Sela et al., 2008; Heydarian et al., 2009; Thomas et al., 2009). As shown in Figure 9, these splice variants share significant sequence homology with the full-length VEGF-R1, differing only at their C-terminus.

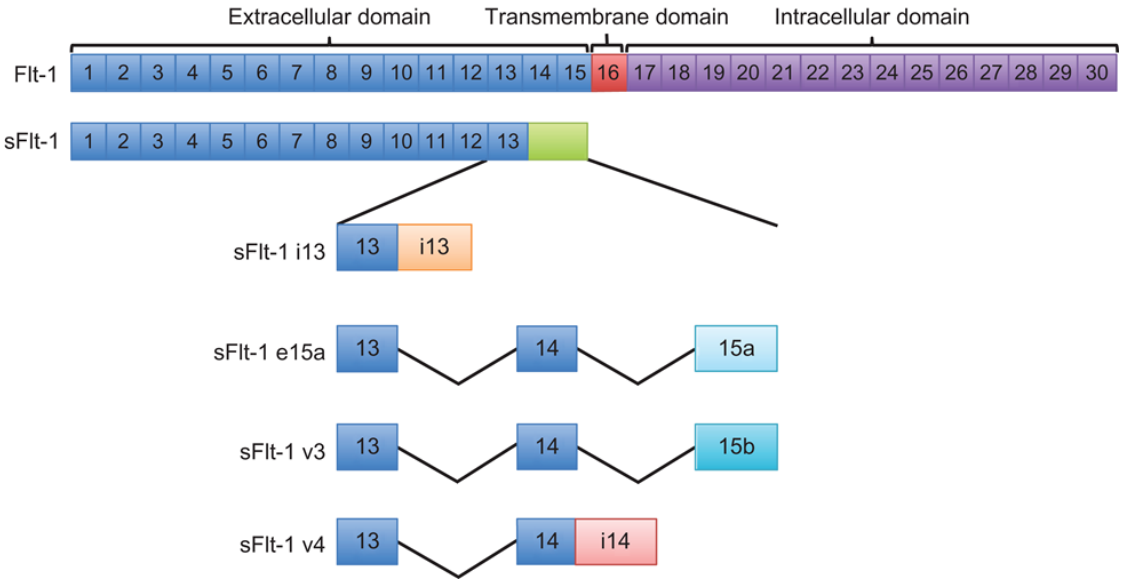


Figure 9. Schematic representations of VEGF-R1 splice variants. *The Flt-1 gene encodes the full-length membrane-bound Flt-1 receptor VEGF-R1, as well as the four soluble alternative sFlt-1 splice variants. All transcripts are identical to exon 13, with the soluble transcripts each identified by a unique C-terminal region. (Palmer et al. 2015).*

3.3.3. VEGF Receptor-2

With its highly specific expression in endothelial cells (Asahara et al., 1998), and as being the main functional receptor of VEGF-A, VEGF-R2 plays a leading role in VEGF mediated signaling and vascular endothelial growth. VEGF-A bound to VEGF-R2 activates a series of intracellular downstream signal molecules related to mitogenic, chemotactic and anti-apoptotic effects. Under stimulation of VEGF-A, tyrosine residues at different locations on VEGF-R2 can bind to multiple proteins, which mediate signals and induce phosphorylation and initiation of a signal cascade reaction, regulating the biological characteristics of endothelial cells through different activation pathways (e.g., endothelial cell migration, cell proliferation, actin remodeling and anti-apoptosis). Eventually, VEGF-A- VEGF-R2 signaling promotes angiogenesis. Coupled with this effect on angiogenesis, VEGF-R2 can also participate in VEGF-A-mediated increases in vascular permeability (Li et al., 2000; Gille et al., 2001; Larrivee & Karsan, 2000).

VEGF-C and VEGF-D also bind to VEGF-R2. Even though VEGF-E is absent in humans and other mammals, VEGF-E can bind specifically to VEGF-R2 and mediate biological effects similar to VEGF-A. For example, in the rat tumor and cornea models, angiogenesis mediated by VEGF-E can be detected and new vessels are

similar to those produced by VEGF-A mediated signaling in their form, structure and function.

3.3.4. VEGF Receptor-3

VEGF-R3 is mainly expressed on endothelial cells of lymphatic vessels (Kukk et al., 1996; Kaipainen et al., 1995) and is the specific receptor of VEGF-C and VEGF-D. VEGF-C and VEGF-D bound VEGF-R3 activate downstream signaling pathways and induce lymphatic endothelial cell proliferation, migration and formation of the lymphoid sinus (Joukov et al., 1997), thus causing lymphatic hyperplasia. VEGF-R3 also plays a vital role in cardiovascular formation during embryonic development. During late embryonic development VEGF-R3 is expressed mainly in lymphatic vessels and to a small degree in blood vessels. Only one layer of endothelial cells with an irregular basement membrane forms the lymphatic capillaries (Karkkainen et al., 2002; Achen et al., 2005). Malignant tumor cells can therefore enter the lymphatic vessels easily, whereas tumor cells need to penetrate a well-aligned basement membrane to intravasate blood vessels (Liotta et al., 1980).

3.4. VEGF and pain

Our nervous system is capable of detecting a wide range of stimuli which can evoke pain. These can generate a short-term sensation (acute pain) which usually resolves. However, sometimes this pain becomes persistent. Constant stimulation provokes alterations in nociceptive transmission, enhancing pain signals and increasing sensitivity. If this state persists for more than 3 months, it is defined as chronic pain. It affects over one-quarter of people worldwide and is more prevalent in women than in men. The mechanisms that sustain and drive chronic pain have been comprehensively reviewed (Woolf and Salter, 2000; Basbaum et al., 2009; von Hehn et al., 2012). The current analgesic treatments (opioids or NSAIDs) do not meet patients needs or are inefficient. In addition, their side effects limit their use. Therefore, the development of new drugs is urgently required. Preclinical research studies have identified an array of molecular targets that are involved in the establishment and maintenance of chronic pain and may represent interesting targets for pharmacological intervention. Among these mediators, Vascular Endothelial Growth Factor (VEGF) has been postulated as a key factor.

3.4.1. VEGF as a therapeutic target

Alterations in the VEGF system, characterized by changes in the expression of its components, have been related to a plethora of diseases. Some of these diseases can occur concomitantly with pain, such as cancer, rheumatoid arthritis or diabetic complications (Ferrara, 2004; Maharaj & D'Amore, 2007; Rosenstein et al., 2010). There are currently several anti-VEGF-related drugs used in clinical settings for the cancer treatment in combination with chemotherapy. Anti-VEGF drugs are also used to attenuate neovascularization in age-related macular degeneration and diabetic macular edema (Ferrara, 2004; Kim and D'Amore, 2012). However, in recent years, the role of the VEGF family in neuroprotection and nociception has received increased attention (Beazley-Long et al., 2013, 2018; Hulse et al., 2014, 2015, 2016; Selvaraj et al., 2015; Hulse, 2017; Lai et al., 2017). The involvement of VEGF in the pathophysiology of pain is not fully understood, however, the association between this growth factor and some of the main hallmarks of painful diseases warrants the investigation of VEGF as a therapeutic target for pain treatment.

3.4.2. Involvement of VEGF in several types of pain

Inflammation is a common feature in different painful syndromes and its components sensitize nociceptors which mediate pain sensation. VEGF is one of the most important mediators participating in this pro-inflammatory milieu. The significance of VEGF-R1 and VEGF-R2 in the pathophysiology of two of the most prevalent chronic inflammatory diseases that are concurrent with pain, namely rheumatoid arthritis (RA) and osteoarthritis (OA), has previously been reported by Hamilton et al. in 2016. Inhibition of VEGF signalling pathways and angiogenesis emerged as a promising approach demonstrating reduced destruction of joints and associated pain in OA (Hamilton et al., 2016). However, the role of each member of the VEGF family, isoforms or alternative splicing in alleviating chronic inflammatory pain is yet to be clarified.

In osteoarthritis, anomalous VEGF expression in synovial fluids has been associated with higher pain scores (Takano et al., 2018) and worse prognosis. VEGF seems to mediate cartilage degeneration, bone and neurovascular invasion of articular cartilage, increased migration and/or activity of macrophages, fibroblasts, and neutrophils. These cells, in turn, increase levels of cytokines and VEGF, amplifying the

inflammatory response (Hamilton et al., 2016; Nagao et al., 2017). VEGF is able to evoke pain by several pathways in synovium, osteochondral junction and meniscus, through both VEGF-R1 and VEGF-R2 (Nagao et al., 2017). Both signalling axes seem to be directly associated with nociceptor sensitization, and accordingly, VEGF signalling inhibition led to a decreased pain (Hamilton et al., 2016). In addition, other VEGF approaches have been experimentally tested and successfully counteracted pain responses and/or improved cartilage degeneration, synovitis and osteophyte formation (Nagai et al., 2014; Hamilton et al., 2016). Taking all of the aforementioned, it seems plausible that proper VEGF therapies targeting ligands or receptors could counteract osteoarthritis progression and its associated pain. In other painful chronic diseases with an autoimmune component, such as rheumatoid arthritis (RA), one of the most potent factors that seems to be responsible for the typical hypertrophied synovium (pannus), oedema, swelling, and chondrolytic and osteolytic reactions, is VEGF (Afuwape et al., 2002; Malemud, 2007). VEGF is expressed in synovial fibroblasts, fibroblasts close to microvessels, vascular smooth muscle and macrophages, but not in endothelial cells of patients with RA (Nagashima et al., 1995). VEGF is augmented in patients' serum and is tightly correlated with TNF-α and some other pro-algesic cytokines (IL-1ß, IL-17, IL-18) which in turn reduce VEGF expression, except in patients who are refractory to TNF-α therapy (Nowak et al., 2008; Beazley-Long et al., 2018). At experimental level, an increased expression of VEGF, VEGF-R1 and VEGF-R2 was described in an RA animal model and the treatment with an anti-VEGF-R1 efficiently blocked pain. However, the neutralization of either the VEGF ligand or VEGF-R2 did not induce the same anti-nociceptive effect (De Bandt et al., 2003). Contrastingly, other authors suggested that VEGF-R2 acts as a positive transducer in vascular proliferation during RA and its pharmacological blockade reduces mechanical sensitivity in an animal model of RA (Beazley-Long et al., 2018). While Beazley-Long and colleagues stated that when VEGF-R2 is inhibited allodynia is reduced and/or prevented (Beazley-Long et al., 2018), De Bandt and colleagues showed VEGF-R2 suppression was insufficient for resolution of this type of pain (De Bandt et al., 2003). Further studies aimed to address this discrepancy are needed. The putative role of VEGF in the relief of pain has been most extensively studied in neuropathic pain compared to other types of pain. VEGF-A has been strongly linked with neuroprotection and its neutralization was found to exacerbate neuropathic damage and pain in a retrospective clinical study (Matsuoka et al., 2016). Contrary to this,

experimental approaches of VEGF blockade have successfully alleviated nociceptive responses in a model of chronic constriction injury, sciatic nerve ligation or diabetic neuropathy. These approaches included the suppression of VEGF-R2 signalling, spinal serine arginine protein kinase 1 (SRPK1) inhibition, and the administration of VEGF-Axxxb. The anti-nociceptive effect derived from VEGF-R2 blockade in painful neuropathies has been reported to be mediated via the interaction with P2X2/3 receptors (De Bandt et al., 2003; Liu et al., 2012), or TRPA1 and/or TRPV1 (Hulse et al., 2015, 2016; Zeng et al., 2018). In vitro studies revealed that in injured peripheral nerves there is an upregulation of VEGF-A in infiltrated cells that seems to mediate angiogenesis, a key component of chronic inflammation and peripheral sensitization (Kiguchi et al., 2014). Blocking VEGF-A has been shown to reduce nociception in rodents and to exert a neuroprotective effect by improving neuronal restoration and conduction, decreasing pro-apoptotic Caspase-3 levels in sensory neurons, preventing neural perfusion and epidermal sensory fiber loss (Taiana et al., 2014; Hulse et al., 2015; Zeng et al., 2018). Another plausible strategy evaluated is SRPK1 inhibition, as this would reduce the pro-nociceptive and pro-angiogenic forms of VEGF (Hulse, 2017). Several studies indicate that administration of VEGF-A165b (the reported anti-nociceptive form of VEGF-A), could constitute an interesting therapeutic strategy for pain, considering that it also has neuroprotective effects. Contrastingly, a recent study demonstrated in an animal model of oxaliplatin-induced pain, that VEGF-A165b expression is augmented in spinal cord, and the intrathecal administration of bevacizumab or VEGF-A165b antibody reversed the hypersensitivity symptoms (Di Cesare Mannelli, …, Vona, et al., 2018). Most studies at an experimental level seem to suggest a pro-nociceptive effect induced by VEGF-A in several types of pain. However, in neuropathic pain due to a partial saphenous nerve ligation injury, Hulse and colleagues focused on the anti-nociceptive effect of VEGF-Axxxb isoform and the relevance of targeting its alternative splicing so as to modulate the balance between the pro- and anti-nociceptive VEGF isoforms. VEGF-A165a (pro-nociceptive) and VEGF-A165b (anti-nociceptive) isoforms have opposing actions on vascular permeability, angiogenesis, and vasodilatation (Hulse, 2016). This had been shown extensively in several papers (Hulse et al., 2014, 2015, 2016; Hulse, 2017; Beazley-Long et al., 2018). However, in a model of oxaliplatin-induced pain, Di Cesare Mannelli et al., (Di Cesare Mannelli, …, Vona, et al., 2018), clearly showed the pro-nociceptive role of VEGF-Axxxb isoform. Further studies are urgently needed in order

to clarify the role of VEGF-Axxxb and the mechanisms underlying the paradoxical effects reported. These disparate functions raise the possibility that different isoforms may have varying pro- and anti-nociceptive role. Among chronic neurologic diseases, migraine is the third most prevalent and disabling. Current treatments are usually unsuccessful. The meningeal and brain mast cells involved can degranulate and release vasoactive substances that can activate trigeminovascular mechanisms inducing pain. Among these mediators, VEGF is one of the most important as it also stimulates nitric oxide synthase and therefore increases nitric oxide levels (Bussolati et al., 2001). Therefore, VEGF plays a direct role in the endothelial cells in the trigeminovascular system. Indeed, increased levels of VEGF have been showed in migraineurs suggesting endothelial alterations (Rodríguez-Osorio et al., 2012). However, decreased serum concentrations of VEGF were found during interictal period (Michalak et al., 2017). In addition, several VEGF haplotypes have been described to be associated with variable susceptibility to migraine (Gonçalves et al., 2010). A better understanding of VEGF fluctuations, genetic profiling and the potential protective role in migraines could constitute an interesting approach for prophylactic intervention. The importance of VEGF in cancer pathophysiology and therapy has been extensively reported (Carmeliet, 2005; Lal Goel and Mercurio, 2014). However, the potential anti-nociceptive effect of VEGF in cancer-induced pain is poorly understood. VEGF-R1 is augmented in humans and in an animal model of osteosarcoma-induced pain. The modulation of VEGF-VEGF-R1 axis signalling by an anti-VEGF-R1 antibody or the administration of the VEGF-R1 soluble form (sFlt-1) that decoys VEGF from binding VEGF-R1, effectively counteracted pain (Selvaraj et al., 2015). Perturbing expression, activation, or signalling of VEGF-R1, but not of VEGF-R2, in peripheral sensory nerves disrupts attenuated cancer-induced pain and tumor-induced remodelling of nerves in mice in vivo (Selvaraj et al., 2015). Additional studies using experimental models of cancer-induced pain that address the role of VEGF-R2 are urgently required in order to delineate the role for this integral mediator. This will inform the design and development of new pharmacological strategies.

4. Aim of the thesis

According to the increasing interest for mesenchymal stem cell (MSCs) efficacy in immune-related diseases and painful neuropathies, the recent data obtained underling the role of VEGF in oxaliplatin neuropathic pain and the effect of stem cells (Di Cesare Mannelli et al., 2018), the purpose of the present study has been to evaluate the possible mechanism of action by which RASCs can reduce VEGF pro-algesic effect. We hypothesized that RASCs could directly release sFlt-1 (therefore dumping VEGF effect) and, on the other side, they could stimulate the VEGFR-VEGF complex internalization through the endocytosis for recycling or/and degradation.

Moreover, we decided to investigate the presence of VEGF receptors (VEGF-Rs) and the intracellular signal induced by VEGF in RASCs in order to better characterize OXA induced pain and the action of the growth factor.

In parallel experiments, oxaliplatin effect was evaluated in mice to ascertain if VEGF action can be a common mediator of oxaliplatin induced neuropathy in different animal species and which one specific cell/anatomical structure can be involved in VEGF nociception.

5. Materials and methods

5.1. Animals

For all the experiments described below, male Sprague-Dawley rats (Harlan, Varese, Italy) and male CD-1 albino mice (Envigo, Varese, Italy) weighing approximately 200 – 250 g or 20-25 g at the beginning of the experimental procedure were used. Animals were housed in CeSAL (Centro Stabulazione Animali da Laboratorio, University of Florence) and used at least one week after their arrival. Ten mice were housed per cage (size 26 × 41 cm); Four rats were housed per cage (size 26 × 41 cm); animals were fed a standard laboratory diet and tap water ad libitum, and kept at 23 ± 1°C with a 12 h light/dark cycle, light at 7 a.m. All animal manipulations were carried out according to the Directive 2010/63/EU of the European parliament and of the European Union council (September 22, 2010) on the protection of animals used for scientific purposes. The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described was obtained from the Italian Ministry of Health (No. 54/2014-B) and from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guidelines (McGrath and Lilley, 2015). All efforts were made to minimize animal suffering and to reduce the number of animals used.

5.2. Rat ASCs preparation

Retrosternal, thighs and aortic fat pads from Wistar rats weighing approximately 550–600 g, were harvested in a sterile fashion, washed with phosphate-buffered saline (PBS), minced and digested with 2 mg/mL collagenase type I (from Clostridium histolyticum, Sigma-Aldrich, St. Louis, MO, USA) in PBS, for 30 min at 37°C in a shaking water bath. The sample was filtered through a 100 µm mesh filter to remove debris. Enzyme activity was neutralized with medium containing 10% fetal bovine serum (FBS; GIBCO, Life Technologies – Thermo Fisher Scientific, Waltham, MA, USA) and sample was centrifuged at 1680 r.p.m. for 10 min to obtain a high-density cell pellet. The pelleted stromal vascular fraction containing ASCs was suspended and plated in Nutrient Mixture F-12 Ham (Sigma-Aldrich, Milan, Italy) supplemented with

20% FBS and 1% penicillin-streptomycin (P/S, Sigma-Aldrich, Milan, Italy) and incubated at 37°C in 5% CO_2 atmosphere. After 4 h, non-adherent cells were removed, and adherent cells were washed twice with medium. The medium was replaced every 2-3 days, and cells with 80-90% confluence were detached with the use of trypsin/ethylenediaminetetraacetic acid (EDTA) (0.5%) and passed. Rat ASCs at early passages with 80-90% confluence were detached by light trypsinization and used for surface marker characterization. Rat ASCs at passage 1 (P1) were frozen in a 10:1 ratio of FBS/dimethyl sulfoxide (Sigma-Aldrich, Milan, Italy) and cryopreserved in liquid nitrogen until required for the *in vivo* studies. In all experiments, only cells at P2 of culture were used.

5.3. Cell cytocharacterization

Immunophenotypical analysis of cultured cells was performed by using the FITC-, PE, APC or eFluor 450- conjugated monoclonal antibodies (mAbs) against CD90, CD79a, CD29, CD45 (e Biosciences, San Diego, CA, USA) respectively.

Briefly, cells at early passages with 80-90% confluence were detached using EDTA buffer pH 7.2, washed, and suspended in buffer EDTA + FBS 1%. Cells were incubated with the specific mAbs at room temperature for 30 min.; cells were then washed with EDTA + FBS 1% buffer, centrifuged, suspended in 0.8 mL of the same buffer and analyzed by flow cytometry CyFlow Space (Sysmex Partec Italia s.r.l., Carate Brianza (MB), Italy). Data were analyzed on DOT-PLOT bi-parametrical diagrams using FlowMax (Sysmex Partec Italia s.r.l., Carate Brianza (MB), Italy). Rat adipose stem cells (RASCs) at passage 1 (P1) were frozen in a 10:1 ratio of FBS/dimethyl sulfoxide (Sigma-Aldrich, Milan, Italy) and cryopreserved in liquid nitrogen until use. Different sources of RASCs were evaluated by processing rat thighs, sternal and aortic fat pads. The stemness of isolated cells was assessed in terms of percentage of CD29-, CD90-positive cells, as markers for mesenchymal stem cells, and CD45-, CD79α-positive cells, as markers for hematopoietic stem cells. All cell lines obtained from different anatomical fat pads exhibited the typical phenotype of mesenchymal stem cells (CD90+ CD29+ CD45- CD79α-) (Table 1). The retrosternal fat pad held a higher percentage of CD90- and CD29-positive cells (83.4% and 92.5%, respectively) than thighs (86.7% and 87.6% respectively) and aortic fat pads (59.9% and 88.7% respectively). The expression of CD45 and CD79α was lower in sternal fat-

derived cells (7.02% and 7.7% respectively) than in thighs (12.3% and 12.7% respectively) and aortic fat-derived cells (11.2% and 11.8% respectively) (Table 1). Furthermore, several cell lines at different serial passages (from P1 to P4) and after thawing and culture until confluence were cytocharacterized by flow cytometry (Table 2). Rat ASCs at P2 showed the better stemness phenotype and cell cryopreservation did not change it. On these bases of these results, sternal-derived RASCs at P2 were chosen to perform all experiments.

Table 1. Immunophenotypic characterization of RASCs isolated from three different anatomical regions at passage 2

Positive cells %			
Marker	**Sternal fat (n=2)**	**Thighs fat (n=2)**	**Aortic fat (n=2)**
CD90	83.4 ± 1.7	86.7 ± 2.5	59.9 ± 2.8
CD29	92.5 ± 1.9	87.6 ± 3.6	88.7 ± 4.7
CD45	7.02 ± 2.05	12.3 ± 1.5	11.2 ± 1.8
CD79α	7.7 ± 1.9	12.7 ± 1.7	11.8 ± 2.8

The expression of surface markers of retrosternal, thighs and aortic fat-derived ASCs was characterized by means of flow cytometry with anti-CD45, CD79α, CD90, CD29 antibodies from fats explanted and prepared from 2 different rats. Results are expressed in percentage of positive cells for each antibody ± SEM.

Table 2. Immunophenotypic analysis of RASCs at early passages

Positive cells %					
Marker	**P1 (n=7)**	**P2 (n=6)**	**P3 (n=2)**	**P4 (n=2)**	**P2 thawed cells (n=2)**
CD90/CD29	79.9 ± 2.2	85.1 ± 2.9	84.2 ± 2.1	84.0 ± 2.9	84.5 ± 2.1
CD45/CD79	0.7 ± 0.2	2.4 ± 0.1	4.1 ± 0.3	13.1 ± 0.8	2.6 ± 0.5

Rat ASCs within 1-4 passages were harvested and detected for specific cell surface antigens by means of flow cytometric analysis. Results are expressed in percentage of positive cells for CD90/CD29 and CD45/CD79α ± SEM.

5.4. Preparation of rat coronary endothelial cells (RCEs)

Rat CEs were obtained from rat hearts (Failli et al 2000). Briefly, hearts were digested with 0.1% type I collagenase (Sigma-Aldrich, Italy), cut in small pieces and cells were obtained by centrifugation (250 x g for 10 min). Cell suspension was stirred for 30 min at 37°C in 0.025% trypsin (Sigma-Aldrich, Italy) in PBS. Cells, obtained by centrifugation (250 x g for 10 min), were plated in complete culture medium and washed twice after 4 h. Three different preparations were used at P1 for experiments.

5.5. Oxaliplatin-induced neuropathic pain model

Neuropathic pain was induced by i.p. injection of 2.4 mg kg^{-1} oxaliplatin dissolved in a 5% glucose and administered i.p. for 4 consecutive days every week for 2 weeks (Cavaletti et al., 2001; Di Cesare Mannelli et al., 2013 with minor modifications). Control animals received an equal volume of 5% glucose.

5.6. Rat ASCs administration

Rat ASCs were suspended in 400 μL of DMEM without phenol red and with 500 I.U. heparin and injected into the tail vein in the amount of $2x10^6$ per rat; control rats received the same amount of vehicle or RASCs conditioned medium i.p.
Animals were randomly divided into the following experimental groups of 10-12 rats each, receiving according to scheme 1 (Figure 10):

- control + DMEM: control (oxaliplatin vehicle i.e. 5% glucose), DMEM (RASCs medium);
- oxaliplatin + DMEM: oxaliplatin, DMEM at day 13 (single administration) or DMEM at days 6, 13, 17 and 20 (repeated administrations);
- control + RASCs: control (oxaliplatin vehicle), $2x10^6$ RASCs at day 13 (single administration);
- oxaliplatin + RASCs: oxaliplatin, $2x10^6$ RASCs at day 13 (single administration) and $2x10^6$ RASCs at day 6, 13, 17 and 20 (repeated administrations).

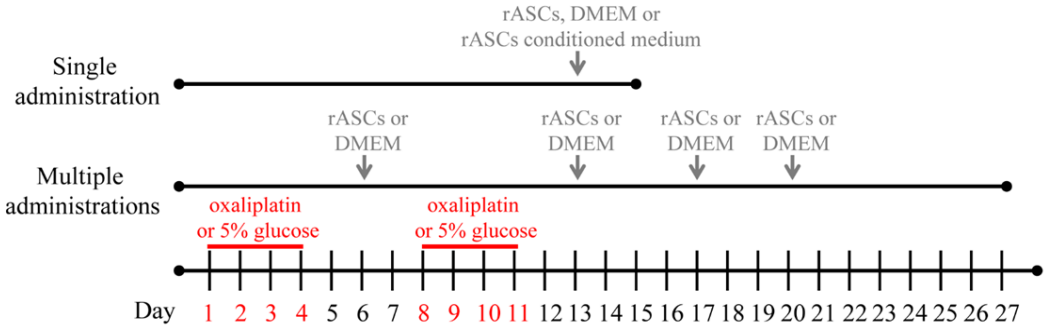


Figure 10. RASCs administrations in the neuropathy model induced by oxaliplatin. *Oxaliplatin (2.4 mg kg^{-1}) was administered intraperitoneally (i.p.) for 4 consecutive days every week for 2 weeks. Control group received 5% glucose. RASCs were administered intravenously (2x10^{6} cells suspended in 400 µL of DMEM containing 500 I.U. heparin. (Di Cesare Mannelli, …, Vona et al, 2018).*

5.7. Cytokines, growth factors, pain related peptides evaluation

IL-1α, IL-6, IP-10, TNF-α, Pan-VEGF-A (without discrimination among isoforms), EGF, α-MSH, β-Endorphin, Substance P and TGF-β1, were measured into plasma samples of groups using quantitative Milliplex Luminex (MilliPlex MAP Rat Cytokine Panel 6-plex and Rat TGF-β single plex, Millipore, Melbourne, Victoria, Australia) assays according to the manufacturer's instructions. In brief, 96-well plates were pre-wet with 200 µL assay buffer (provided by the manufacturer) for 10 minutes. The content of the wells was discarded by inverting the plate and tapping it smartly onto absorbent towels. Standards and plasma samples (30 µL) were added to appropriate wells, followed by the addition of assay beads. Plates were incubated for 2 h with mild agitation at room temperature (RT); the fluid was then removed using a magnetic plate and wells were washed twice. Detection antibodies were added to each well, and incubated for 1 h at room temperature, the fluorescent conjugate streptavidin-phycoerythrin was added to each well and plates incubated for 30 min at room temperature. Fluid was then removed using a magnetic plate and wells were washed twice. Each sample was analyzed in duplicate. Identical positive and negative quality controls were included on each assay in duplicate. Assays were performed on one batch, with samples randomly mixed. The minimum detectable concentration (pg mL^{-1}) was 4.2 IL-1α, 30.7 IL-6, 1.4 IP-10, 1.9 TNF-α, 2.6 VEGF, 0.3 EGF, 86 α-MSH, 70 β-Endorphin, 2 Substance P, and 9.8 TGF-β1, while the intra-assay variability was less than 10%. Data were collected and analyzed using a BioPlex 200 instrument equipped with BioManager analysis software (BioRad, Segrate, Milano, Italy). Nerve growth

factor (NGF) was measured into 100 µL plasma samples using the ELISA kit (Sigma-Aldrich, Milan, Italy) assay according to the manufacturer's instructions. The minimum detectable concentration was 20 pg mL^{-1}.

5.8. Intracellular Ca2+ measurement

Intracellular cytosolic Ca2+ dynamic ([Ca2+]i) was evaluated in fura-2AM loaded RASCs and RCEs as described (Failli et al. 2000). Briefly, cells were plated on round glass coverslips (25 mm diameter) and seeded for 1-2 days in complete medium. Cells were loaded with 4 µM fura-2AM (Molecular Probes-Invitrogen Life technologies, San Giuliano Milanese, Italy) for 45 min at 37°C and then washed with standard HEPES buffer. Coverslips were mounted in a perfusion chamber and placed on the stage of an inverted reflected light fluorescence microscope (Zeiss Axio Vert. A1 FL-LED) equipped with fluorescence excitation (385 nm) based on LED. Soon after recorded the basal fluorescence, cells were stimulated with different agonists: VEGF(100ng/ml) and ATP (1µM) and cells were observed for at least 2 min after agonist administration. Fura-2 fluorescence was recorded with a Tucsen Dhyana 400D CMOS camera (Tucsen Photonics Co, Ltd, Fuzhou, China) with a frame rate of 4 Hz and a resolution of 1024x1020 pixels2. Ca2+ dynamic was measured by single cell imaging analysis at 35°C. Images were recorded using Dhyana software SamplePro and dynamically analysed with the open source community software for bio-imaging Icy (Institute Pasteur, Paris, France). A signal-to-noise ratio of at least 5 A.U. (fluorescence arbitrary units) was considered as the low limit to define a calcium increase and cells presenting a calcium increase as "responders cells". All cells (identified by transmitted light microscopy) found in an optical field (using 40X magnification objective) were analysed. From 15 to 20 cells were evaluated blindly every experimental day for each experimental treatment.

5.9. Rat paw-pressure test

The nociceptive threshold of rats was determined with an analgesimeter (Ugo Basile, Varese, Italy), according to the method described by (Leighton et al., 1988). In the experiments, a constantly increasing pressure was applied to a small area of the dorsal surface of the hind paw using a blunt conical probe by a mechanical device. Mechanical pressure was increased until vocalization or a withdrawal reflex occurred

while rats were lightly restrained. Vocalization or withdrawal reflex thresholds were expressed in grams. For analgesia measures, mechanical pressure application was stopped at 120 g.

5.10. Mice paw-pressure test

Mechanical hyperalgesia was determined by measuring the latency in seconds to withdraw the paw away from a constant mechanical pressure exerted onto the dorsal surface (Russo R, et al., 2012). A 15 g calibrated glass cylindrical rod (diameter = 10 mm) chamfered to a conical point (diameter = 3 mm) was used to exert the mechanical force. The weight was suspended vertically between two rings attached to a stand and was free to move vertically. A single measure was made per animal. A cut off time of 40 s was used.

5.11. Cold plate test

The animals were placed in a stainless-steel box (12 × 20 × 10 cm) with a cold plate as floor. The temperature of the cold plate was kept constant at 4 °C ± 1 °C. Pain-related behaviour (licking of the hind paw) was observed, and the time (seconds) of the first sign was recorded. The cut-off time of the latency of paw lifting or licking was set at 30 s. The results were expressed by the licking latency resulting from the compounds acute administration.

5.12. Blood collection

Sample of venous blood were collected, using heparinized syringes (Westmed, Arizona, USA) of control group, oxaliplatin and oxaliplatin+RASCs rat groups 24 hours after DMEM or RASCs administration.
At time 24 h, blood was used to prepare plasma which was stored at –80°C until Pan-VEGF and sFlt1 levels analysis.

5.13. Bevacizumab and sFlt-1 administrations

Bevacizumab (Avastin®, Genetech, South San Francisco, CA, USA) and sFlt1 (OriGene Technologies GmbH, Germany) were dissolved in physiological solution and injected intrathecally (5 μg/5 μL and 25ng/5 μL, respectively) on day 13 measuring

the pain threshold (Paw pressure test) 15, 30 min and 1, 3, 6 h after injection in oxaliplatin treated groups (on day 13 after the beginning of oxaliplatin treatment).

5.14. VEGFR agonists administration

Human recombinant VEGF165b (R&D Systems, Minneapolis, MN, USA), VEGF-E ((Orf Virus), Prospec, Israel) and PlGF-2 (R&D Systems, Minneapolis, MN, USA) were administered intrathecally (30 and 100 ng/5 μL) in naïve mice. Mechanical and thermal hypersensitivity (Paw pressure and cold plate test) were evaluated before and 30 min, 1, 3, 6 and 24 h after injection.

5.15. VEGF receptors inhibition in vivo

The scrambled siRNA or the specific VEGFR siRNA (VEGFR1-VEGFR2 siRNA, ambion life technologies, Italy) were intrathecal injected twice distance to 24h (3.3 μg/5 μL per mouse) at level of the lumbar region of the mice spinal cord. On the third day, were conducted behavioural analysis after VEGF administration. Mice were sacrificed between the fourth and fifth days after injection. Scramble controls were used.

5.16. Western blot analysis

The spinal cord was explanted in control and oxaliplatin groups of rats 24 hours after drug administration and immediately frozen with liquid nitrogen. Plasma was obtained by centrifugation from whole blood as previously described. Alternatively, the samples were obtained from confluent cell cultures of RASC or RCE after washing twice with DMEM. The frozen tissues or cells were homogenized with lysis buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100 and complete protease inhibitor (Roche, Milan, Italy). The suspensions were sonicated on ice using three high intensity 10s bursts with a cooling period of 10s each burst and centrifuged at 13.000xg for 10 minutes at 4 ° C. Protein concentrations were quantified by bicinchoninic acid test. Fifty μg of tissue homogenate, 20 μg of plasma or 40 μg of cell culture homogenate were resolved with prefabricated polyacrylamide gel (BOLT 4-12% Bis-Tris Plus gel; Thermo Fisher Scientific, Italy) before electrophoretic transfer to nitrocellulose membranes (Bio-Rad, Milan, Italy). The membranes were blocked with 1% BSA and 5% fat-free powdered milk in PBS containing 0.1% Tween

20 (PBST) and then probed overnight at 4 ° C with specific primary antibody compared to VEGF165b (1: 500; 22 kDa; Merck Millipore, Italy), VEGF-R1 (1: 1000; 180 kDa, Abcam, Italy), Pan-VEGF (1: 1000; 25 kDa; BD Pharmingen, Italy), VEGF-R2 (1: 1000; 200 kDa; BioCell, Italy), VEGFR2 (1: 1000; 150 kDa; Abcam, Italy), sFlt-1 (1: 500; 55-130 kDa; Thermo Fisher, Italy), GAPDH (1: 1000; 36 kDa, Cell Signaling Technology, Italy), α-Tubulin (1: 10000; 55 kDa; Sigma-Aldrich , Milan, Italy). The membranes were then incubated for 1 hour in PBST containing the appropriate secondary anti-goat conjugated with horseradish peroxidase (1: 5000; Sigma-Aldrich, Milan, Italy) or anti-mouse antibody (1: 2000; Santa Cruz, USA). ECL (Enhanced Chemiluminescence Pierce, Rockford, IL, USA) was used to visualize peroxidase-coated bands. Densitometric analysis was performed using the "ImageJ" analysis software (ImageJ, NIH, Bethesda, MD, USA). Ponceau stained membranes were used as loading control for plasma sample analysis. Normalization of α-tubulin or GAPDH was performed for nerve tissue samples, RASCs and RCEs. The values were reported as percentages of controls arbitrarily set at 100%.

5.17. Statistical analysis

Results were expressed as means ± S.E.M. and the analysis of variance was performed by ANOVA test and Students t- test. A Bonferroni's significant difference procedure was used as post hoc comparison. P values less than 0.05 were considered significant. Data were analysed using "Origin® 10" software.

6. Results

6.1. Cell cytocharacterization

Different sources of RASCs were evaluated by processing rat thighs, sternal and aortic fat pads. The stemness of isolated cells was assessed in terms of percentage of CD29, CD90-positive cells, as markers for mesenchymal stem cells, and CD45-, CD79α-positive cells, as markers for hematopoietic stem cells. All cell lines obtained from different anatomical fat pads exhibited the typical phenotype of mesenchymal stem cells ($CD90^+$ $CD29^+$ $CD45^-$ $CD79\alpha^-$) (Table 1). The retrosternal fat pad held a higher percentage of CD90- and CD29-positive cells (83.4% and 92.5%, respectively) than thighs (86.7% and 87.6% respectively) and aortic fat pads (59.9% and 88.7% respectively). The expression of CD45 and CD79α was lower in sternal fat-derived cells (7.02% and 7.7% respectively) than in thighs (12.3% and 12.7% respectively) and aortic fat-derived cells (11.2% and 11.8% respectively) (Table 1). Furthermore, several cell lines at different serial passages (from P1 to P4) and after thawing and culture until confluence were cytocharacterized by flow cytometry (Table 2). Rat ASCs at P2 showed the better stemness phenotype and cell cryopreservation did not change it. On bases of these results, sternal-derived RASCs at P2 were chosen to perform all experiments. Figure 11 shows the surface positive CD90/CD29 and negative CD79α/CD45 markers as indexes of mesenchymal phenotype of a representative RASCs preparation (P2). There were 91% CD90 positive cells which also expressed CD29 (Figure 11A) and 99% of the CD79α and CD45 negative cells (Figure 11B). In order to highlight mesenchymal characteristics, RASCs were cultured *in vitro* and differentiated under specific conditions to mesenchymal cell lineages such adipocytes and osteocytes (Figure 11C and D).

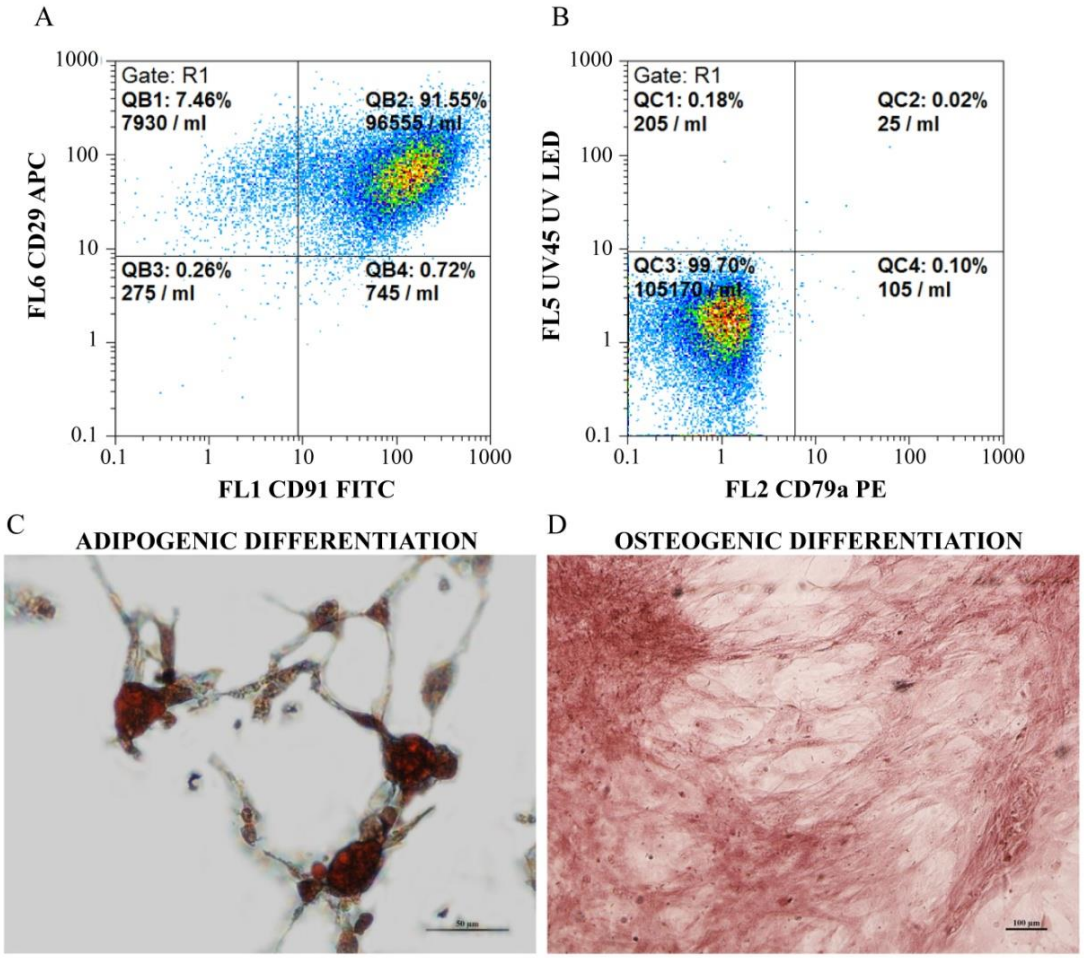


Figure 11. Characterization of isolated RASCs. *A) and B) Immunophenotypic characterization of a representative isolated P2 RASCs. Panels show the expression of RASCs surface markers characterized by means of flow cytometry with anti-CD29, CD90, CD45 and CD79a specific antibodies. C) and D) In vitro rat mesenchymal stem cell differentiation to mesodermal lineages. P2 RASCs were used. C) Adipogenic differentiation. Oil Red O staining was performed to detect lipid droplets. Scale bar: 50 µm. D) Osteogenic differentiation. Alizarin red staining was performed to detect calcium accumulation. Scale bar: 100 µm. (Di Cesare Mannelli, …, Vona et al, 2018).*

6.2. Effect of RASCs administration in oxaliplatin-treated rats

Neuropathic pain was induced in rats by repeated treatments with oxaliplatin. The anticancer drug was administered (2.4 mg kg-1 i.p.) for four consecutive days in two consecutive weeks. On day 13, the response of oxaliplatin-treated rats to a noxious mechanical stimulus (Paw pressure test) was altered and the weight tolerated on the posterior paw significantly decreased from the control value of 64.1 ± 1.6 g to 43.4 ± 0.6 g for oxaliplatin-treated rats (Figure 10). On the same day, $2x10^6$ RASCs were injected into the caudal vein of control + RASCs and oxaliplatin + RASCs groups. Rat ASCs induced a significant reduction of oxaliplatin-induced hypersensitivity 1 h after

injection with peaked at 6 h (Figure 12). The effect of RASCs lasted at least 48 h. In control rats, the caudal vein RASCs administration ($2x10^6$) did not alter pain sensitivity. Moreover, the administration of RASCs conditioned medium was ineffective in reducing pain in oxaliplatin-treated rats, suggesting that stem cells are necessary to exert the antineuropathic effect.

In order to better understand the effect of RASCs on neuropathic pain, repeated administrations of RASCs were performed (Figure 10). Di Cesare Mannelli et al demonstrated that RASCs determined a significant, comparable reduction of hypersensitivity induced by mechanical noxious (Paw pressure test) and non-noxious (Von Frey test) stimuli each time that cells were administered. This analgesic effect lasted for 5 days after injection.

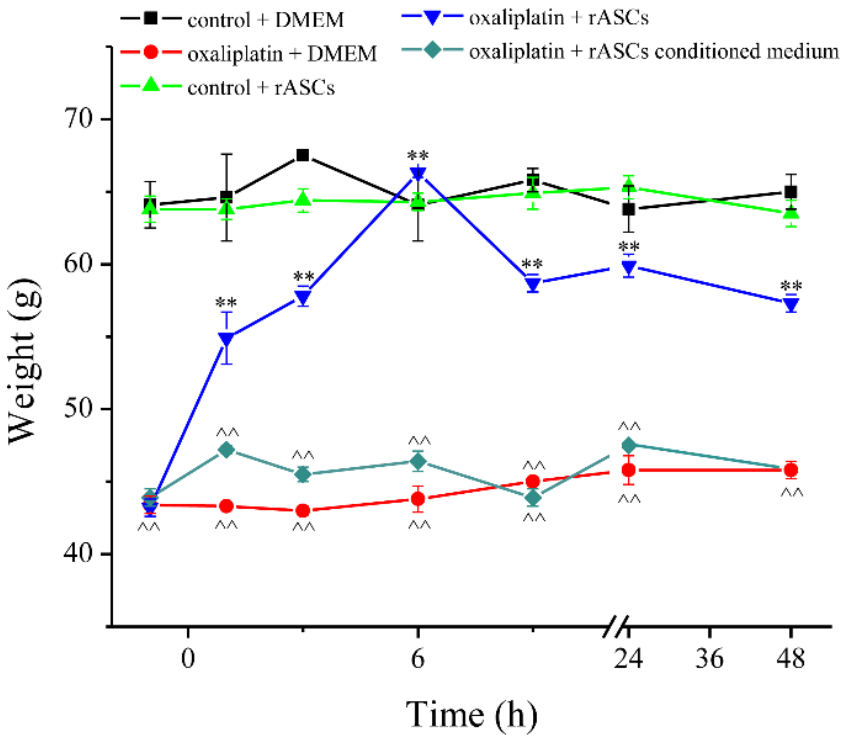


Figure 12. Pain reliever effect of RASCs administration in oxaliplatin-treated rats. *Paw pressure was used to evaluate hypersensitivity to mechanical noxious stimulus. On day 13, when neuropathy was established $2x10^6$ RASCs were i.v. injected in rats of the control + RASCs and oxaliplatin + RASCs groups. Each value represents the mean ± SEM of 10 rats per group, performed in 2 different experimental sets. ^^P<0.01 vs control + DMEM treated animals; **P<0.01 vs oxaliplatin + DMEM treated animals. (Di Cesare Mannelli, ..., Vona et al, 2018).*

6.3. Cytokines and growth factors measurement

To evaluate the biomolecular response evoked by RASCs, plasmatic time course concentrations of IL-1α, IL-6, IP-10, TNF-α, Pan-VEGFA, EGF, α-MSH, β-

Endorphin, Substance P, TGF-β1 and NGF were assayed. Pan VEGF-A significantly increased in oxaliplatin-treated rats compared to control animals. After RASCs administration in oxaliplatin-treated rats, VEGF concentrations decreased in a significant way, while administration of RASCs to control animals did not influence VEGF concentrations (Figure 13). TGF-β1 and EGF concentrations were not altered in any group compared to control (data not shown). No other analysed peptides were detectable in the plasma.

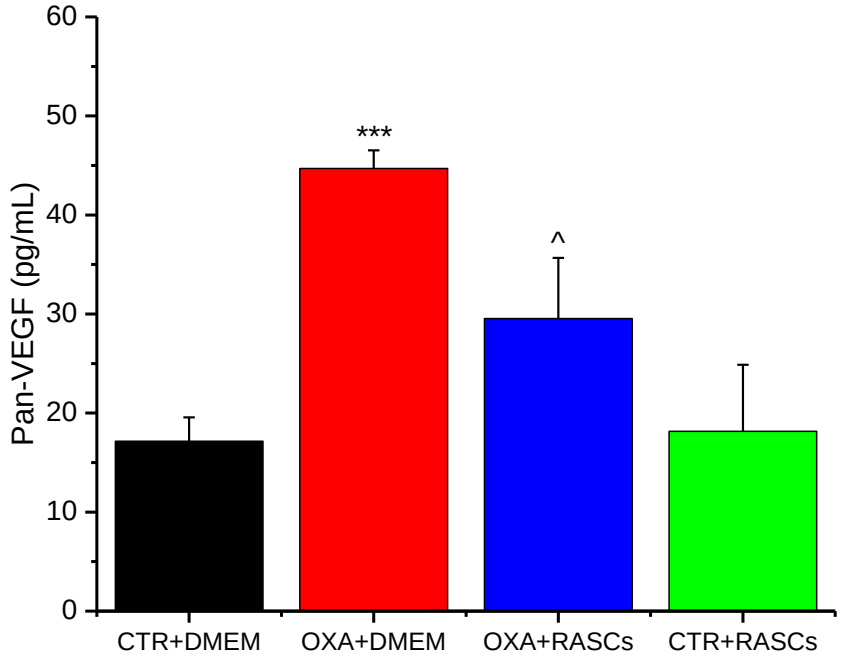


Figure 13. Pan-VEGF plasma concentration. *On day 13, when neuropathy was established, 2x10^6 RASCs were i.v. injected in rats of the control + RASCs and oxaliplatin + RASCs groups. Before and 3, 6, 9, 24 and 48 hours after i.v. cell administration, blood was collected, plasma separated by centrifugation and VEGF was measured by Multiplex kit. Each value represents the mean ± SEM of 4 rats per group, performed in 2 different experimental sets. ^P<0.05 vs OXA+DMEM and ***P<0.001 vs CTR + DMEM. Di Cesare Mannelli, …, Vona et al, 2018).*

These data suggest an involvement of Pan-VEGF level modulation in oxaliplatin-induced neuropathic pain.

6.4. VEGF-R1 and VEGF-R2 expression in RASCs

Since Pan-VEGF increased in OXA treated rats and RASCs administration decreased it, we studied the expression of VEGF receptors in *"in vitro"* RASCs. The study of the relevance of Pan-VEGF modulation in RASCs activity begun by the evaluation of

the protein expression of the VEGF specific receptors VEGF-R1 and VEGF-R2. As shown in Figure 14, RASCs expressed VEGF-R1 in high quantities (comparable to that detected in rat coronary endothelial cells, data not shown), while VEGF-R2 receptor was slightly expressed.

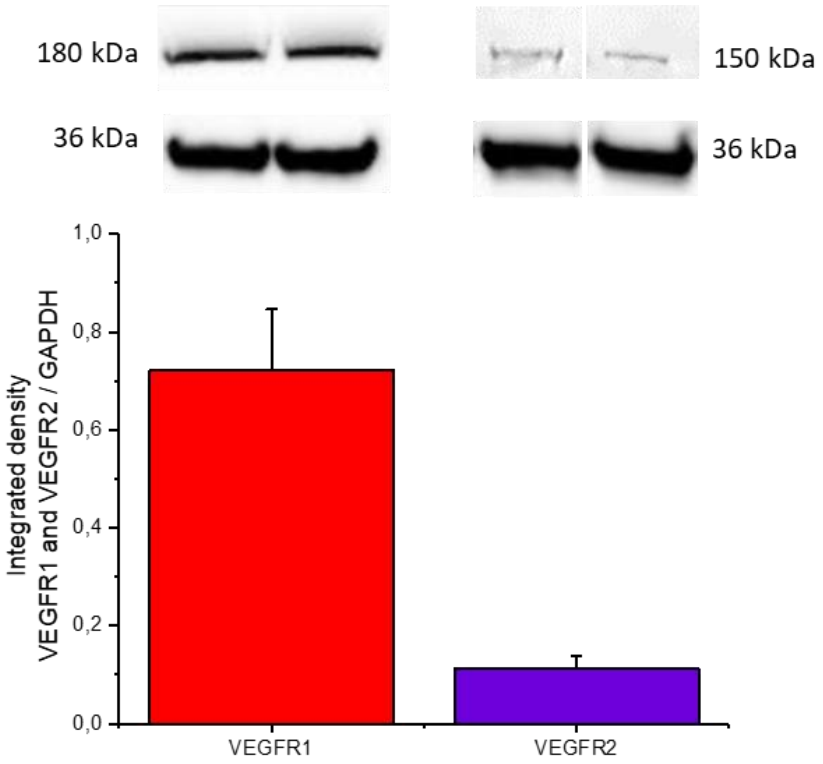


Figure 14. VEGF-R1 and VEGF-R2 expression in RASCs. *Protein fractions (40 µg) from rat adipose stem cells (RASCs) were analyzed by western blot to evaluate VEGF-R1and VEGF-R2 level (n=5). Representative blots are shown, as well as their respective densitometric analysis. GAPDH normalization was performed. The validity of the VEGF receptor antibody was tested on RCE.*

6.5. VEGF stimulation induced a Ca^{2+} rise in RASCs

To characterized the intracellular signaling of VEGF receptor stimulation we measured intracellular calcium dynamic in RASCs.

Intracellular calcium dynamic was measured using FURA-2 imaging analysis.

As shown in figure 15, VEGF165b (100ng/ml) induced a small increase in intracellular calcium in RASCs. As comparison the signal obtain in RCEs is also reported. The rise of the intracellular calcium induce by VEGF was somehow slow and not synchronized in all analyzed cells in both cell types and showed an oscillatory trend. Between stimulus and onset of the first Ca2+ peak we observed variability in the delay time of

the calcium rise. The profile of the calcium increase was similar in RASCs and RCEs. Even if, as shown, in RASCs the calcium signaling was smaller than in endothelial cells. Responsive RASCs were almost at the culture serial passage P2 (40%). RCEs responders cell were 41%.

In same experiments, we used ATP as positive control of calcium rise in both cell types, since purinergic ATP receptors are well described in endothelial cells and mesenchymal stem cells. On the contrary of VEGF, ATP induced a massive calcium signal in both cell lines characterized by oscillations and a steeply, rapid rise (Figure 16). The maximum of the calcium increase was synchronized in RCEs (Tmax from 12,5 to 15,7 s), while in RASCs the time to the maximum was observed after different intervals from agonist administration (from 3,3 to 21 s P2-P4). Moreover, ATP was very effective in RASCs at P2-P3 (76%- 81% respectively) whereas P1 cells were less responders (8%, Tmax 55-59 s) and the number of responders cells decreased at P4 (14%).

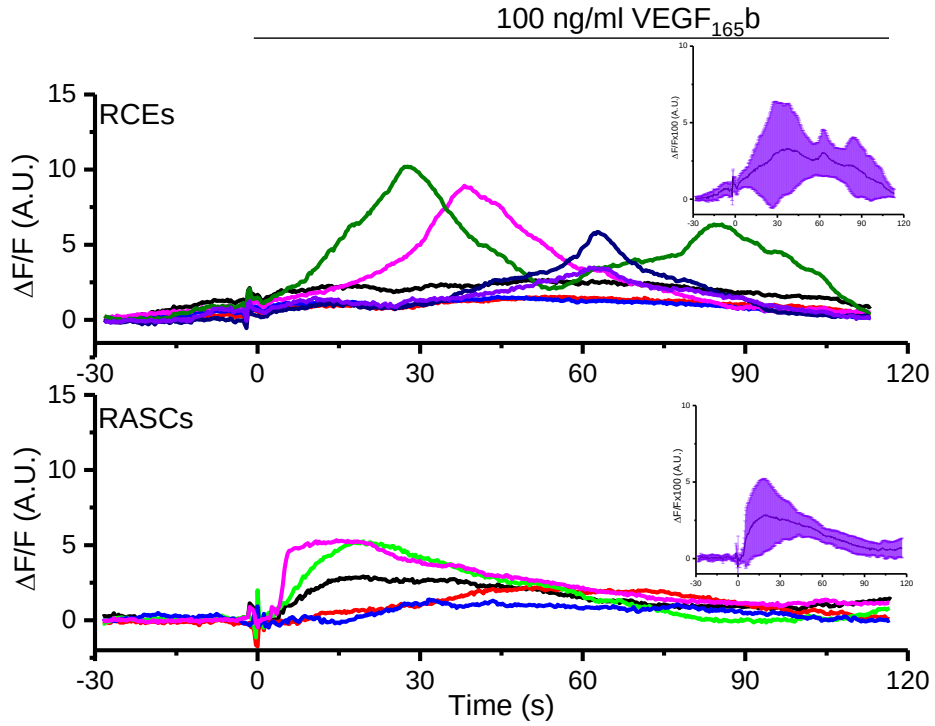


Figure 15. VEGF165b stimulation induces a Ca^{2+} rise in RCEs (A) and RASCs(B).
Typical time courses of Ca^{2+} increase induced in RCEs and RASCs (P3). Each trace is the time course of a single "responder"cell. Inserts show the mean ± s.e.m. of responder cells.

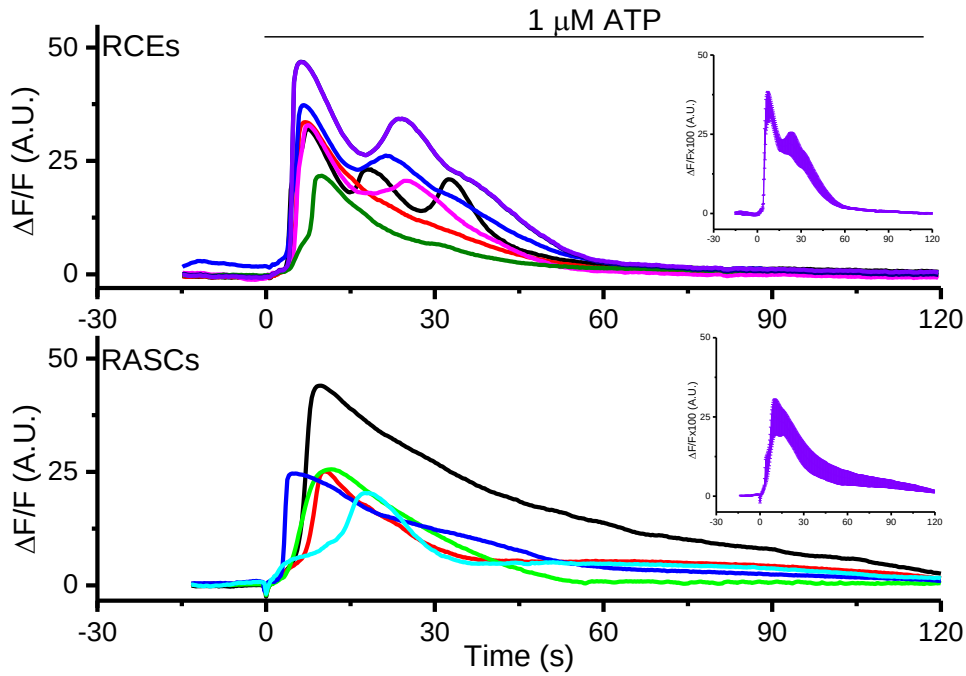


Figure 16. ATP stimulation induces a Ca^{2+} rise in RCEs (A) and RASCs(B). *Typical time courses of Ca^{2+} increase induced in RCEs and RASCs (P3). Each trace is the time course of a single "responder"cell. Inserts show the mean ± s.e.m. of responder cells.*

Furthermore, to investigate the role of both VEGF receptors in calcium signalling, we performed experiments using VEGF-E (agonist VEGF-R2) and PlGF-2 (agonist VEGF-R1). As shown in figure 17, calcium signalling is almost dependent on the stimulation of R2 receptor. In fact, the calcium signal induced by VEGF-E (100ng/ml) was rapid and observed in 45% of RASCs (P2-P3).

PlGF-2 (100ng/ml) signal on the contrary was at the limit of detectability.

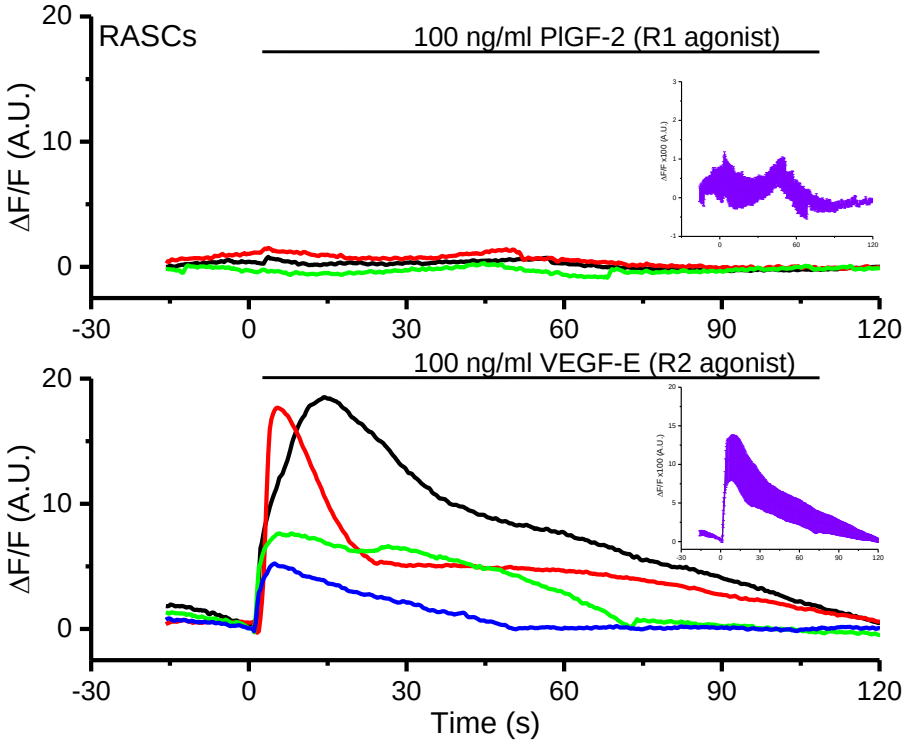


Figure 17. VEGF-E and PlGF-2 stimulation induces a Ca^{2+} rise in RASCs.
Typical time courses of Ca^{2+} increase induced in RASCs (P3). Each trace is the time course of a single "responder"cell. Inserts show the mean ± s.e.m. of responder cells.

6.6. Evaluation of sFlt-1 content in cell cultures of RASCs or RCEs

A mechanism of dumping VEGF activity is the autocrine production of sFlt-1. sFlt-1 is the extracellular fragment of R1 and an endogenous neutralizing ligand of the agonist. Therefore, we performed experiments aimed to investigate if VEGF activated RASCs can produce sFlt-1 *"in vitro"*. As shown, in control condition sFlt-1 was present in RASCs cytosol. One hour after VEGF165b administration (1ng/ml), sFlt-1 level significantly decreased in RASCs, while it was restored after 24h from VEGF administration (Figure 18). In RCEs (used as positive control), only the band at 55 kDa of sFlt-1 was significantly reduced (Figure 19).

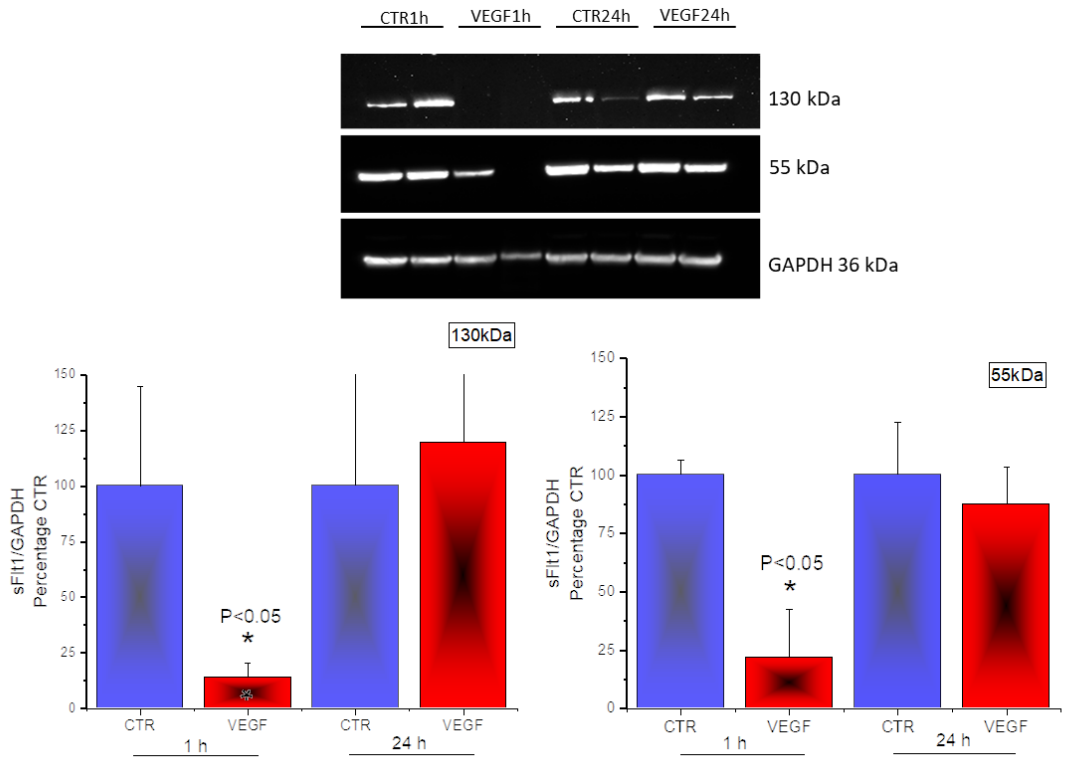


***Figure 18**. **sFlt-1 expression in RASCs homogenates in control condition and after stimulation with 1ng/ml of VEGF165b**. Both 55 kDa and 130 kDa are quantified. sFlt-1 was quantified by western blot analysis. Forty µg of proteins were blotted and revealed by specific monoclonal antibodies. Densitometric levels of the recognized bands were calibrated on GAPDH normalization. Representative Western blot are shown, as well as their respective densitometric analysis. *P<0.05 vs CTR Students t- test for unpaired data.*

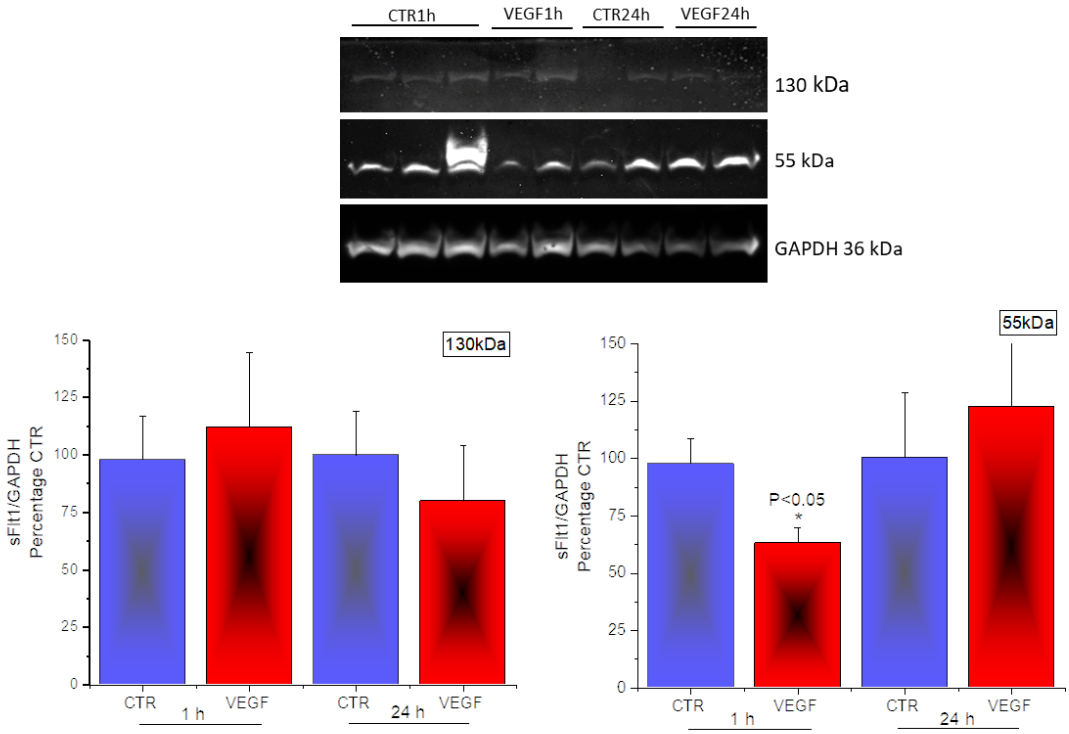


***Figure 19**. **sFlt-1 expression in RCEs homogenates in control condition and after stimulation with 1ng/ml of VEGF165b.** Both 55 kDa and 130 kDa are quantified. sFlt-1 was quantified by western blot analysis. Forty µg of proteins were blotted and revealed by specific monoclonal antibodies. Densitometric levels of the recognized bands were calibrated on GAPDH normalization. Representative Western blot are shown, as well as their respective densitometric analysis. *P<0.05 vs CTR Students t- test for unpaired data.*

In the same experiments the effect of VEGF stimulation on R1 expression was quantified in RASCs and RCEs. As shown in figure 20, in RASCs the VEGF-R1 type was well expressed in control condition. One hour after VEGF165b administration (1ng/ml), VEGF-R1 level decreased in RASCs. In RCEs the VEGF-R1 was slightly reduced. After 24h from VEGF165b administration, receptor expression was totally restored.

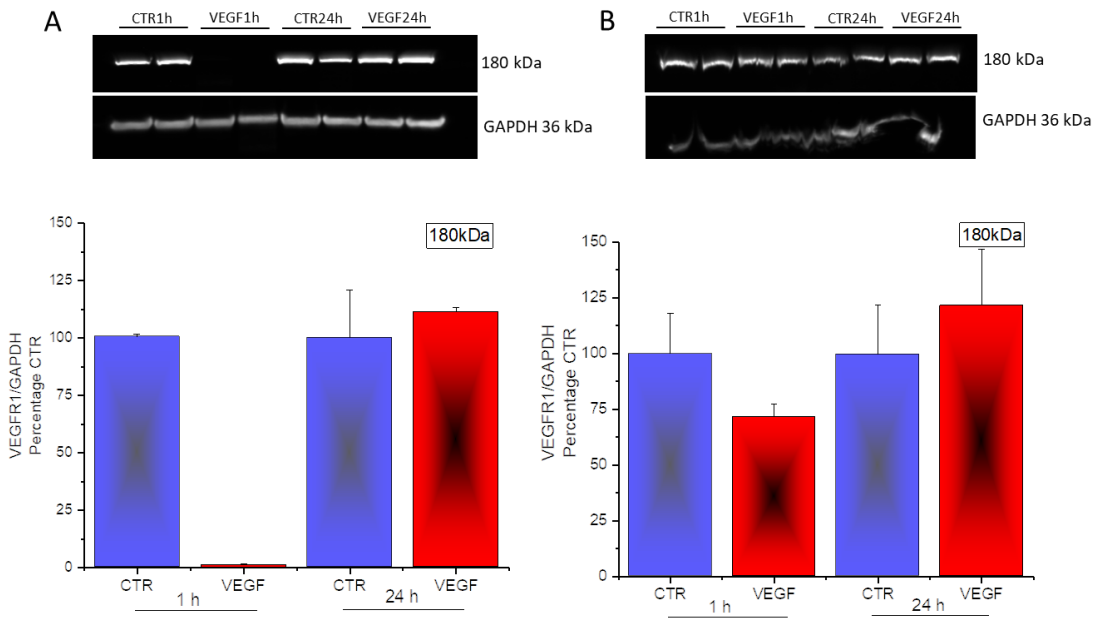


Figure 20. VEGF-R1 expression in RASCs (A) and RCEs (B) homogenates in control condition and after stimulation with 1ng/ml of VEGF165b. *VEGF-R1 was quantified by western blot analysis. Forty µg of proteins were blotted and revealed by specific monoclonal antibodies. Densitometric levels of the recognized bands were calibrated on GAPDH normalization. Representative Western blot are shown, as well as their respective densitometric analysis.*

6.7. Evaluation of sFlt-1 and Pan-VEGF in plasma

sFlt-1 and Pan-VEGF were evaluated in plasma of control, oxaliplatin and oxaliplatin + RASCs groups 24h after cell injection by western blot analysis. In plasma of control, OXA-treated and RASCs injected OXA-treated rats, the level of sFlt-1 was very similar (Fig. 21A), while Pan-VEGF was significantly higher in OXA treated group than in control and RASCs injected OXA-treated rats (Fig. 21B).

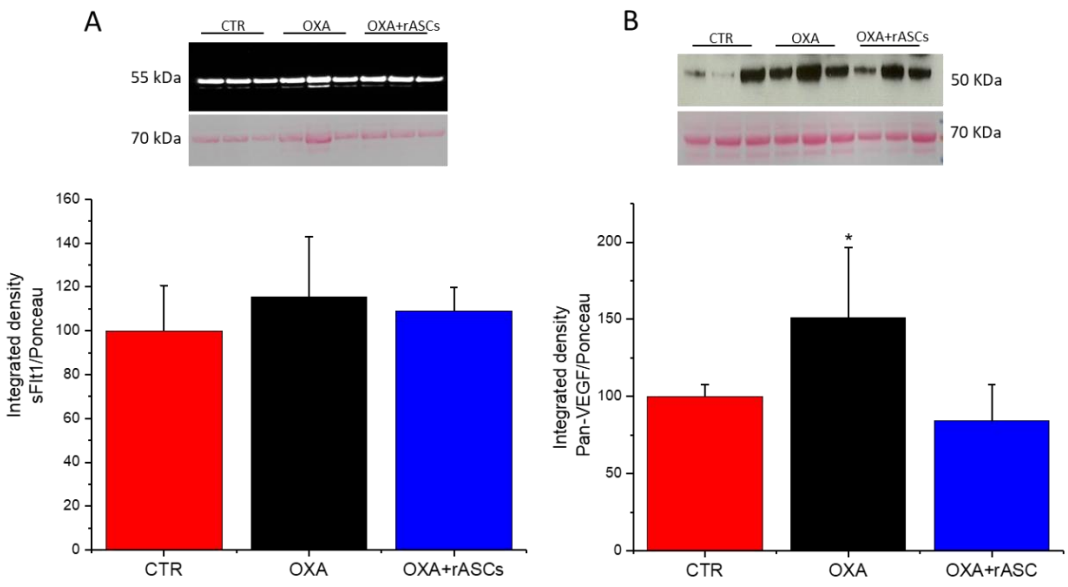


Figure 21. sFlt-1 and Pan-VEGF expression. *sFlt-1(A) and Pan-VEGF (B) expression in plasma of control, OXA-treated and RASCs injected OXA-treated rats. sFlt-1 and Pan-VEGF were quantified by western blot analysis. Forty µg of proteins were blotted and revealed by specific monoclonal antibodies. Densitometric levels of the recognized bands were calibrated on ponceau normalization. Representative Western blot are shown, as well as their respective densitometric analysis. Values are the mean ± s.e.m. of 4-6 animals. *P<0.05 vs CTR Students t- test for unpaired data.*

6.8. VEGF directly induces pain in rats

Aimed to evaluate the pro-nociceptive potential of VEGF, we injected recombinant VEGF165b, a splice variant of VEGF-A into the right paw of naïve rats (Selvaraj et al., 2015). Paw pressure test was used in order to evaluate the pain threshold induced by a mechanical insult (Di Cesare Mannelli, …, Vona, et al., 2018). As shown (Figure 22), starting from 30 min after the intraplantar administration of 10, 30 or 100 ng, VEGF165b dose-dependently reduced the weight tolerated on the paw, being the maximal pain perception reached after 3 h. Bevacizumab (15 mg kg^{-1} i.p.) fully prevented the pro-algesic effect.

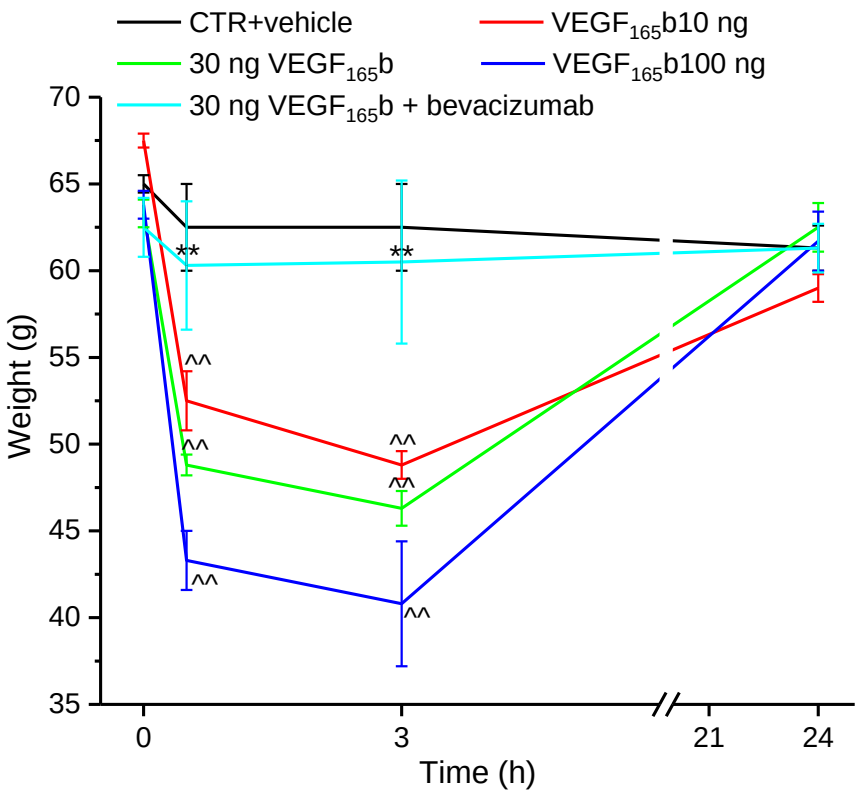


Figure 22. Effect of recombinant VEGF165b on pain threshold. Intraplantar injection. *Naïve rats were treated with 10, 30 or 100 ng of VEGF165b in comparison to vehicle (saline). A final volume of 50 µl was administered by intraplantar injection in the right paw. Bevacizumab (15 mg kg−1 i.p.) was administered 15 min before VEGF165b. The response to mechanical noxious stimulus was evaluated by the paw pressure test over time (30 min–24 h). Each value represents the mean ± SEM of 10 rats per group, performed in 2 different experimental sets. ^^P<0.01 vs control + vehicle; ** P<0.01 vs VEGF treatments. ANOVA followed by Bonferroni's test (Di Cesare Mannelli et al., 2018 modified).*

Moreover, intrathecally administered VEGF165b (30 and 100 ng) decreased pain threshold 1 – 6 h after treatment (Paw pressure test), demonstrating a pain profile of VEGF in central nervous system (Figure 23)

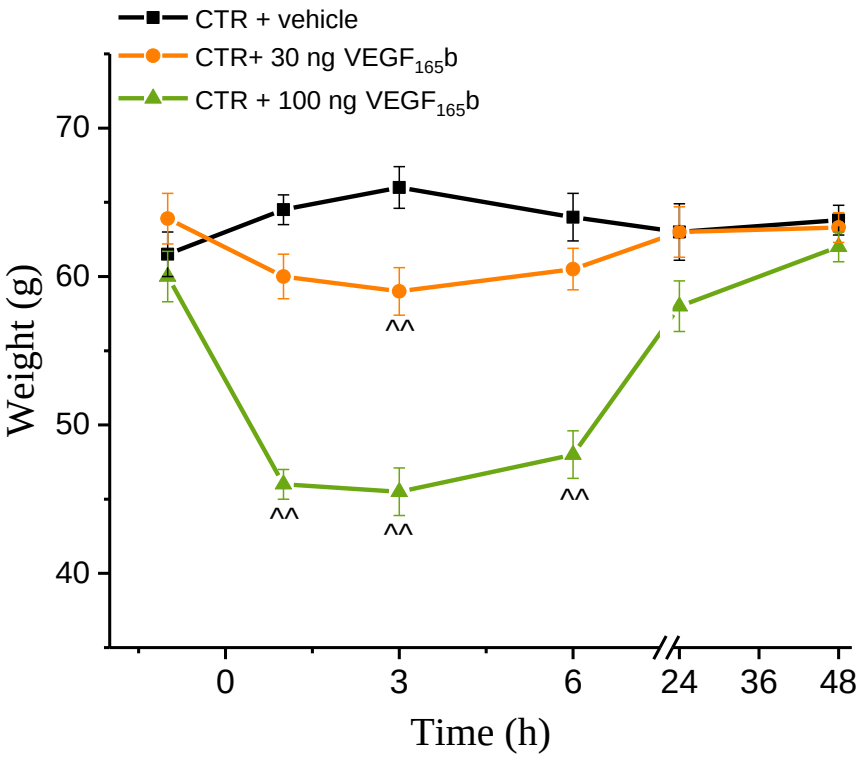


Figure 23. Central nervous system modulation of pain: dose-dependent effect of VEGF165b. *Intrathecal injection. Naïve rats were treated with 30 or 100 ng of VEGF165b in comparison to vehicle (saline). The response to a mechanical noxious stimulus was evaluated by the paw pressure test over time (1–48 h). Each value represents the mean ± SEM of 10 rats per group, performed in 2 different experimental sets. ^^P <0.01 vs control + vehicle. ANOVA followed by Bonferroni's test (Di Cesare Mannelli et al., 2018).*

In other evaluated if VEGF exerts an algogenous effect by central nervous system injection in other animal species, in parallel experiments, the painful effect of $VEGF_{165}b$ was evaluated in mice.

6.9. Modulation of pain threshold by VEGF agonists in mice

After the observation of the central role of VEGF in OXA induced pain and the painful direct effect of VEGF, we decided to investigate if VEGF agonists can mimic this behavior in mice. As shown in figure 24, the directed administration VEGF165b (100ng) by intrathecal injection (5 µl total volume) rapidly induced a long-lasting mechanical hyperalgesia. After 1h from injection withdrawal latency in paw pressure test was significantly reduced from 21 to 7 seconds. This hyperalgesia behavior lasted at least 6h.

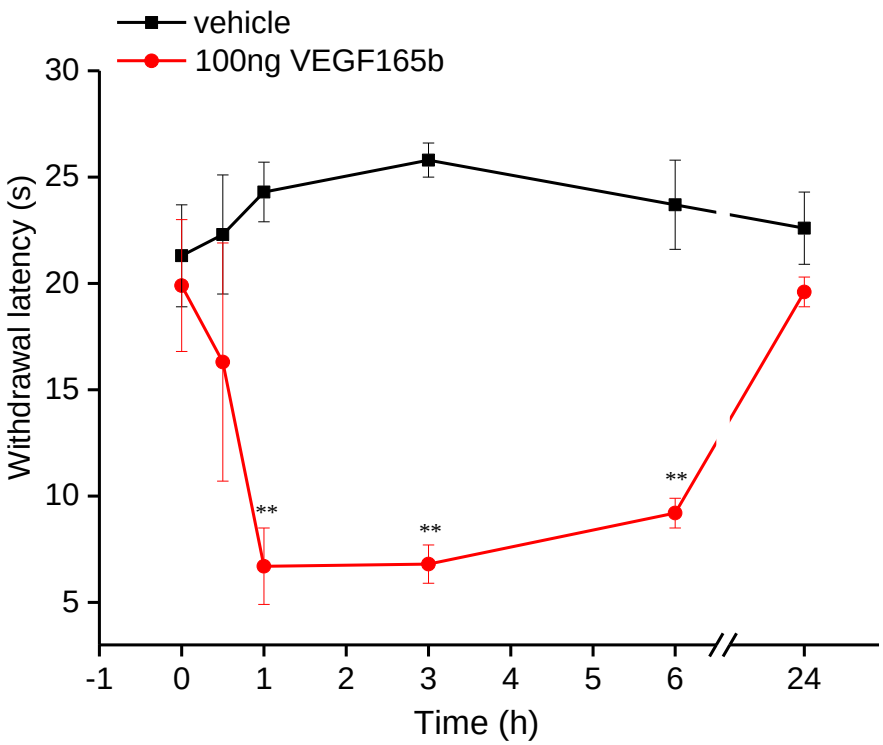


Figure 24. Effect of VEGF165b in mice. *Intrathecal administration of 100ng (5µl of total injection) induced mechanical hyperalgesia (paw pressure test). Values are the mean ± s.e.m. of 6 animals. **P<0.01 vs CTR ANOVA followed by Bonferroni's test.*

Moreover, VEGF165b (30ng/5µl, i.t.) induced cold allodynia (Figure 25). In order to investigated the role of different VEGF receptors in cold allodynia, in parallel experiment the agonists for R1 and R2 receptor were intrathecally administered. All agonists induced a significantly decrease of licking latency 30 minutes after administration. The liking latency on cold plate lasted 3h independently to the agonist used. In the case of PlGF-2 the cold allodynia was not reverted after 6h.

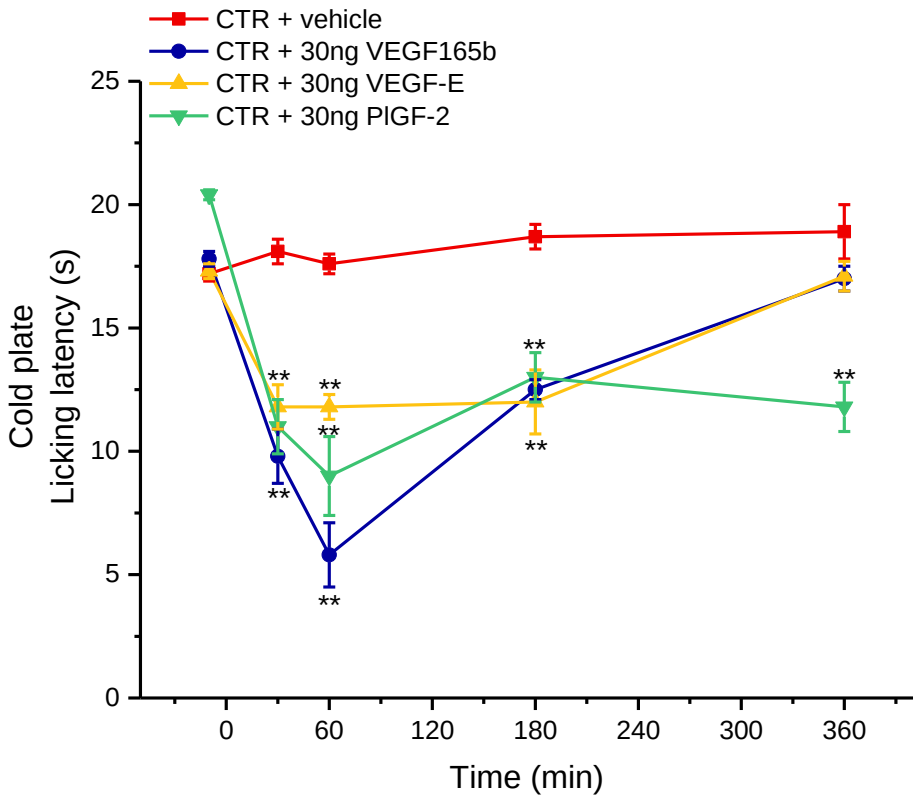


Figure 25. Effect of VEGF agonists in mice. *Intrathecal administration of 30ng (5µl of total injection) of VEGF165b (VEGF-R1-R2 agonist), VEGF-E (VEGF-R2 agonist) and PlGF-2 (VEGF-R1 agonist) induced cold allodynia. Pain-related behavior (licking of the hind paw) was observed, and the time (seconds) of the first sign was recorded. Values are the mean ± s.e.m. of 5-6 animals. **P<0.01 vs CTR. ANOVA followed by Bonferroni's test.*

6.10. Characterization of oxaliplatin induced pain in mice

Neuropathic pain was induced by repeated administrations of oxaliplatin in mice (Figure 26). The response of oxaliplatin-treated mice to a noxious mechanical stimulus (Paw pressure test) was altered and animals tolerated for a reduced time the noxious stimulus on posterior paw. Indeed, as shown in figure 26, the withdrawal latency was significantly decreased from the control mean value of 20.3 ± 0.35 seconds (control) to 6.4 ± 0.51 seconds for oxaliplatin-treated rats (oxaliplatin). These values were similar repeating the measure for several time (Figure 26A).

Moreover, OXA-treated mice developed a cold allodynia (figure 26B) and in the treated animals, licking latency was reduced from 18.1 ± 0.32 seconds (control mean value of the successive measure) by 50%.

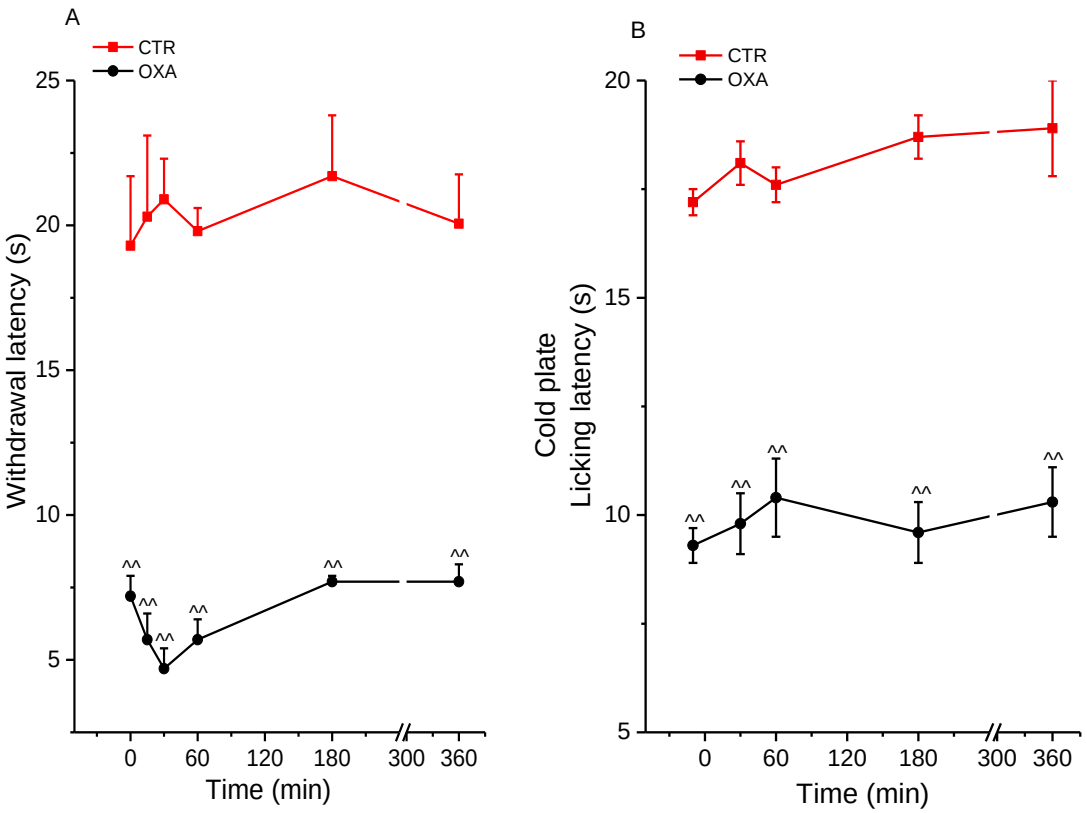


Figure 26. Effect of OXA in mice. A) mechanical hyperalgesia and B) cold allodynia. *Oxaliplatin-induced neuropathic pain in comparison to control animals. Panel A shows mechanical hyperalgesia (paw pressure test); Panel B shows cold allodynia (cold plate licking latency). The temperature of the cold plate was kept constant at 4°C ± 1°C. Pain-related behavior (licking of the hind paw) was observed, and the time (seconds) of the first sign was recorded. The results are expressed by the licking latency. Oxaliplatin (2.4 mg kg-1 i.p.) was administered for 4 consecutive days on weeks 1 and 2. Values are the mean ± s.e.m. of 8 (panel A) and 12 (panel B) animals. ^^P<0.01 vs CTR. ANOVA followed by Bonferroni's test.*

6.11. Bevacizumab and sFlt-1 effects on oxaliplatin-treated mice

According to our experimental findings in rats, the involvement of VEGF in neuropathic pain induced by OXA in mice was evaluated by the efficacy of bevacizumab and sFlt-1. Indeed, intrathecal bevacizumab and sFlt-1 administration reduced oxaliplatin-dependent hypersensitivity induced by mechanical noxious stimulus, as shown in figure 27. The beneficial effect of both VEGF-A ligands was observed soon after i.t. injection (15 min) and lasted at least 1h. To note the very low quantity of sFlt-1 active as compared to bevacizumab.

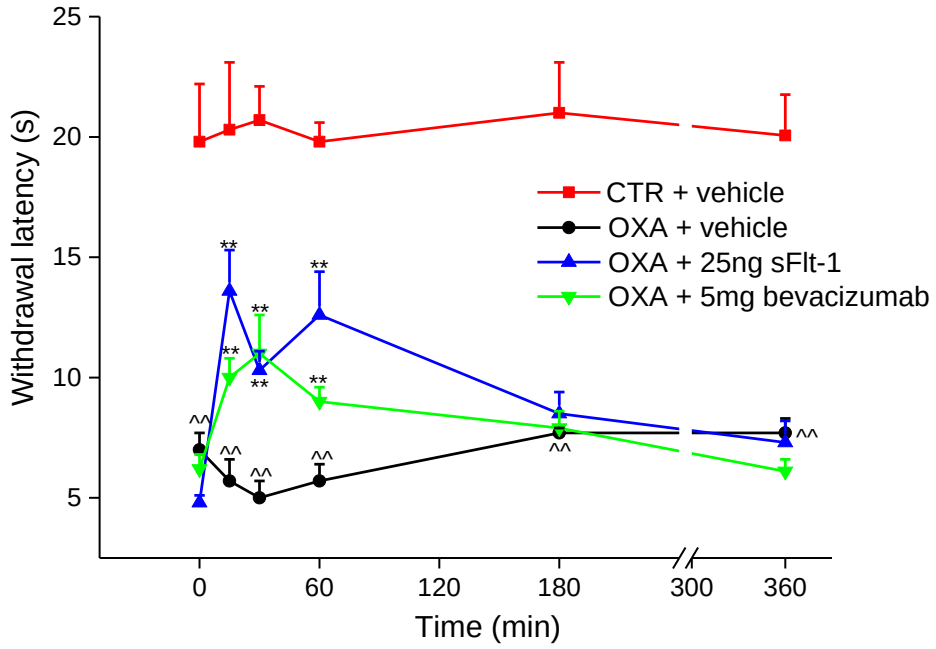


Figure 27. Bevacizumab and sFlt-1 effects in mice. Paw pressure. *The intrathecal administrations of bevacizumab and sFlt1 (5µg/5µl and 25ng/5µl, respectively), reduced mechanical hypersensitivity to starting 15 minutes after administration. Oxaliplatin (2.4 mg kg-1 i.p.) was administered for 4 consecutive days on weeks 1 and 2. Values are the mean ± s.e.m. of 10-12 animals. **P<0.01 vs OXA + vehicle; ^^P<0.01 vs CTR. ANOVA followed by Bonferroni's test.*

6.12. Evaluation of VEGF receptors and Pan-VEGF in mice spinal cord

VEGF receptors expression were evaluated in spinal cord of control and oxaliplatin animals. In spinal cord, the level of VEGF-R1 was very similar in control and OXA group (Figure 28A), while the level of VEGF-R2 was significantly increased in oxaliplatin mice group (Figure 28B).

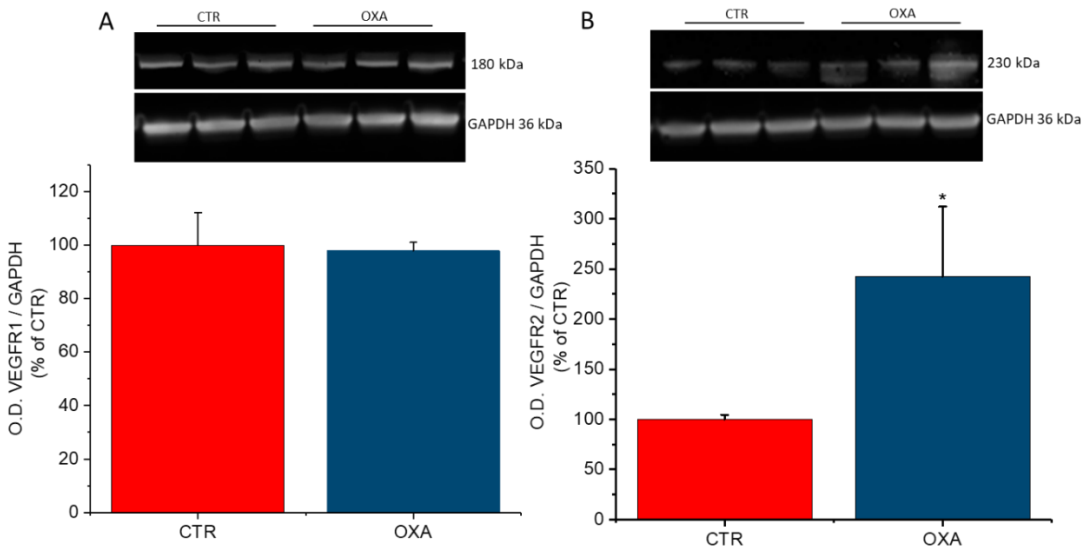


Figure 28. VEGF-R1 and VEGF-R2 expression in control and OXA mice spinal cord*. Receptors were quantified by western blot analysis. Forty µg of proteins were blotted and revealed by specific monoclonal antibodies. Densitometric levels of the recognized bands were calibrated on GAPDH normalization. Representative Western blot are shown, as well as their respective densitometric analysis. Values are the mean ± s.e.m. of 4-6 animals. *P<0.05 vs CTR Students t- test for unpaired data.*

In parallel experiment we evaluated Pan-VEGF level in spinal cord of control and oxaliplatin animals. In spinal cord, Pan-VEGF protein levels were significantly increased in OXA group as compared to control (Figure 29).

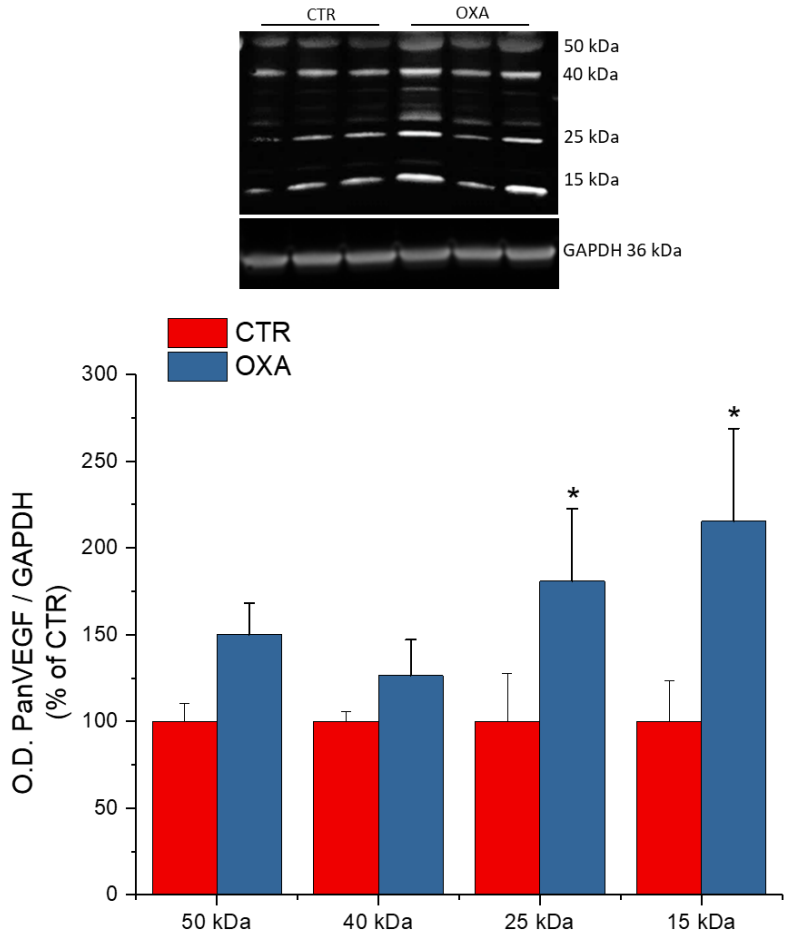


Figure 29. Pan-VEGF expression in control and OXA mice spinal cord. *Pan-VEGF was quantified by western blot analysis. Forty µg of proteins were blotted and revealed by specific monoclonal antibodies. Densitometric levels of the recognized bands were calibrated on GAPDH normalization. Representative Western blot are shown, as well as their respective densitometric analysis. Values are the mean ± s.e.m. of 4-6 animals. *P<0.05 vs CTR Students t- test for unpaired data.*

6.13. Silencing of VEGF-R1 and VEGF-R2 expression in mice spinal cord

Since specific tools for blocking VEGF-R1 and VEGF-R2 receptors are not validated in mice, we decided to investigate the relevance of VEGF-R1 and VEGF-R2 receptors in oxaliplatin induced neuropathy using silencing methodology. Therefore, experiments were started controlling siRNA R1 and siRNA R2 efficacy in reducing VEGF-R1 VEGF-R2 receptors in different parts of spinal cord. After injection of siRNA R1 or siRNA R2 at lumbar level a reduction of VEGF-R1 (Figure 30A) and VEGF-R2 (Figure 30B) was observed in the lumbar segment. Scramble RNA used as control did not significantly reduced receptor's expression. In the same western blot

Pan-VEGF was also evaluated, but no significant differences were observed (not shown).

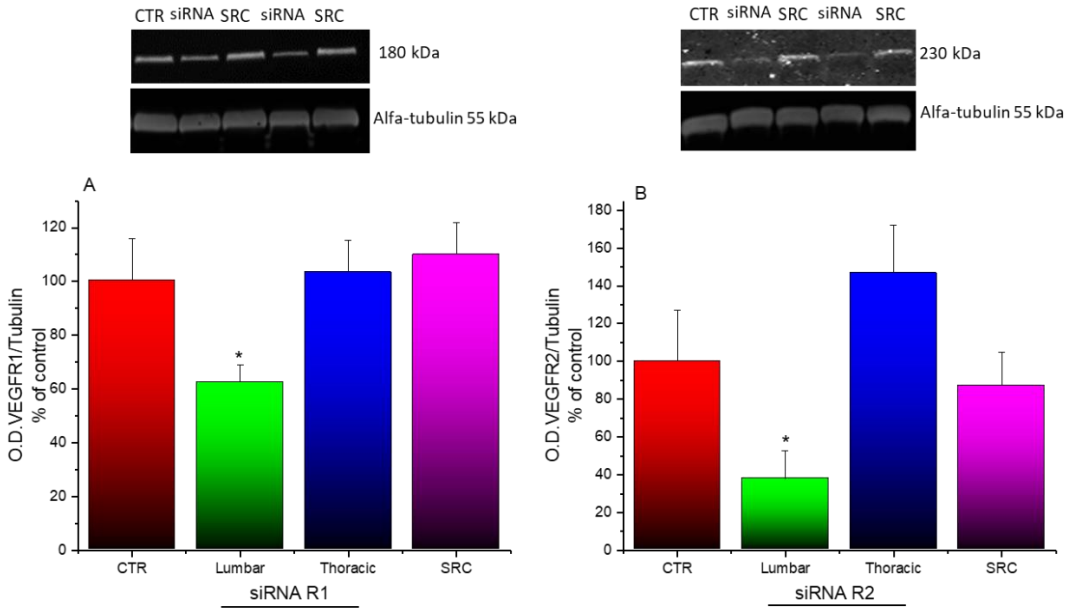


Figure 30. VEGF-R1 and VEGF-R2 expression in mice spinal cord. *VEGF-R1 (A) and VEGF-R2 (B) were quantified by western blot analysis in control mice and after administration of siRNA R1, siRNA R2 or scramble. Forty µg of proteins were blotted and revealed by specific monoclonal antibodies. Densitometric levels of the recognized bands were calibrated on alfa-tubulin normalization. Representative western blot is shown, as well as their respective densitometric analysis. Values are the mean ± s.e.m. of 4-6 animals. *P<0.05 vs CTR Students t- test for unpaired data.*

7. Discussion

Adipose-derived stem cells are a class of mesenchymal stem cells mainly isolated from the stromal vascular fraction (SVF) of the adipose tissue. For many years the interest for MSCs of scientific community was concentrated on their ability to differentiate into mesodermal (chondrocytes, adipocytes, osteocytes and myocytes) and non-mesodermal (neurons, cardiomyocytes and endothelial cells) cells (Zuk et al., 2002; De Ugarte et al., 2003; Bunnel et al., 2008). By virtue of these features, ASCs might be used in a cell-based therapy in which organ-specific tissue engineering and tissue reconstruction are the main purpose. Nevertheless, the recent scientific literature proposes an adjunctive mechanism of action for MSCs, based on their modulatory properties. Indeed, in ischemic, neurodegenerative and traumatic pain, their beneficial effect seems to be dependent on their modulatory and stimulating properties of mechanisms involved in tissue repair induced by secreted factors (Chan et al., 2014). In several anatomical localised neuropathic pain models such as chronic constriction injury (Franchi et al., 2012), spared nerve injury (Siniscalco et al., 2011) or spinal cord injury (Watanabe et al., 2015), the administration of stem cells by i.v. or spinal injection can reduce pain sensitivities with different time-courses and intensities.

Mesenchymal stem cells can be primed to express a “medicinal phenotype” (Caplan et al., 2011) following a lesion and they secrete bioactive molecules that establish a microenvironment sustaining a regeneration of injured tissues. Secreted bioactive molecules can stimulate angiogenesis (Sorrel et al., 2009), inhibit apoptosis and activate progenitor tissue-intrinsic stem cells to divide and differentiate appropriately (Rehman et al., 2004; Wagner et al., 2009). Evidence indicates that the modulation of pro- and anti-inflammatory cytokines and growth factors is an important way of explaining a possible mechanism of action of stem cells (Gonzalez-Rey et al., 2010; Sacerdote et al., 2013; Franchi et al., 2012; Siniscalco et al., 2011).

The data reported above describe the pain-relieving efficacy of RASCs in a rat model of oxaliplatin-induced neuropathy. Rat ASCs administration (both intravenously and intrathecally) reduced significantly the pain threshold alteration starting 1 h and lasting up to 4- 5 days after injection. Other i.v. administered cells type (rat vascular smooth cells) did not possess a similar anti-neuropathic effect. Repeated RASCs administrations determine pain relief with the same time-course and intensity as the first one.

As already described (Cavaletti et al., 2001; Di Cesare Mannelli, …, Vona, et al., 2018), daily oxaliplatin administrations (for 5 days per week) evoke mechanical hypersensitivity (measurements of hyperalgesia and allodynia).

On the contrary, in other models of pain characterised by severely damaged restricted areas, oxaliplatin-induced chronic neuropathy is characterised by high sensitivity of different stimuli (pressure, cold), suggesting a whole involvement of altered pain mechanisms with low inflammatory components. Therefore, the effect of RASCs seems to be dependent on the particular secretome able to respond to this OXA environment.

In our oxaliplatin model, we detected an increase in the plasmatic concentration of Pan-VEGF. Other cytokines/growth factors such as IL-1α, IL-6, TGF-β1 and EGF were not altered. Cytokines are locally produced by damaged, inflamed areas. Therefore, it is not surprising that in our model, cytokines are not involved.

VEGF levels could be increased in response to hypoxia induced by the reduction of red blood cell number that we found in OXA rats. The administration of bevacizumab i.t. in this model decreased pain demonstrating a pharmacodynamic relevant role of VEGF-A in oxaliplatin-induced neuropathy.

Since RASCs administration in our experiments reduced plasma Pan-VEGF concentration, the cells analgesic effect can be linked to VEGF; and VEGF by itself might be a co-responsible factor priming the stimulation of RASCs secretome in OXA environment.

The implication of VEGF in nociception has been recognised in several forms of pain even if conflicting data in literature suggest both pro- and anti-nociceptive effects for this growth factor. Verheyen and colleagues (Verheyen et al., 2012) demonstrate that neutralisation of all endogenous VEGF isoforms, or VEGF receptor antagonism, increase pain sensitivity in paclitaxel-induced neuropathy. Conversely, in chronic constriction injury rats anti-VEGF receptor treatment alleviates neuropathic pain by decreasing the expression of VEGF and purinergic P2X2/3 receptors in DRG neurons (Lin et al., 2010); the VEGF increase in synovial fluid is correlated with the degree of osteoarthritis pain (Hamilton et al., 2016). VEGF-A is extensively and alternatively spliced into two families of splice variants (Woolard et al., 2004) and an opposite effect of VEGF165a (pro-nociceptive) and VEGF165b (anti-nociceptive) has been noticed in rats with saphenous nerve injury (Hulse et al., 2014) where VEGF165a is overexpressed. VEGF165b exerts anti-nociceptive and protective effects in

streptozotocin-induced diabetic rats (Hulse et al., 2015). On the other hand, both splice variants (VEGF165a and VEGF165b) can prevent cisplatin-induced sensory neurodegeneration and cell death (Vencappa et al., 2015). Selvaraj and co-author (2015) have not distinguish VEGF-A isoform in their experiments. Indeed, the plantar injection of VEGF-A dose-dependently increases hypersensitivity to von Frey mechanical and noxious heat stimuli apply to the ipsilateral paw (Selvaraj et al., 2015). Moreover, in the same research, neutralisation of VEGF and VEGF pathway reduces paw pressure pain as well as pain induced by experimental tumors.

The complexity of VEGF role in pain is also underlined by conflicting data; in particular, transplantation of neural stem cells expressing VEGF enhances functional recovery and remyelination and induces pain reduction in a rat sciatic nerve injury model (Lee et al., 2015). But to our knowledge, the main purpose of our work is to delineate a link between VEGF, RASCs and neuropathic pain.

As reported in this thesis, VEGF receptors were present in RASCs; in particular, VEGF-R1 was well expressed as compared to the level found in RCEs, while VEGF-R2 receptor was slightly expressed.

For this reason, we characterised the intracellular calcium dynamic of VEGF receptor stimulation. After VEGF165b administration RASCs showed a typical calcium dynamic particularly in RASCs early culture passages characterised by a wavy form of low intensity. A similar calcium signaling was measured in endothelial cells, even if endothelial cells demonstrated a more sustained increase.

Furthermore, to investigate the role of both VEGF receptors in calcium signaling, we performed experiments using VEGF-E (agonist VEGF-R2) and PlGF-2 (agonist VEGF-R1). Calcium signaling was almost dependent on the stimulation of VEGF-R2 receptor. In fact, the calcium signal induced by VEGF-E was rapid, PlGF-2 signal, on the contrary, was at the limit of detectability. The small increase in intracellular calcium induced by VEGF in RASCs suggests that almost VEGF-R2 receptors is actively linked to an active intracellular signaling. This is not surprising because the VEGF-R2 strongly activates intracellular signaling while the VEGF-R1 has a negative regulatory role in vascular biology (Claesson-Welsh, 2016). Nevertheless, according to current literature, VEGF-R1 is linked to pain.

Concerning our experimental setting, ATP (used as positive control) determined a strong calcium rise; purinergic ATP receptors are well described in mesenchymal stem cells and endothelial cells (Kaebisch et al., 2014). In parallel experiments, purinergic

ATP receptors able to induce calcium transient in RASCs were characterised as P2X4 and P2X7.

In literature, the autocrine production of sFlt-1 is reported as a mechanism of dumping VEGF activity. Therefore, we investigated if VEGF activated RASCs can produce and release sFlt1 "*in vitro*". One hour after VEGF165b administration, sFlt-1 level in RASCs cytosol was significantly decreased, while it was restored 24h after VEGF administration. sFlt1 is the extracellular fragment of R1. sFlt1 derives predominantly from alternative splicing of mRNA VEGF-R1 gene (Kendall et al., 1993), but also from proteolytic cleavage of full-length VEGF-R1 (Cai et al., 2006; Palmer et al., 2015).

Since sFlt-1 may derive from cleavage of the ectodomain of VEGF-R1 (potentially due to the actions of proteolytic enzymes, Palmer et al., 2015), in the same experiment the level of the R1 receptor in RASCs was also determined after VEGF165b stimulation.

One hour after VEGF165b administration, VEGF-R1 expression was decreased in RASCs. 24h after administration, receptor expression was full restored. Similarly, in RCEs VEGF-R1 stimulation induced a release of sFlt-1.

These experiments suggest that sFlt1 could be possibly released from cell or from an internal store and the cleavage of full membrane receptor. After 24h, the VEGF-R1 and sFlt-1 were re-synthesised, demonstrating that RASCs are full responsive to the growth factor.

We were unable to detect an increase of sFlt-1 in the plasma of OXA-RASCs treated rats.

However, according to our protocol, the blood was drawn 24h after cell administration. Therefore, it could be possible that the sFlt-1 released/produced from RASCs is completely destroyed at this time. Further experiments will clarify this point.

These experimental results open a new therapeutic horizon for the use of mesenchymal stem cells and anti-VEGF agents in the relieve of neuropathic pain.

In order to ascertain if VEGF plays a central painful role in other animal models (by itself or in OXA neuropathy) we continued experiments on mice.

The intrathecal administration of VEGF165b increased pain as demonstrated by the reduced tolerance to paw pressure and the effect of VEGF (i.t.) lasted at least 6h. VEGF165b (i.t.) also induced cold allodynia and this behaviour was imitated by the PlGF-2 (R1 agonist) and VEGF-E (R2 agonist) at the same dosage. Using PlGF-2, the

cold allodynia was still present after 6h, whereas VEGF-E and the aspecific agonist VEGF165b were less long-lasting even if the intensity was similar.

VEGF seems to be involved in OXA neuropathic pain in mice, since bevacizumab and sFlt1 administration reduced oxaliplatin-dependent mechanical noxious stimulus sensitization, supporting the hypothesis that the link of VEGF and OXA in the development of neuropathic pain is independent of a particular specie. sFlt1 was effective at a lower dose than bevacizumab in relieve pain according to its high affinity of VEGF.

In spinal cord, type 1 or type 2 VEGF receptors were both expressed in control condition and we detected an increased level of VEGF-R2 in OXA treated mice. Moreover, Pan-VEGF was increased in OXA group, further supporting the relevance of VEGF signaling in pain.

Since specific tools for blocking VEGF-R1 and VEGF-R2 receptors are not validated in mice, we decided to use siRNA-R1 and siRNA-R2 for better distinguishing the predominance of either receptor in OXA induced neuropathy. siRNA-R1 or siRNA-R2 injected at lumbar level specifically reduced VEGF-R1 or VEGF-R2 in the definite anatomical spinal cord segment. Future experiments will clarify the predominance of type 1 or type 2 VEGF receptors.

8. Conclusion

These data support the importance of secretome as mediator of stem cell efficacy; moreover, make MSCs very suitable for use as therapeutic agents in vivo. In particular, adipose stem cells are appropriate for autologous transplantations and they can be isolated without or with less discomfort for patients as compared to blood cell precursors.

The stem cell secretome is very multifaceted. Therefore, results presented here could not be simply extended to other pathologies and/or other types of pain.

The anti-neuropathic effect of adipose stem cells in oxaliplatin-induced neuropathic pain seems to be mainly dependent on VEGF blockade.

Even if the role of VEGF and its receptors in some pathologies has been extensively studied, their function in nociception is not fully understood yet.

Anti-VEGF therapies are used in cancer for their anti-angiogenic effects; but this approach could play a positive role in cancer pain itself or in chemotherapy induced pain.

Current studies exemplify the successful alleviation of pain through the targeting of VEGF or VEGF receptors, but some considerations have to be taken:

- The different role of splicing variants of VEGF-A in neuropathic pain must be clarified since researchers have observed anti-nociceptive or pro-nociceptive effects of the two VEGF-A splicing variants. Therefore, it is essential to understand alternative splicing mechanisms in order to facilitate specific targeting and, hence, to provide effective analgesia without decrease trophic effect of the growth factors.
- For therapeutic aims, it could be different to block the agonist (VEGF) or the intracellular signaling induced by VEGF on one of its specific receptors.
- The localisation on specific cell/structure involved in VEGF nociception as well as genetic or epigenetic factors influencing VEGF effects should be studied in order to specifically address effective therapies.

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