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The Study of the Adhesion, Growth and Differentiation of Stem Cells
for Bone and Cardiovascular Tissue Engineering

Studium adheze, růstu a diferenciací kmenových buněk pro účely
kostního a kardiovaskulárního tkáňového inženýrství

Dissertation Thesis

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Abstrakt

Úvod: Tkáňové inženýrství je nadějným a rychle se rozvíjejícím oborem regenerativní medicíny. Jsou studovány různé druhy autologních a alogenních buněk za účelem nalezení vhodného zdroje buněk využitelných k osazení nově vytvářených tkáňových konstruktů či k využití v rámci buněčných terapií.

Cíle: Práce se zaměřuje na možné využití mesenchymálních kmenových buněk, především pak kmenových buněk z tukové tkáně (ADSCs), v tkáňovém inženýrství. V rámci dizertační práce byly sledovány tři hlavní oblasti: (i) vliv faktorů přítomných během získávání tukové tkáně liposukcí na pozdější charakteristiku ADSCs, (ii) interakce ADSCs s nově připravenými biomateriály pro účely tkáňového inženýrství cév, (iii) interakce ADSCs s nově připravenými biomateriály pro účely tkáňového inženýrství kostí.

Výsledky: (i) Zjistili jsme, že oblast liposukce mírně ovlivnila výtěžek buněk, tj. množství adherovaných ADSCs. Nicméně v dalších buněčných pasážích nebyly pozorovány signifikantní rozdíly v proliferaci, viabilitě, průměru či fenotypu buněk v závislosti na oblasti liposukce či na použitém negativním tlaku během liposukce. (ii) V další studii jsme pomocí ADSCs a cévních hladkých svalových buněk (VSMCs) potvrdili vysokou biokompatibilitu nově připravených poly(L-laktidových) folií variabilně modifikovaných plasmatem, polyethylenglykolem či dextranem. Navíc, dříve optimalizovaná metoda diferenciaci ADSCs směrem k VSMCs ověřila možnost využití těchto folií osazených diferencovanými ADSCs v cévním tkáňovém inženýrství. (iii) Ve třetí studii jsme pomocí ADSCs a mesenchymálních kmenových buněk z kostní dřeně potvrdili vysokou biokompatibilitu vrstev amorfního uhlíku podobného diamantu, které byly dále pro zlepšení fyzikálně-chemických vlastností obohaceny chromem či titanem v různých koncentracích. Tyto vrstvy zároveň podpořily osteogenní diferenciaci obou typů testovaných mesenchymálních kmenových buněk.

Závěr: Zdá se, že pro budoucí využití ADSCs v tkáňovém inženýrství není limitujícím faktorem ani oblast liposukce ani negativní tlak použitý během liposukce. Potvrdili jsme, že pomocí vhodného složení kultivačního média a nově připravených biomateriálů je možné ADSCs využít pro aplikace v tkáňovém inženýrství potenciálních náhrad cév a kostí.

Klíčová slova: cévní tkáňové inženýrství, kostní tkáňové inženýrství, mesenchymální kmenové buňky, kmenové buňky z tukové tkáně, buněčná diferenciaci, vrstvy z amorfního diamantu podobného uhlíku, poly(L-kyselina mléčná)

Abstract

Introduction: Tissue engineering is a fast developing and very promising field of regenerative medicine. New sources of autologous or allogenic cells have been studied to find or to confirm suitable sources of cells for enhancing tissue-engineered constructs or for applying in cell-based therapies.

Aims: This thesis focuses on the possible use of adult mesenchymal stem cells, mainly of adipose tissue-derived stem cells (ADSCs), in tissue engineering. Three main aspects have been studied: (i) the influence of harvesting factors during liposuction on further ADSC characteristics, (ii) the interaction of ADSCs with newly fabricated biomaterials for vascular tissue engineering purposes, (iii) the interaction of ADSCs with newly developed biomaterials for bone tissue engineering purposes.

Results: (i) Harvesting site slightly influenced initial ADSC yields after isolation procedure. However, in further passages, we did not observe significant influence of the harvesting site or of negative pressure used during liposuction on ADSC characteristics (i.e. cell proliferation, viability, diameter or phenotype). (ii) In the next study, the ADSCs and vascular smooth muscle cells (VSMCs) proved suitable biocompatibility of new variably modified poly (L-lactic acid) foils (i.e. modified by plasma treatment, by polyethylene glycol or by dextran). Moreover, previously optimized differentiation of ADSCs towards VSMCs on these foils demonstrated the possibility to use ADSCs for reconstruction of the blood vessel wall. (iii) Further, ADSCs and bone marrow mesenchymal stem cells (bmMSCs) proved good biocompatibility of diamond-like carbon (DLC) layers doped with chromium or titanium in various concentrations. Moreover, successful osteogenic differentiation of the cells was obtained on these layers.

Conclusion: For the further use of ADSCs in tissue engineering, it seems that neither the harvesting site nor the negative pressure is a key determinant factor. With appropriate composition of culture media and newly developed biomaterials, we proved that the use of ADSCs for vascular and bone tissue engineering applications is possible.

Key words: vascular tissue engineering, bone tissue engineering, mesenchymal stem cells, adipose tissue-derived stem cells, cell differentiation, diamond-like carbon layers, poly (L-lactic acid)

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Abbreviations

AA	ascorbic acid
ADSCs	adipose tissue-derived stem cells
ALP	alkaline phosphatase
α SMA	α smooth muscle actin
B2M	β 2 microglobulin
BDNF	brain-derived neurotrophic factor
BGCP	β -glycerophosphate
BMI	body mass index
bmMSCs	bone marrow mesenchymal stem cells
BMP	bone morphogenic protein
BSA	bovine serum albumin
CAL	cell-assisted lipotransfer
CD	cluster of differentiation
CFU	colony-forming unit
Cr	chromium
CSF	colony-stimulating factor
Dex	dextran
DLC	diamond-like carbon
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DT	doubling time
DXM	dexamethasone
ECM	extracellular matrix
ECs	endothelial cells
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGM2	endothelial cell growth medium 2
ESCs	embryonic stem cells
EVs	extracellular vesicles
FBS	foetal bovine serum
FGF1	fibroblast growth factor 1, acidic fibroblast growth factor
FGF2	fibroblast growth factor 2, basic fibroblast growth factor

FSCs	fetal stem cells
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GDNF	glial-derived neurotrophic factor
HGF	hepatocyte growth factor
HSCs	hematopoietic stem cells
IGF	insulin-like growth factor
IL	interleukin
iPSCs	induced pluripotent stem cells
IQ range	interquartile range
MSCs	mesenchymal stem cells
NGF	nerve growth factor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PLLA	poly (L-lactic acid)
PLLA240	poly (L-lactic acid) treated by plasma for 240 s
PS	tissue culture polystyrene
PEG	polyethylene glycol
RANKL	receptor activator of NF- κ B ligand
RNA	ribonucleic acid
RUNX2	runt-related transcription factor 2
Saos-2	sarcoma osteogenic cell line
SD	standard deviation
SEM	standard error of mean
SMGM2	smooth muscle cell growth medium
SM-MHC	smooth muscle myosin heavy chain
SVF	stromal vascular fraction
Ta	tantalum
TGF	transforming growth factor
Ti	titanium
VEGF	vascular endothelial growth factor
VSMCs	vascular smooth muscle cells

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1. Introduction

The current lifestyle mainly in developed countries is associated with so-called diseases of civilization. These diseases include a large group of cardiovascular diseases, metabolic diseases, rheumatic and musculoskeletal diseases, tumor diseases, autoimmune diseases, and psychiatric disorders. Nowadays, the life expectancy is increasing, but many patients suffer from various age-related and degenerative diseases that are also influenced by current lifestyle habits (i.e. diet, exercise, smoking, alcohol, and mental stress). Age-related and degenerative diseases can be mitigated and sometimes also prevented by changing the patient's habits, but pharmacotherapy often becomes necessary. However, in situations, where the pharmacotherapy is not sufficient, and severe complications occur, tissue replacement can be essential.

In clinical practise, tissue replacements are realized with biological grafts or with artificial engineered grafts. Biological grafts can be of autologous, allogenic or xenogeneic origin. They are still considered as the “golden standard” for tissue replacements because they usually possess superior biocompatibility and biomechanical properties. However, allogenic and xenogeneic grafts can trigger undesirable host immune response that can decrease the successful rate of tissue engraftment. Biological grafts are usually not available in sufficient quantities. Moreover, harvesting autologous tissue can cause higher morbidity of the patient. Artificial engineered grafts are composed of natural or synthetic biomaterials. Depending on the replaced tissue, the material should mimic specific biomechanical properties of an original tissue. Another advantage of some of these materials can be their variable porosity mimicking extracellular matrix and their possible gradual biodegradability. The artificial engineered grafts can be alternatively enriched with cellular components. Acellular artificial grafts are still widely used; however, new approaches dealing with a use of patient's autologous cells can accelerate the implant integration into human body, since there is a lower immune response of the body. Similarly, patient's autologous cells can be used for seeding of biological grafts that previously underwent decellularization, i.e. the process of removing original cells.

Patient's autologous cells for graft seeding can be of different origin (i.e. stem cells or specialized mature cells). Specialized mature cells are usually highly tissue-specific elements. Nevertheless, for regenerative medicine purposes, they are usually not available in large quantities and their proliferation capacity under *in vitro* conditions is often

limited. Tissue engineering therefore focuses on the potential use of various stem cell types. Depending on their specific origin, stem cells are undifferentiated or partially differentiated cells with high proliferation capacity. They can be differentiated depending on *in vitro* culture conditions. Adult mesenchymal stem cells seem to be a promising source for various tissue replacements as they can be quite easily harvested in large quantities, they can be used for autologous application and they are loaded with less ethical issues than other stem cell types.

This work is focused on the use of adult mesenchymal stem cells in tissue engineering. Adipose tissue-derived stem cells (ADSCs) are studied as one of the most promising sources of adult mesenchymal stem cells. Firstly, ADSCs are characterized with a respect to harvesting techniques during liposuction procedure. Secondly, ADSCs are studied concerning their potential use in soft tissue engineering, i.e. mainly in cardiovascular tissue engineering. Thirdly, ADSCs and bone marrow mesenchymal stem cells (bmMSCs) are studied concerning their potential application in hard tissue engineering, i.e. mainly in bone tissue engineering. In the performed experiments, the ADSCs are compared with mature cells specific to those tissue types.

The following chapters summarizing a current state of the art are mainly based on the published review article written by Trávníčková and Bačáková (2018). The chapters have been updated and variably extended or shortened.

1.1. Stem cells

Stem cells are generally defined as clonogenic cells capable of both self-renewal and (multi)lineage differentiation into phenotypically mature cells (Weissman 2000). Although most of the stem cells meet the criteria for multilineage differentiation, this feature is not mandatory and depends on a degree of “potency” (Visvader and Clevers 2016).

The degree of “potency” of stem cells, i.e. the range of differentiation options for generating different cell types, is used to classify stem cells as follows: (i) totipotent cells, (ii) pluripotent cells, (iii) multipotent cells, (iv) oligopotent cells, and (v) unipotent cells (for a review, see Bissels *et al.* 2016, Bačáková *et al.* 2018c) (**Fig. 1**). Totipotent cells are capable of generating all cells, including extraembryonic cell types (e.g. placental cells). The only totipotent cells are zygote and the cells within the first couple of cell divisions after zygote formation. Pluripotent cells are able to generate all body cells, including

germ cells. These cells include embryonic stem cells derived from morula and blastocyst, and also induced pluripotent stem cells prepared artificially by reprogramming somatic cells. Multipotent cells are capable of generating all or at least some tissue cell types and their origin is either from endodermal, mesodermal or ectodermal line (e.g. hematopoietic stem cells, mesenchymal stem cells). Oligopotent stem cells are some of the progenitor cells having the ability to differentiate into a few cell types (e.g. lymphoid or myeloid progenitor cells). Unipotent cells are able to generate only a single cell type (e.g. spermatogonial stem cells, adult muscle (satellite) stem cells, or basal cells in epidermis) (Bissels *et al.* 2016, Bačáková *et al.* 2018c).

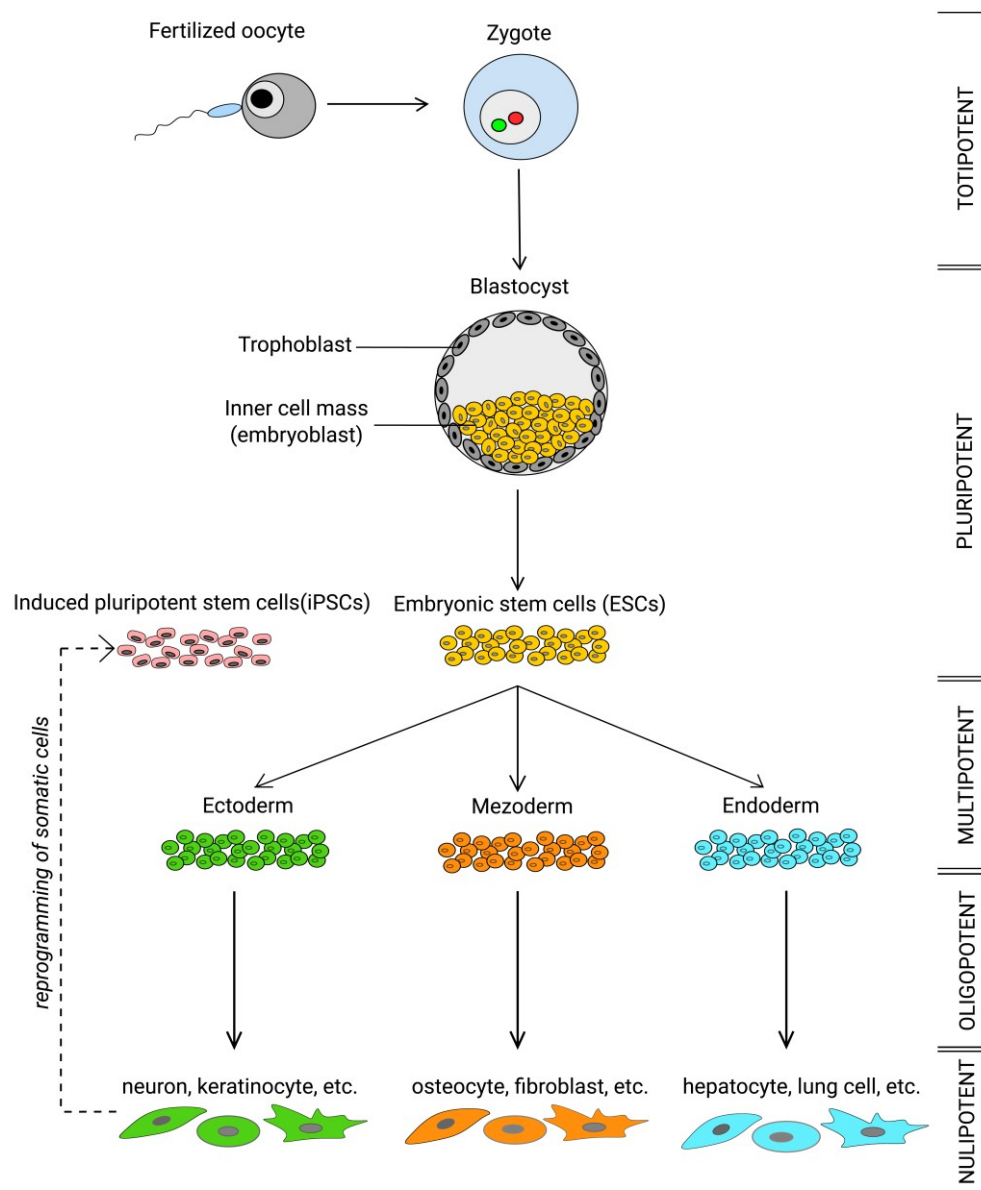


Fig. 1. The hierarchy of stem cells with a respect to their origin and “potency” characteristics. ©2021 Martina Travníková

Besides categorizing them by the degree of “potency”, stem cells can be classified according to their source. In recent years, various studies have suggested that stem cells can be obtained in different quantities and qualities from all parts of various kinds of tissue. Four main groups of sources can be identified: (i) embryonic stem cells, (ii) fetal stem cells, (iii) adult stem cells, and (iv) induced pluripotent stem cells (**Table 1**). Moreover, depending on specific tissue source, the stem cells can be present either in quiescent (dormant) state or in actively dividing state (Visvader and Clevers 2016).

Table 1. *Classification of stem cells according to their origin and their source (adapted from Trávníčková and Bačáková 2018)*

Origin of stem cells	Source of stem cells
Embryonic stem cells	morula, blastocyst (Gaskell <i>et al.</i> 2016)
Fetal stem cells	fetal tissue (O'Donoghue and Fisk 2004), placenta (Igura <i>et al.</i> 2004), amniotic fluid (In't Anker <i>et al.</i> 2003), Wharton jelly (Wang <i>et al.</i> 2004), umbilical cord blood (Kern <i>et al.</i> 2006)
Adult stem cells	bone marrow (Wakitani <i>et al.</i> 2002), adipose tissue (Zuk <i>et al.</i> 2001, 2002), dental pulp (Stanko <i>et al.</i> 2018), skeletal muscle (Hinken and Billin 2018), skin (Ojeh <i>et al.</i> 2015), peripheral blood (Esposito 2018), liver (Willemse <i>et al.</i> 2017), neural tissue (Zhang <i>et al.</i> 2016), heart (Klopsch <i>et al.</i> 2017), intestine (Barisas and Stappenbeck 2018), cancer tissue (De Francesco <i>et al.</i> 2018)
Induced pluripotent stem cells	differentiated mature somatic cells (Takahashi and Yamanaka 2006)

Stem cells are a very promising source for tissue engineering, as they have higher proliferation capacity, a lower tendency to senescence, and they withstand more passages in a cell culture than mature differentiated cells (for a review, see Atala 2005). Their multilineage potential can be used in differentiation into a desired phenotype, and their paracrine secretion of various growth factors can have a positive influence on the cells around (for a review, see Gneccchi *et al.* 2008). However, there are many ethical and regulatory constraints surrounding the use of stem cells, especially totipotent and pluripotent stem cells, i.e. cells derived from human embryonic or fetal tissues.

The following sections will cover all four main groups of stem cells, with special emphasis on adult mesenchymal stem cells – adipose tissue-derived stem cells.

1.1.1. Embryonic stem cells

Embryonic stem cells (ESCs) are defined as cells derived from the inner cell mass of pre-implantation stage blastocyst, 5-7 days after fertilization (Gaskell *et al.* 2016). These cells have great potential for regenerative therapies because of their pluripotency (i.e. their ability to differentiate into a cell of any type in the human body) and their limitless proliferation capacity. Various studies have been carried out on their use in organ transplantation, in pharmacological drug delivery therapies, and in the treatment of diseases. Although the therapeutic potential of ESCs could be high, the procedure for obtaining them is difficult due to the ethical law in many countries, and the harvested cells are available only in limited quantities. Moreover, as the pluripotent potential of these cells is so high, the potency to teratoma formation also seems to be non-negligible (Ben-David and Benvenisty 2011).

1.1.2. Fetal stem cells

Fetal stem cells (FSCs) are multipotent stem cells of various origins. Among FSCs, haematopoietic and mesenchymal stem cells have been studied most intensively. These stem cells can be harvested from the fetal blood or from the fetal bone marrow. Compared to adult haematopoietic and mesenchymal stem cells, the FSCs have better intrinsic homing and engraftment, greater multipotency and lower immunogenicity (O'Donoghue and Fisk 2004). In spite of these advantages, harvesting stem cells from fetal tissues can be controversial.

In recent years, comparatively harmless new sources of fetal multipotent stem cells have been studied, namely extrafetal tissues, such as placenta, amniotic fluid, Wharton jelly, umbilical cord blood, decidua basalis, decidua parietalis (Igura *et al.* 2004, In't Anker *et al.* 2003, In't Anker *et al.* 2004, Kim *et al.* 2004, Kögler *et al.* 2004, Wang *et al.* 2004, Yang *et al.* 2004). Moreover, some of these tissues can contain subpopulations displaying pluripotency transcription factors (Bryzek *et al.* 2013, García-López *et al.* 2019). In some classifications, these cells are treated as adult stem cell sources, as the origin of the extrafetal tissues is both maternal and fetal.

Nowadays, stem cells of umbilical cord blood origin can also be banked for future allogenic or autologous cellular therapies, especially for umbilical cord blood

transplantation in hematologic disorders (for a review, see Brunstein and Wagner 2006, Ballen *et al.* 2013).

1.1.3. Adult mesenchymal stem cells

Mesenchymal stem cells (MSCs) are a heterogeneous subset of plastic-adherent fibroblast-like cells that can be obtained from various adult tissues, e.g. bone marrow, adipose tissue, peripheral blood, dental pulp, skeletal muscle, endometrium, brain, skin and others (Marquez-Curtis *et al.* 2015, Wuchter *et al.* 2016).

However, the nomenclature of MSCs is not uniform. The term “mesenchymal stem cells” was established by Caplan in 1991 (Caplan 1991). Since that time, there have arisen various names which refer to the same cells. These names – marrow stromal cells, multipotent stromal cells, mesodermal stem cells, mesenchymal stem cells, mesenchymal stromal cells, medicinal signalling cells – refer to the origin of the cells, or to their current use in a given study (Caplan 2017). As mesenchymal stem cells are nowadays mostly used to home in on the sites of injuries or diseases and secrete bioactive factors, Caplan suggested in 2017 that the term “medicinal signalling cells” should be used preferentially.

To meet the minimal criteria of International Society for Cellular Therapy for MSCs, the cells should display adherence to plastic in standard culture conditions (Dominici *et al.* 2006). Moreover, they should have following phenotypic characteristics: CD105, CD90 and CD73 ($\geq 95\%$), and they should lack CD45, CD34, CD14 or CD11b, CD79 α or CD19 and HLA-DR ($\leq 2\%$) (Dominici *et al.* 2006). According to the current definition, MSCs are multipotent cells and they have to display trilineage mesenchymal differentiation. Under *in vitro* conditions, they should be able to differentiate into following cells of mesodermal lineages, i.e. osteoblasts, chondrocytes and adipocytes (Dominici *et al.* 2006). Furthermore, their differentiation capacity is broader in *in vitro* cultures, depending on the biochemical composition of the medium, the mechanical properties of the scaffold, and the dynamic cultivation conditions.

The following sections will focus on bone marrow mesenchymal stem cells (bmMSCs) and mainly on adipose tissue-derived stem cells (ADSCs) that are two most widely studied cell types among MSCs. These cells have been tested in many *in vitro*, *in vivo*, and clinical studies on regenerative medicine and cell-based therapy as they poses some specific undermentioned advantages.

1.1.3.1. Bone marrow mesenchymal stem cells

Bone marrow mesenchymal stem cells (bmMSCs) were the first MSCs to be successfully isolated and characterized in detail. In 1970s, they were investigated as fibroblast-like cells with rapid adherence and they were distinguished from the hematopoietic stem cells in the stromal component of bone marrow tissue (Friedenstein *et al.* 1970). Since that time, they have been widely studied and used in regenerative medicine as a source of adult mesenchymal stem cells. Bone marrow is usually aspirated from the sternum or from the pelvic area as a diagnostic method for blood diseases or during a bone marrow transplantation procedure. This procedure is uncomfortable and painful for patients, and yields of isolated bmMSCs can be limited.

The bmMSCs meet the criteria for multipotent stem cells as they can be differentiated into osteoblasts, chondrocytes, and adipocytes. Moreover, based on the tissue of their origin, the bmMSCs seem to be more prone to differentiate towards these skeletal cell types rather than to other mesodermal cell types, e.g. smooth muscle cells or cardiomyocytes (Bianco *et al.* 2008). The bmMSCs are therefore studied as a source of adult mesenchymal stem cells mainly for bone and cartilage tissue engineering. There have been studies using (i) the secretome of bmMSCs for cartilage regeneration in osteoarthritic knees (Wakitani *et al.* 2002, Yamasaki *et al.* 2014) and in wound healing (Wu *et al.* 2007, Chen *et al.* 2008), or (ii) an interaction of bmMSCs with a suitable scaffolds, e.g. with silk (Meinel *et al.* 2005) or alginate (for a review, see Sun and Tan 2013) for repairing bone and cartilage.

The bmMSCs have been deeply studied and characterized, and they therefore serve as reference MSCs for all other newly isolated types of MSCs in comparative studies.

1.1.3.2. Adipose tissue-derived stem cells

Adipose-derived stem cells (ADSCs) are stem cells of adult origin that were first identified and described in 2001 as an alternative source of autologous or allogenic MSCs for tissue engineering (Zuk *et al.* 2001). In many patients, subcutaneous adipose tissue is more abundant and easily accessible than the bone marrow tissue. ADSCs can be harvested *via* liposuction under local anaesthesia. The liposuction procedure is a relatively painless and reasonably comfortable way for patients to provide enough autologous mesenchymal stem cells. Despite some differences that will be undermentioned, bmMSCs and ADSCs have a similar multilineage differentiation potential (Zuk *et al.* 2002, Strem *et al.* 2005). On the contrary, the yields of isolated stem

cells, the proliferation capacity, and colony forming units were observed to be higher for ADSCs than for bmMSCs (Kern *et al.* 2006, Mohamad-Ahmed *et al.* 2018).

In recent years, adult stem cell research has made a huge progress in the field of cell-based therapy and in regenerative medicine in general. The ADSC secretome includes active molecules such as vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF β), basic fibroblast growth factor (FGF2), granulocyte colony-stimulating factor (G-CSF), interleukin 6 and 8 (IL-6, IL-8), nerve growth factor (NGF), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), brain-derived neurotrophic factor (BDNF), and glial-derived neurotrophic factor (GDNF) (for a review, see Kapur and Katz 2013, Banyard *et al.* 2015).

These active molecules and many others can be secreted out of the ADSCs in small vesicles that are known as extracellular vesicles (EVs). Such EVs can be present in the form of microvesicles (50-1000 nm) or in the form of exosomes (30-100 nm), and their main role is to transport active molecules to target cells by paracrine signalling (for a review, see Wong *et al.* 2019). The EVs are not unique for ADSCs; however, they are very important products of stem cells. In comparison with mature differentiated cells, undifferentiated stem cells produce a very diverse mixture of proteins, lipids, mRNAs, miRNAs, and noncoding RNAs that does not have to be specific for a particular tissue. Thus, the EVs can positively influence the regeneration of various tissues, angiogenesis, inflammation states, and others. Moreover, there is an increasing interest in the EVs as they can be purified and can serve as immunologically inert particles with a low risk of tumorigenicity (for a review, see Wong *et al.* 2019).

The future of ADSCs therefore offers direct use of their secretion of trophic factors, and also their multilineage differentiation potential for regeneration of various tissues (Frese *et al.* 2016, Li *et al.* 2016). However, there still remain some barriers in clinical applications that have to be overcome, namely (i) a precisely-defined source population of ADSCs, (ii) isolation procedures according to regulatory clinical standards, (iii) better understanding of the behaviour of ADSCs in their niche and in their interaction with scaffolds, (iv) an evaluation of the safety and the effectiveness of clinical applications, and (v) long-term follow-up studies on the consequences in patients (Banyard *et al.* 2015, Li *et al.* 2016).

1.1.3.2.1. Sources, isolation procedure, and characterization of ADSCs

There are various deposits of adipose tissue from which ADSCs can be isolated. These deposits can be classified as (i) superficial subcutaneous sources and (ii) deep peri-organ (visceral) adipose deposits, e.g. mesenteric, omental, or intrathoracic deposits. These deposits are not identical and the harvested preadipocytes and ADSCs display distinct expression profiles, phenotype profiles, and differentiation capacity (Tchkonina *et al.* 2007, Russo *et al.* 2014). Due to their easier accessibility, subcutaneous sources are widely used, especially *via* the liposuction procedure. The area where liposuction is performed varies from study to study; however, the abdomen and thigh areas are particularly suitable for liposuction, as there is usually an abundant deposit of adipose tissue in these areas.

Effective approaches to isolate high yields of ADSCs are usually based on digesting the adipose tissue with collagenase solution, i.e. enzymatic isolating techniques (Bora and Majumdar 2017). However, newer non-enzymatic techniques (e.g. vibration and/or centrifugation) are studied, because they can be easier translated to clinical applications (Bora and Majumdar 2017, Raposio *et al.* 2017). The isolating techniques aim to obtain separated fractions, i.e. (i) the uppermost oily layer, (ii) the layer of (digested) adipose tissue with adipocytes, (iii) the infranatant layer composed of blood and digesting/washing solution, and (iv) the lowest layer of so-called stromal vascular fraction (SVF) (**Fig. 2**). After removing all 3 supernatant layers, SVF is obtained, and the cells can be seeded in a growth medium into culture flasks (Estes *et al.* 2010).

The SVF is a heterogeneous cell population with a variable representation of proper ADSCs, fibroblasts, smooth muscle cells, pericytes, preadipocytes, endothelial cells, endothelial progenitor cells, macrophages, and T regulatory cells (Varma *et al.* 2007, Zimmerlin *et al.* 2010, Bora and Majumdar 2017). Depending on culture conditions, by washing away non-adherent cells, and by subsequent passaging, the percentage of ADSCs in cell culture increases. Alternatively, the ADSCs can be sorted out from SVF according to specific CD surface markers.

In subsequent cell passages, it is necessary to perform phenotypical characterization of isolated cells to meet the criteria for mesenchymal origin. According to the International Society for Cellular Therapy, ADSCs in a culture retain the following surface markers: CD105, CD90, CD73, CD29, CD44, and CD13 (>80%), and they lack

CD45, CD31, CD235a (<2%) (Bourin *et al.* 2013). Detailed description of the most commonly studied CD surface markers is explained in **Table 2**.

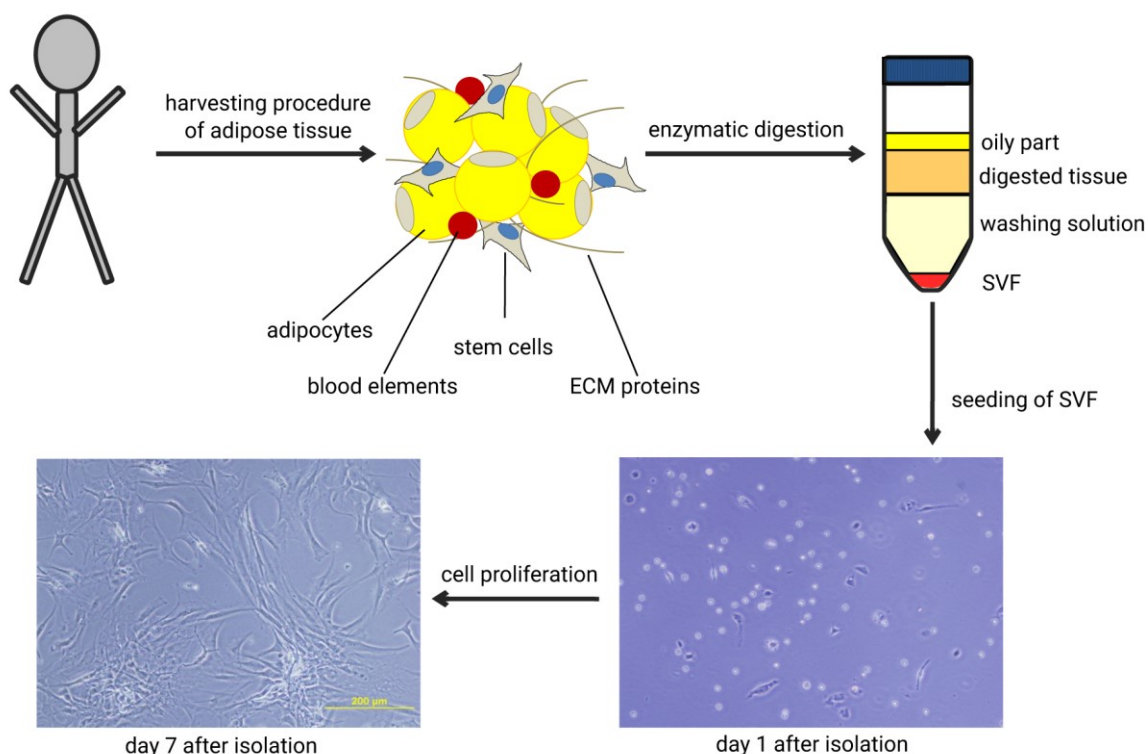


Fig. 2. A scheme of the commonly used procedure for isolation of SVF and ADSCs.

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Table 2. The description, positivity and negativity of the most commonly studied CD surface markers in adipose tissue-derived stem cells (ADSCs). ++ (> 80%), +/- (can be variably present), - (< 2%) (Bourin *et al.* 2013, Bačáková *et al.* 2018c).

Marker	Description	Positivity
CD105	endoglin, part of TGF-beta receptor complex	++
CD90	Thy-1, associated with multipotent progenitor activity	++
CD73	ecto-5'-nukleotidase, converts AMP to adenosine	++
CD44	transmembrane P-glycoprotein 1 (cell adhesion receptor)	++
CD29	integrin beta-1, fibronectin receptor (cell adhesion receptor)	++
CD146	receptor for laminin, MCAM	+/-
CD34	surface glykoprotein, hematopoetic progenitor cell antigen	+/-
CD31	platelet-endothelial cell adhesion molecule (PECAM-1)	-
CD45	leukocyte common antigen	-

Similarly, in agreement with general criteria for MSCs, it is necessary to test ADSC trilineage differentiation potential into osteoblasts, chondroblasts, and adipocytes by specific assays (Bourin *et al.* 2013). In addition, it has been advised to perform colony-forming unit (CFU) assay to calculate the population doubling capacity (Bourin *et al.* 2013).

1.1.3.2.2. Factors influencing SVF and ADSC characteristics

In recent years, as the number of studies using ADSCs in regenerative medicine has been rising, various factors influencing ADSC characteristics have been investigated and discussed. In general, these factors can be divided into (i) factors during liposuction procedure, cell isolation and cell culture and (ii) patient factors. All these variabilities can influence yields of harvested cells, their viability, phenotypic profiles, proliferation abilities, self-renewal potential, migratory capacity, and differentiation capacity. In addition, broad range of patient-to-patient variabilities in obtained results needs to be taken into account. All these influencing factors are therefore important either for direct clinical use of ADSCs or for the use of ADSCs in tissue engineering applications. So far, some of the published articles studying the influencing factors have resulted in contradictory outcomes as different methods have been applied, different number of patients has been studied or the group of patients has not been completely uniform. Moreover, there is the lack of standardization in protocols and the results can vary according to the specific measured outcomes (for a review, see Fontes *et al.* 2018).

Concerning the harvesting techniques, yields or subsequent proliferation of ADSCs are almost the same *via* liposuction and excision procedures (von Heimburg *et al.* 2004, Torio Padron *et al.* 2010). Moreover, liposuction is more comfortable for patients.

There are also factors resulting from ADSC harvesting and isolation methods that can influence the quality and the further use of isolated cells. It was investigated that different values of negative pressure during the liposuction procedure did not influence subsequent adipocyte viability, number of MSCs, or histological evaluation of lipoaspirate after *in vivo* fat grafting (Lee *et al.* 2013, Charles-de-Sá *et al.* 2015). On the contrary, other studies suggested that negative pressure can negatively influence cell characteristics. Higher yields of ADSCs were obtained under lower negative pressure (Mojallal *et al.* 2008, Chen *et al.* 2017). Moreover, the ADSCs obtained under low negative pressure proliferated faster and secreted higher levels of VEGF and FGF2 (Chen *et al.* 2017). Nevertheless, slightly different target cell fractions and populations were

studied (i.e. adipocytes, nucleated cells, SVF or ADSCs) and different anatomical harvesting sites were used in these studies. In addition to the studies on the optimal liposuction pressure, other factors (e.g. differences between liposuction regions, gender, age, BMI) need to be taken into account (for a review, see Varghese *et al.* 2017).

There are some predominantly used anatomical harvesting sites within the liposuction procedure. An abundant adipose tissue is usually located in the abdomen region, in the inner thigh region, and in the outer thigh/hip region. So far, the region of adipose tissue harvesting has been selected on the basis of actual need. Nevertheless, cells from different anatomical regions express different homeobox (Hox) genes and thus the regions suitable for ADSCs harvesting need to be carefully selected depending on specific further application (Dani *et al.* 2017). Similarly to the influence of negative pressure, results of published studies are not uniform and are dependent on specific studied parameters and techniques that were used. Adipose tissue from different donor sites can result in different yields of SVF cells (Padoin *et al.* 2008, Iyyanki *et al.* 2015, Tsekouras *et al.* 2017) or in different ADSCs frequencies in SVF (Jurgens *et al.* 2008). Moreover, the subsequent ADSCs numbers (Tsekouras *et al.* 2017) or CFU (Fraser *et al.* 2007) can differ. On contrary, the total number of nucleated cells can be similar (Jurgens *et al.* 2008). Similarly, further growth kinetics of ADSCs (Jurgens *et al.* 2008, Iwen *et al.* 2014) or cell viability (Rohrich *et al.* 2004, Tsekouras *et al.* 2017) seem to be unaffected by the donor site. Last but not least, depending on the different anatomic regions, the cells may vary in adipogenic, osteogenic and/or angiogenic differentiation potential (Levi *et al.* 2010, Iwen *et al.* 2014, Li *et al.* 2019).

The main patient factors that can vary from patient to patient (i.e. gender, age, BMI, harvest site) were summarized by Varghese and co-workers (for a review, see Varghese *et al.* 2017). They analyzed 41 papers in which some of the patient factors showed significant differences in ADSC characteristics. Namely, they found decreasing proliferation and differentiation potential of ADSCs with increasing age and BMI. Besides these main factors, there are some studies that have suggested a potential influence of radiotherapy, chemotherapy, hormonal treatment, smoking, hypertension, diabetes mellitus, or renal disease on the properties of isolated ADSCs (for a review, see Varghese *et al.* 2017). Recently, some of the other intercurrent diseases such as breast cancer (Parson *et al.* 2018), atherosclerosis (Saad *et al.* 2016, Kizilay Mancini *et al.* 2018), or scleroderma (Griffin *et al.* 2017) have also been reported to alter the secretome, differentiation capacity, migratory capacity, or DNA damage.

Except for the aforementioned factors, many studies have reported non-negligible heterogeneity of measured data among donors. No comprehensive studies have yet been performed that bring together all potentially relevant factors. However, each study provides a better understanding of ADSC behaviour, including patient variability. In addition, most of the already performed studies have been focused on all nucleated cells, adipocytes, SVF or ADSCs for their subsequent or immediate clinical use in plastic surgery and in regenerative medicine in general. However, for tissue engineering purposes, the later *in vitro* cell behaviour in subsequent passages is mainly the subject of interest. Within *in vitro* culture conditions, the ADSCs can be expanded until great numbers of cells are obtained for needs of tissue engineering purposes; however the cells could display further differences in growth kinetics, viability, differentiation capacity and/or phenotypic maturation.

1.1.3.2.3. Current and potential clinical applications of ADSCs

As mentioned previously, clinical applications of ADSCs can be divided into (i) direct use of ADSCs and their secretome, (ii) the use of separate EVs that were harvested from ADSCs, and (iii) the use of differentiated ADSCs for tissue engineering applications.

For the direct use of ADSCs, cell-assisted lipotransfer (CAL) has been used for filling and tissue augmentation purposes in plastic surgery and aesthetic medicine. CAL usually combines different ratio of freshly isolated autologous SVF or ADSCs with an injection of intact lipoaspirate (Yoshimura *et al.* 2008, Li *et al.* 2016).

As has been discussed above, ADSCs secrete a large variety of growth factors and other proteins involved in the trophic effects of ADSCs on the protection, healing, vascularization, survival, repair and differentiation of a variety of tissues (Salgado *et al.* 2010, Wong *et al.* 2019). Physiologically, wound healing is a sequence of 3 steps, i.e. (i) inflammation stage, (ii) proliferation and granularization stage, and (iii) re-epithelization stage. The MSCs are involved mainly in the inflammation stage as they are secreting growth factors and cytokines modulating the signalling cascades in guided immunomodulation (for a review, see Gaur *et al.* 2017). The potential of SVF, ADSCs-secretome, or their EVs for accelerated treatment of chronic limb defects and chronic non-healing wounds is therefore another example of the direct autologous application (Collawn *et al.* 2012, Strong *et al.* 2015, Lombardi *et al.* 2019).

ADSCs-secretome has also been reported to modulate an immune response and inflammatory signs during immune diseases (Singer and Caplan 2011). For example,

ADSCs reduced the immune response in experimental colitis (González *et al.* 2009), experimental autoimmune diabetes (Bassi *et al.* 2012), graft-versus-host disease (Yañez *et al.* 2006) and rheumatoid arthritis (Gonzalez-Rey *et al.* 2010).

Due to the mesodermal origin of the cells, ADSCs seem to be predetermined for bone and cartilage tissue engineering. Using the ADSCs paracrine functions or ADSCs themselves, there have been several studies in which the intraarticular or periarticular injection of SVF or ADSCs was applied to support cartilage regeneration in patients suffering from osteoarthritis. The studies have proved apparent safety and possible minimal manipulation with SVF (Di Matteo *et al.* 2019). In general, the majority of the studies showed short-term positive outcomes (Lopa *et al.* 2019). However, in these studies, different protocols containing many modifications have been used. Thus, the long-term follow up clinical studies containing more cohesive methodology are still need to be performed to ensure long-term efficacy of SVF or ADSCs in various orthopaedic applications (Di Mateo *et al.* 2019, Shariatzadeh *et al.* 2019, Lopa *et al.* 2019). Moreover, separate EVs harvested from ADSCs can also alleviate cartilage damage and downregulate senescence in osteoblasts during osteoarthritis process (Tofiño-Vian *et al.* 2017, Woo *et al.* 2020). ADSCs can also be used in bone tissue engineering to repair bone defects. In 2004, Lendeckel *et al.* reported the treatment of a calvarial defect using a combination of autologous ADSCs and fibrin glue (Lendeckel *et al.* 2004). Another approach to the use of ADSCs in bone repair is their osteogenic differentiation into osteoblasts, which can be seeded into a suitable scaffold. Subsequently, this cell-populated scaffold would be transplanted into the human body to replace damaged bone or a damaged joint (Awad *et al.* 2004, Levi and Longaker 2011). The detailed differentiation cues of ADSCs into osteoblasts will be mentioned in the following chapter.

Current prospects in vascular tissue engineering offer the possibility to differentiate ADSCs towards vascular smooth muscle cells (VSMCs) or endothelial cells (ECs). Although both ADSCs and ECs are of mesodermal origin, the differentiation of ADSCs towards ECs is considered difficult and resembling the trans-differentiation of ADSCs into cells of another germ layer, e.g. keratinocytes that are of ectodermal origin. The main reason for the difficulty is the polarization of ECs. These cells have apical-basal polarity, i.e. functional specialization of their luminal and basolateral membranes (Roberts and Sandra 1993), and planar polarity, which is important for EC movement, proliferation, angiogenesis and the response to shear stress (McCue *et al.* 2006). By contrast, ADSCs

are mesenchymal cells, i.e. they are mesodermal cells that lack polarity. Therefore, an alternative approach is to utilize the paracrine secretion of growth factors from ADSCs and to co-culture them with ECs in order to accelerate the EC growth and phenotypic maturation (Merfeld-Clauss *et al.* 2010). This approach would then be suitable for vascular prosthesis seeding or for vascularization of tissue or of artificially produced scaffolds. The detailed differentiation cues of ADSCs into VSMCs for reconstruction of blood vessel will be mentioned in the following chapter.

In addition to these applications, there have been numerous studies focused on various areas of regenerative medicine, such as hair regeneration (for a review, see Jin and Sung 2016), peripheral nerve regeneration (di Summa *et al.* 2010), cardiovascular repair (for a review, see Madonna and De Caterina 2010, Brenner *et al.* 2016), pancreatic repair (Lin *et al.* 2009) and hepatic repair (Banas *et al.* 2009).

1.1.4. Induced pluripotent stem cells

Induced pluripotent stem cells (iPSCs) were first described by Takahashi and Yamanaka in 2006 (Takahashi and Yamanaka 2006). The key step in iPSC production is the process of reprogramming mature differentiated cells into an embryonic-like state. By influencing mouse adult fibroblasts with four factors (i.e. Oct3/4, Sox2, c-Myc, and Klf4), Takahashi and Yamanaka were able to obtain iPSCs exhibiting similar morphology and growth properties to those of ESCs. Since that time, the number of successful cell reprogramming attempts has been rising, and the range of differentiated cells being reprogrammed (e.g. keratinocytes, fibroblasts, peripheral blood mononuclear cells, renal tubular epithelial cells) has also expanded (Pane *et al.* 2016).

The advantages of iPSCs seem to be (i) their pluripotency, (ii) their potential for gene correction, and (iii) the fact that they are a relatively easily accessible source of autologous cells. However, Riggs *et al.* (2013) studied the differences in transcriptomes between iPSCs and *in vitro* produced tumor cells from the same fibroblast origin. These authors found a significant number of similar genes that were present either in iPSCs or in tumor cells. These results suggested similar processes while inducing pluripotency and tumorigenicity in mature cells. Hence, similarly to pluripotent ESCs, the potential to form ectopic and tumorigenic tissue seems to be non-negligible, and needs to be further investigated in detail (Ben-David and Benvenisty 2011).

1.2. Tissue engineering and mesenchymal stem cells

Tissue engineering is a promising subfield of regenerative medicine. The aim of this recently-established field of medicine is to deal with regenerating and replacing human tissues and even organs that have been impaired by degenerative diseases, by trauma or that are part of congenital abnormalities. Tissue engineering involves studies of biologically compatible materials that can be implanted into the human body. Due to the constant lack of suitable biological tissues, natural-based or synthetic biomaterials mainly in a form of scaffolds are widely studied. These materials themselves can be directly used for tissue replacements; furthermore, they can be seeded with different cell types (i.e. mature cells or stem cells) to better mimic cellular component of original tissue. The advantage of using stem cells is their self-renewal potential and their versatility in differentiation towards a specific mature cell type. Moreover, seeding with the patient's autologous cells seems to be an advantageous way to accelerate the integration of implants into the human body, since there is a lower immune response of the body.

Following chapters will focus on the possible use of ADSCs in soft tissue engineering (namely in vascular tissue engineering) and in hard tissue engineering (namely in bone tissue engineering).

1.2.1. Soft tissue engineering

Soft tissue engineering covers replacements of “soft tissues” in a human body. The partitioning of tissue engineering into “soft” and into “hard” is based on the mechanobiology characteristics of an original tissue that can be highly specific. Soft tissue engineering therefore includes neural tissues, cardiovascular tissues, adipose tissue, muscle tissue, gastrointestinal tissues, cartilage tissue or parenchymal organs.

1.2.1.1. Vascular tissue engineering

Out of soft tissue engineering, vascular tissue engineering is one of the most studied because many patients suffer from degenerative blood vessels disorders. Atherosclerosis and all its consequences often result in blood vessel replacement. The following chapter will briefly summarize the vascular anatomy and physiology. Then, the main emphasis will be put on current replacements used in vascular tissue engineering and on differentiation of ADSCs towards VSMCs.

1.2.1.1.1. Vascular anatomy and physiology

Blood vessels (i.e. arteries and veins of various diameters) are composed of three concentric layers: *tunica intima*, *tunica media*, and *tunica adventitia* (**Fig. 3**). The thickness of each of the layers varies among individual blood vessel types.

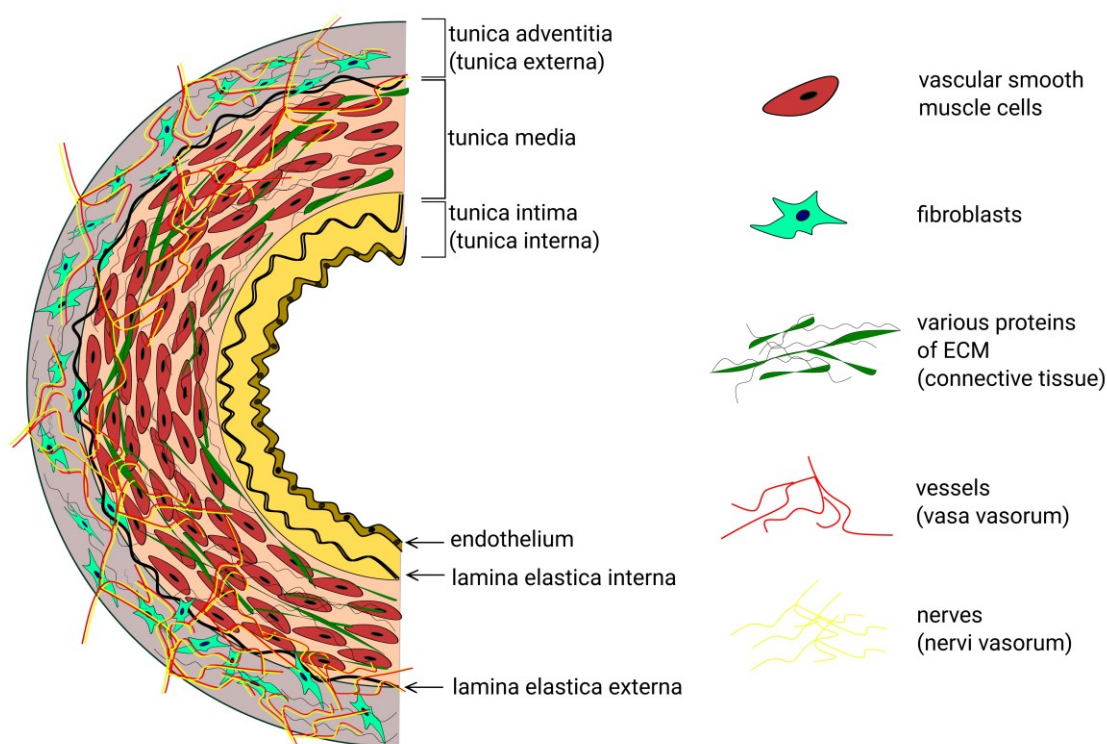


Fig. 3. The illustrative cross-section of the arterial wall. ©2021 Martina Travnickova

Tunica intima (tunica interna) is the inner layer, which comes into direct contact with the blood flow. This layer is composed of a monolayer of polygonal ECs, the basal membrane, connective tissue, and there may also be a supportive internal elastic lamina (in the arterial wall) (**Fig. 3**).

Tunica media is the middle layer of blood vessels. It is composed of VSMCs and fibroblasts, each of them produce the extracellular matrix (**Fig. 3**). The extracellular matrix is composed mainly of elastic and collagen fibres (i.e. collagen (type I, III, IV), laminin, and elastin) that are very important for imparting strength to the vascular wall (Wagenseil and Mecham 2009). Arteries can be divided into elastic and muscular, depending on the amount of smooth muscle and elastic fibres. *Tunica media* is usually the thickest layer of the artery wall. The media in the vein wall is thinner, and contains a bigger amount of extracellular matrix than of smooth muscle (Pometlová *et al.* 2015).

Tunica adventitia (tunica externa) is the external layer of blood vessels. The adventitia is composed of fibroblasts, collagen connective tissue and, in arteries, also of

external elastic lamina (**Fig. 3**). Small blood vessels, referred to as *vasa vasorum*, and nerves are also present to support nutrition and innervation of the vessel wall. The adventitia is also a source of pericytes (in capillaries and microvessels) and adventitial cells (around large vessels), which are, in fact, multipotent mesenchymal stem cells able to differentiate into various cell types (Corselli *et al.* 2012).

Physiologically, the endothelial layer is continuous and intact, and is composed of mature ECs. The properties of ECs are as follows: (i) non-proliferating, (ii) non-thrombogenic, (iii) non-immunogenic, and (iv) active in secreting various molecules. The secreted molecules can regulate the blood flow (e.g. endothelin, angiotensin, NO, prostacyclins), haemocoagulation (e.g. von Willebrand factor, tissue factor, trombomodulin, prostacyclins), the growth of vascular smooth muscle cells (e.g. FGF, platelet-derived growth factor - PDGF, heparin, TGF, epidermal growth factor - EGF), and the production of extracellular matrix, particularly heparin-like glycosaminoglycans, which keep the VSMCs in their quiescent, non-proliferative and differentiated contractile phenotype (Pometlová *et al.* 2015; for a review, see Bačáková *et al.* 2014).

1.2.1.1.2. Substitutes used in vascular tissue engineering

Vascular grafting is a surgical procedure in which a damaged or occluded vessel is replaced by a biological graft or by a synthetic prosthesis. An ideal vascular graft should be characterized by (i) zero or at least low immunogenicity, (ii) anti-thrombogenicity or at least non-thrombogenicity, (iii) anti-microbial effect, (iv) appropriate bioactivity, (v) appropriate biodegradability, and (vi) suitable biomechanical properties.

Human grafts can be of arterial origin (e. g. from *arteria thoracica interna* or *arteria radialis*) or venous origin (e.g. *vena saphena magna*). They usually are of good mechanical properties, high infection resistance and low antigenicity for patients. Although autografts are the first choice when replacing small or medium calibre vessels, smaller grafts (i.e. < 5-6 mm in diameter) are associated with a high risk of thrombosis and usually fail within 5 years after surgery (for a review, see Leon and Greisler 2003, Carrabba *et al.* 2018). Moreover, autografts and allografts are available only in limited quantities, due to the age of the patients and donor site morbidity. The lack of autologous or allogenic grafts could be solved by the use of decellularized vessels of xenogeneic, e.g. porcine, origin (Badylak 2004). The optimal decellularization procedure can offer a source of biocompatible scaffolds in which the mechanical properties are very similar to

those of autografts, while the immunogenicity is very low due to the absence of xenogenous cells (for a review, see Tapias and Ott 2014, Simsa *et al.* 2018).

Until now, vascular grafting has widely used acellular scaffolds with a polymer-based origin. Polymer-based biomaterials are elastic, so they mimic the biomechanical properties of vessels. Various modifications of polyethylene terephthalate (PET) and polytetrafluoroethylene (PTFE) have been used in blood vessel replacements (Kannan *et al.* 2005, Chlupáč *et al.* 2009). PET and PTFE are non-toxic *per se*, but they are hydrophobic and non-degradable which limit possible cell-material interactions and remodeling of the materials. Thus, possible disadvantages of currently used synthetic vascular prostheses are (i) limited incorporation, (ii) graft rejection, (iii) risk of infection, (iv) dilatation over time, and (v) surface thrombogenicity (Chlupáč *et al.* 2009).

New compounds of natural or synthetic origin have been studied and fabricated to ameliorate graft properties (i.e. gradual biodegradability, bioactivity, anti-thrombogenicity, non-immunogenicity, and biomechanical properties). The natural compounds include various forms of polysaccharides (e.g. alginate, chitosan, dextran, pullulan, pectin, cellulose, xanthan, or starch) (for a review, see Bačáková *et al.* 2014) and some of the extracellular matrix (ECM) proteins (e.g. collagen, fibrin, or fibronectin). The synthetic polymer-based materials include forms of polyurethanes (PU), polycaprolactone (PCL), poly (L-lactic acid) (PLLA), poly (glycolic acid) (PGA), polyvinyl alcohol (PVA) or polyethylen glycol (PEG) (for a review, see Pashneh-Tala *et al.* 2016, Hielscher *et al.* 2018). All these biomaterials can be used to create mono-, bi- or tri-layer scaffolds (for a review, see Goins *et al.* 2019). They can also be further loaded with some growth factors (e.g. VEGF, FGF2, PDGF, etc.) or with some other bioactive molecules (e.g. heparin, sirolimus, simvastatin, etc.) to improve graft bioactivity (for a review, see Strobel *et al.* 2018). The newly developed scaffolds could serve as a temporary structure for attaching the cells. The attached cells could start to produce new layers of the blood vessel, while the degradable scaffold would slowly resorb (for a review, see Venkatraman *et al.* 2008). However, these biomaterials have usually inadequate mechanical properties (Ravi *et al.* 2009), and there is a risk that the scaffolds degrade in the human body more rapidly than the blood vessel forms.

Last but not least, there are scaffold-free approaches suggesting the use of cell sheets only (i.e. confluent layers of vascular cells that can be shaped into blood vessel) (Carrabba *et al.* 2018). Cells in these sheets would subsequently produce ECM proteins needed for the vessel strength.

In order to improve the biocompatible properties of vascular replacements, there are strategies that attempt to combine abovementioned scaffolds or decellularized biological grafts with patient's autologous cells (Harris *et al.* 2011, Quint *et al.* 2012). This combination could provide higher acceptance of the graft and suitable mechanical properties similar to those of autografts (for a review, see Murphy and Atala 2013). Depending on the reconstructed blood vessel layer, autologous ECs or VSMCs, i. e. the fully mature cells of vascular origin, can be used. Nevertheless, these autologous mature cells are available in limited quantities and they do not withstand many passaging within *in vitro* conditions. Thus, new sources of immature pluripotent or multipotent stem cells have been studied. Among stem cells, adult mesenchymal stem cells are considered to be one of the most promising sources for vascular grafts (Zhang *et al.* 2017, Hielscher *et al.* 2018).

As mentioned above, a natural blood vessel is composed of 3 layers. Most of the studies (if dealing with a cellular component) are focused on reconstructing *tunica intima*, because the intact confluent layer of ECs would mimic internal lumen and would prevent undesirable adhesion of blood elements. So far, the reconstruction of *tunica media* has been avoided, because VSMCs under *in vitro* conditions can dedifferentiate (Bačáková *et al.* 2018b). This would therefore lead to impaired contractile function of VSMCs and to excessive growth of VSMCs on the prosthesis surface. Nevertheless, *tunica media* having a proper contractile function is needed for complex constructs of blood vessel wall (i.e. bi-layer or tri-layer constructs) in modern tissue engineering. Such advanced bi-layer or tri-layer constructs can be fabricated in separate functional-specific layers (Yokoyama *et al.* 2017) or in simultaneous layer-specific cell differentiation achieved by separate flow perfusion (Pennings *et al.* 2019) (for a review, see Goins *et al.* 2019).

1.2.1.1.3. Differentiation of ADSCs towards VSMCs

In general, cells under *in vitro* conditions are influenced by various stimuli influencing cells in changes of morphology, secretome, proliferation rate, and differentiation. Stem cells are particularly responsive to every stimulus, as it is natural for them to receive signals *in vivo* in the human body. The role of these signals is either to retain them in “stem condition” or to trigger differentiation into the desired phenotype. In tissue engineering, differentiation of cells can be triggered and guided mainly by (i) biochemical composition of culture medium, by (ii) biomaterial/scaffold properties, and by (iii) dynamic culture conditions (Bačáková *et al.* 2018c). Different culture conditions

can be used separately, or can be combined to multiply these effects. Similar approaches can be applied on ADSCs when differentiating them towards VSMCs for the vascular tissue engineering purposes (**Fig. 4**).

ADSCs differentiated towards VSMCs seem to be an ideal source of cells contributing to the formation of *tunica media*. Moreover, their paracrine function could positively influence ECs in *tunica intima*. Such newly differentiated VSMCs could be used either for cell sheets in scaffold-free approaches or during cell seeding in scaffold-based approaches.

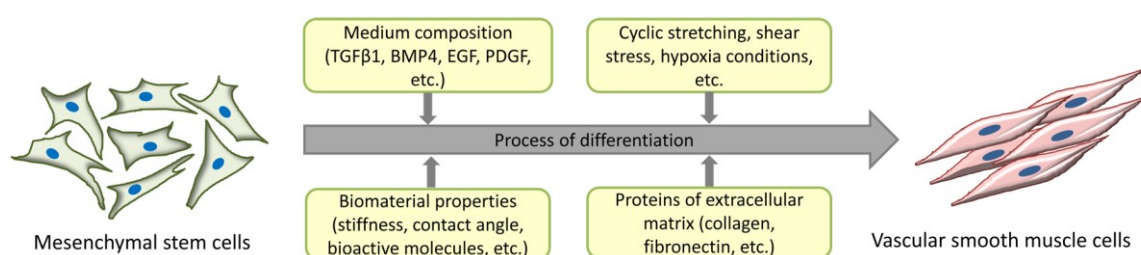


Fig. 4. *In vitro* culture conditions influencing the differentiation of ADSCs towards VSMCs. ©2021 Martina Travnickova

There are many studies referring to the various biochemical composition of differentiation medium. The use of specific growth factors and other molecules is usually based on the embryonic development of VSMCs from appropriate progenitors. VSMCs in the human body do not have a single embryonic origin. Thus, their function and phenotype can differ as they arise from various multipotent progenitors (Majesky 2007). There are two main sources of VSMCs, namely the mesoderm and the neuroectoderm (for a review, see Bačáková *et al.* 2018a). Transforming growth factor beta (TGFβ) and PDGF are important chemoattractants in VSMCs signalling and development. Their correct function seems to be crucial for embryonic VSMCs differentiation as well as for VSMCs phenotype switching/dedifferentiation/re-differentiation (Guo *et al.* 2012, Wang *et al.* 2015). The impaired function of these signalling pathways can play a role in some cardiovascular diseases (Guo *et al.* 2012). Concerning the *in vitro* stem cell differentiation towards VSMCs, a strongly positive effect of TGFβ1 *per se*, or in a combination with bone morphogenic protein 4 (BMP4), has been proved in many studies (Brun *et al.* 2015, Elçin *et al.* 2017, Zhang *et al.* 2017). Except for these two factors, PDGF-BB, epidermal growth factor (EGF), thromboxane A2, angiotensin II, sphingosylphosphorylcholin, heparin, or ascorbic acid (AA) in various concentrations and combinations have been reported to support differentiation towards VSMCs (Narita *et al.* 2008, Kim *et al.* 2009,

Harris *et al.* 2011, Brun *et al.* 2015, Zhang *et al.* 2017). Nevertheless, it should be taken into account that TGF β 1 and some other factors are also involved in some pathological states of the cardiovascular system (Guo *et al.* 2012). The proper differentiation effect of TGF β 1 towards VSMCs phenotype should be simultaneously supported by appropriate scaffold properties and particularly by appropriate mechanical stimulation.

Smooth muscle cell differentiation is an aspect of “soft tissue engineering”, and is supported by “soft” biomaterials. Basic components of extracellular matrix, i.e. collagen, poly-L-lysine or fibronectin (Cooper and Sefton 2011, Sgarioto *et al.* 2012, Wei *et al.* 2014), are used to fabricate fibrillar scaffolds or at least to coat biomaterials for faster cell adhesion and also for cell differentiation (Filová *et al.* 2014). Nanofibrillar scaffolds can better mimic 3D microenvironment *in vivo* where cell-cell and cell-material interactions are better developed than in 2D plastic dish. The cell adhesion is realized through integrin-binding receptors and these receptors can convert important mechanical stimuli from ECM into specific biochemical signals (Hussey *et al.* 2018). Not only the 3D microenvironment, but also the organisation of fibres or material surface structure contributes to a proper ADSCs alignment within differentiation (Liguori *et al.* 2019). The ideal scaffold should mimic biomechanical properties of a native blood vessel. The elasticity and stiffness of a vessel wall is directly dependent on ECM components, and VSMCs *in vivo* are able to increase synthesis of specific ECM protein if needed (Wagenseil and Mecham 2009). The elastic modulus, which is approximately 0.3-1.0 MPa for aortic wall, is therefore specific for different types of vessels located in cardiovascular system (Wagenseil and Mecham 2009).

In the human body, blood vessels are exposed to permanent cyclic strain stress with an average frequency of 60 to 100 heartbeats per minute. It has been reported that similar dynamic stimulation under *in vitro* culture can cause cell alignment and can enhance mRNA expression of contractile proteins in mature VSMCs in 3D collagen gells (Asano *et al.* 2018). Moreover, cyclic stretch (amplitude of 5 - 10%, frequency of 0.5 – 1.5 Hz) supports the differentiation pathways of progenitor cells towards VSMCs (for a review, see Dan *et al.* 2015, Zhang *et al.* 2017). Biomechanical stimulation alone was reported to induce cell alignment and to support expression of early and/or mid-term markers of VSMCs in differentiated ADSCs (Parvizi *et al.* 2016, Walters *et al.* 2017, Rabbani *et al.* 2017). The differentiation can be further enhanced by a synergic effect of biochemical stimulation (i.e. composition of media) and mechanical stimulation. This synergic effect

seems to play a key role in expression of late VSMCs markers and in synthesis of ECM proteins (Wang *et al.* 2010, Helms *et al.* 2020).

The differentiation is mainly detected by increased mRNA expression and observed by formation of the α smooth muscle actin (α SMA), SM22 α , calponin, caldesmon, desmin, smoothelin-B, and smooth muscle myosin-heavy chain (SM-MHC) protein structures that are involved in smooth muscle cell differentiation (for a review, see Zhang *et al.* 2017). The detailed description and code genes of the most commonly studied markers are given in **Table 3**. Except for these main differentiation markers, specific integrins (α 1, α 7, β 1) (i.e. integrins anchoring proteins typical for contractile type of VSMCs) can be a sign of mature VSMCs (Zhang *et al.* 2017). Interestingly, some of the VSMCs markers, such as α SMA, calponin isoforms, and desmin, can also be present in undifferentiated MSCs (Liu *et al.* 2013).

Table 3. The early, mid-term and late markers of vascular smooth muscle cells (VSMCs). The specificity for VSMCs is higher with increasing rate of maturation from α SMA to SM-MHC (Zhang *et al.* 2017, Bačáková *et al.* 2018b).

Marker	Gene	Description
α SMA	ACTA2	Isoform typical for smooth muscle cells, myofibroblasts
SM22 α	TAGLN	Calponin related protein
SM-calponin	CNN1	Calcium binding protein, regulates ATPase activity
h-caldesmon	CALD1	Calmodulin binding protein, regulates ATPase activity
desmin	DES	Intermediate filament protein, muscle-specific protein
Smoothelin-B	SMTN	Associated with stress fibres, part of cytoskeleton
SM-MHC	MYH11	Smooth muscle myosin heavy chain, contractile protein

So far, the studies have suggested many different culture conditions supporting the differentiation of ADSCs towards VSMCs. The great diversity of medium composition (i.e. various components in various concentrations), of material types and properties, and of dynamic conditions causes difficult comparison of successful ADSCs differentiation among the studies. Moreover, some studies are focused only on very early changes at mRNA expression of early and/or mid-term markers. Detecting only the early markers can result in later failure/difficulties in obtaining fully mature VSMCs, because these markers are not exclusively expressed only in VSMCs. Last but not least, it is necessary to take into account potential variation in the differentiation response in ADSCs mainly

due to high donor-to-donor variability. Thus, there is still need to optimize the procedure of ADSCs differentiation towards VSMCs, and to observe the cell behaviour on appropriate biomaterials in a longer-term follow up studies.

1.2.2. Hard tissue engineering

Hard tissue engineering covers replacements of “hard tissues” in a human body. Joint and bone defect replacements are considered as a part of hard tissue engineering as the original bone tissue is one of the hardest tissue in human body.

1.2.2.1. Bone tissue engineering

Bone tissue engineering represents an extensive chapter of tissue engineering. Ameliorating current biomaterials or fabricating new ones is very desirable as many patients suffer from osteoarthritis or traumatic damage of the bone tissue. Moreover, bone tissue engineering covers oral and maxillofacial surgery. The following chapter will briefly sum up the bone anatomy and physiology. Then, the main emphasis will be put on current replacements used in bone tissue engineering and on differentiation of ADSCs towards osteoblasts.

1.2.2.1.1. Bone anatomy and physiology

Bone is a compact tissue consisting of cancellous and cortical bone. The functions of bones are as follows: (i) to provide a load-bearing skeleton of the body, (ii) to protect the internal organs, (iii) to store minerals, (iv) to provide movement, and (v) to produce haematopoietic stem cells in the bone marrow.

Most of the bone tissue is created by ECM. The ECM is composed of inorganic salts and organic matrix. The organic matrix is predominantly composed of collagenous proteins, mainly of type I collagen. Other organic components are represented by osteocalcin, osteopontin, osteonectin, fibronectin, family of BMPs, and other growth factors (Florencio-Silva *et al.* 2015). Inorganic salts are represented by hydroxyapatite (i.e. main inorganic part composed of calcium and phosphate ions) and by other ions and compounds (i.e. carbonate, sodium, potassium, bicarbonate, magnesium, fluorite, etc.) (Florencio-Silva *et al.* 2015). Type I collagen provides tensile strength, and hydroxyapatite provides compressive stiffness (Reichert *et al.* 2016). Besides these main components, the ECM contains proteoglycans and glycosylated proteins. Each of the

proteins has a role in influencing bone mineral deposition and in regulating the cell activity (for a review, see Clarke 2008).

There are four main types of cells in the bone tissue: (i) osteoprogenitor cells, (ii) osteoblasts, (iii) osteocytes, and (iv) osteoclasts (**Fig. 5**) (Downey and Siegel 2006, Florencio-Silva *et al.* 2015). Osteoprogenitor cells are stem cells of mesenchymal origin capable of differentiation into osteoblasts. Osteoblasts are derived from osteoprogenitor cells. They are responsible for the synthesis and mineralization of the bone tissue. Their role is important during new bone formation and also in bone remodelling. Osteocytes are mature cells that occupy bone lacunae that are surrounded by mineralized bone matrix. These long-living cells formed by transition from osteoblasts represent 90-95% of all bone cells. Osteoclasts are terminally differentiated multinucleated cells involved in bone resorption. They are derived from mononuclear cells belonging to hematopoietic stem cell lineage (Downey and Siegel 2006, Florencio-Silva *et al.* 2015). In addition, bone marrow is the main source of hematopoietic stem cells (HSCs) during adulthood (Morrison and Scadden 2014). These HSCs are precursor cells necessary for production of cellular blood components.

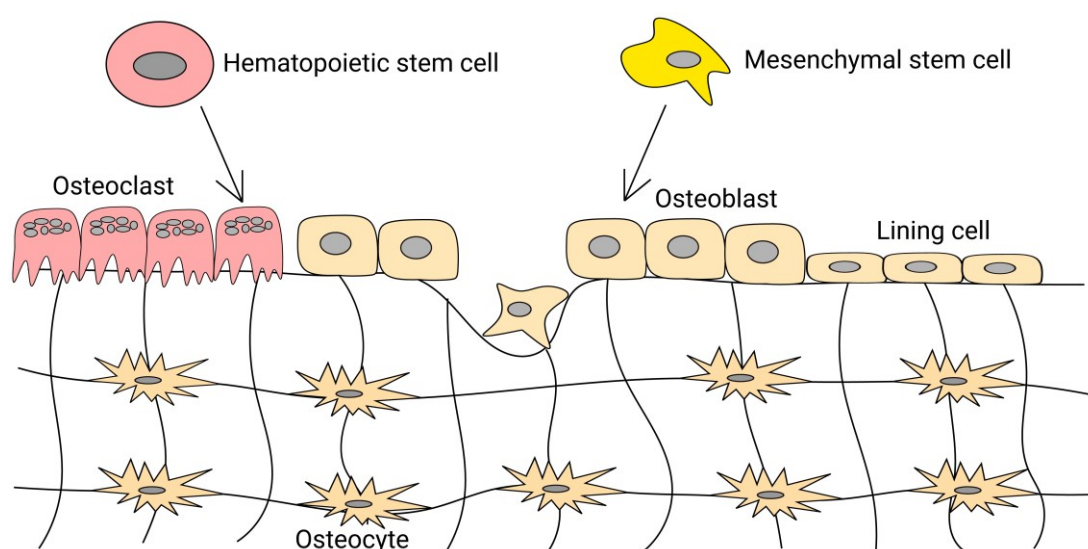


Fig. 5. The main types of bone cells (i.e. osteoblasts, osteocytes, and osteoclasts) and their origin. ©2021 Martina Travnickova

Both in childhood and in adult age, the bone underlies the remodelling cues in a dynamic response to physiological influences and mechanical forces (Clarke 2008). During the remodelling process, the old cells are resorbed by osteoclasts and new bone tissue is formed by osteoblasts to keep mineral homeostasis stable (Reichert *et al.* 2016).

Osteoblasts and osteoclasts can influence and communicate with each other via cell-cell contacts, specifically via gap junctions (Chen *et al.* 2018). In the first phase of bone remodelling, osteoclast formation and activation is regulated by receptor activator of NF- κ B ligand (RANKL), colony-stimulating factor (CSF), osteoprotegerin, proteins of the interleukin family, parathyroid hormone, 1,25-dihydroxyvitamin D, and calcitonin (Clarke 2008, Martin and Seeman 2008, Raggatt and Partridge 2010). Subsequent bone tissue formation is influenced and regulated by bone matrix-derived factors, such as FGF1, FGF2, TGF (β 1 and β 2), BMPs (2, 3, 4, 6, and 7), PDGF, insulin-like growth factor (IGF) I and II, and potentially other molecules (Bonewald and Mundy 1990, Clarke 2008, Martin and Seeman 2008, Raggatt and Partridge 2010).

1.2.2.1.2. Substitutes used in bone tissue engineering

Many patients suffer from degenerative or traumatic diseases of the skeletal system. Degenerative diseases are mainly represented by osteoarthritis (in the scientific literature also referred to as osteoarthrosis), which is a widespread chronic age-related joint disorder. Osteoarthritis preferentially alters big joints that are loaded during activities of daily living (Kutzner *et al.* 2010). The first damage usually occurs in articular cartilage of the affected joint. Subsequently, pathological changes expand to subchondral bone, bone marrow, synovium, menisci, ligaments and periarticular muscles (for a review, see Man and Mologhianu 2014). Earlier stages of the disease can be ameliorated by medication; however later stages can lead into a total joint replacement. Other bone tissue replacements are necessary to treat consequences of various traumatic injuries, of oncological lesions or of other degenerative disorders.

Current therapies in bone diseases are focused on autografts and allografts, or on the implantation of artificial biomaterial implants (Reichert *et al.* 2016). The strategies for fabricating an appropriate biomaterial are directed at tissue conduction and induction. Tissue conduction refers to the migration of cells of a given tissue and blood vessel formation that can be reached in 3D scaffolds. An induction strategy refers to support for the proliferation and differentiation of progenitor cells to a desired phenotype (Reichert *et al.* 2016). So far, biological bone grafts remain the best choice concerning osteoconductive and osteoinductive properties, i.e. they stimulate the ingrowth of osteoblasts inside the graft material and, at the same time they induce differentiation of local stem cells into osteoblasts (García-Gareta *et al.* 2015, Baldwin *et al.* 2019).

However, these grafts can be harvested only in limited quantities, and donor site morbidity also has to be taken into account (Baldwin *et al.* 2019).

Following chapters are devoted to fabricated substitutes in bone tissue engineering that are, for better comprehension, divided into joint replacements that are usually of metal or ceramics origin and into cancellous bone replacements that are usually of natural/polymeric origin mimicking porous 3D structure.

1.2.2.1.2.1. Metallic and ceramics implants

For total big joint replacements, metallic biomaterials are still used as the “gold standard”. Out of the metallic compounds, stainless steels, cobalt-based alloys (mainly CoCrMo), and titanium alloys (mainly Ti-6Al-4V) have been widely used. Nevertheless, some of their properties that are different from those of the healthy bone tissue can impair later osseointegration (for a review, see Saini *et al.* 2015). Osseointegration is a complex process during which an artificial biomaterial is being gradually integrated into a surrounding bone tissue. Successful osseointegration plays a key role in long-term stability of implants. The main properties influencing the suitable osseointegration of an implanted biomaterial can be divided into (i) physicochemical, (ii) mechanical and (iii) biological properties. Physicochemical properties involve characteristics of the material such as wettability, surface roughness, corrosion resistance or potential release of ions. Mechanical properties include Young’s modulus and wear resistance. Last but not least, biological properties include high biocompatibility, osteoconductivity, osteoinductivity, antimicrobial activity, non-immunogenicity and potential biodegradability (Chen and Thouas 2015, Reichert *et al.* 2016).

There are several main limitations of conventional metallic implants: (i) wear of metallic implants, (ii) mechanical failure, (iii) variable biocompatibility, (iv) surface colonization and biofilm formation (Prasad *et al.* 2017). Current bone tissue engineering aims to prevent undesirable rejection that can occur as a result of impaired osseointegration. Some imperfections of above mentioned traditional alloys can be solved or at least ameliorated by surface modifications. The surface modifications cover e.g. changes of the material surface roughness at micro or nanoscale to support cell-material interactions (Feller *et al.* 2015, Zhou *et al.* 2017) or additional material coating to prevent corrosion and consequent ion release (Dehghanghadikolaei and Fotovvati 2019).

Moreover, to ameliorate biological properties of metals, bio-inert conventional metals can be coated with ceramics. Ceramics by definition are inorganic compounds

formed at high temperature. These compounds having a crystalline structure contain either metallic (e.g. alumina, zirconium) or mainly non-metallic (e.g. hydroxyapatite, calcium phosphates, carbon) elements (Roberts and Rosenbaum 2012, Shanmugam and Sahadevan 2018). The most frequently studied ceramics compounds having at least bioinert or better bioactive properties are e.g. carbons, bioactive glass, Al_2O_3 , ZrO_2 , hydroxyapatite or tricalcium phosphates (Shanmugam and Sahadevan 2018).

To support the osteoinduction, materials can be loaded with various biological factors that are mainly represented by growth factors and cytokines. Similarly to natural bone formation, FGFs, TGF β s, BMPs, IGFs, and PDGF can be used as the main factors regulating and supporting osteoblast and osteocyte maturation (Prasad *et al.* 2017).

Recently, some other metals or metal ions have been involved in alloy preparation and surface modifications. Namely, nickel, magnesium, tantalum, zirconium, copper, strontium, zinc or silver have been studied, due to their suitable physical, mechanical, chemical or biological properties (Chen and Thouas 2015, Przekora 2019a). Interestingly, alloys of magnesium, zinc or iron can also be fabricated in biodegradable forms (Prasad *et al.* 2017, Su *et al.* 2019). The gradual degradation provides sufficient initial mechanical support and enables later tissue growth during regeneration (Prasad *et al.* 2017).

To sum up, most of the metallic alloys provide superior mechanical properties; however, it is still necessary to ameliorate their biological properties to obtain better results in their long-term use.

1.2.2.1.2.2. Porous natural (bioinspired)/polymeric bone replacements

Engineered porous 3D scaffolds are desirable as substitutes of small defects in a cancellous bone tissue, because biological grafts are available only in limited quantities. The main role of porous scaffolds is to mimic composition and architecture of a bone tissue that is naturally formed by ECM proteins. To maintain authentic architecture of a cancellous bone tissue, various bioinspired and polymeric scaffolds can be fabricated by electrospinning, 3D printing or other methods enabling formation of 3D structured scaffolds (for a review, see Przekora 2019b). Such a porous 3D scaffold enables natural adhesion, proliferation and differentiation of osteoprogenitor cells (for a review, see Przekora 2019b). In a human body, a cortical bone tissue is formed by Haversian systems having size about 250 μm in diameter (Maggiano *et al.* 2016). The minimal pore size of the materials enabling cell ingrowth is about 100 μm (for a review, see Marrella *et al.* 2018). However, materials having a larger pore size, especially the materials having a

pore size from 200 μm to 400 μm , seem to be the most suitable to support subsequent graft vascularization and innervation mimicking Haversian channels (Marrella *et al.* 2018).

Bioinspired scaffolds can be made of natural polymers (e.g. collagen, gelatin, chitosan, silk fibroin, alginate or hyaluronic acid) or of synthetic polymers (e.g. polycaprolactone, polyurethane, polylactide, polyglycolide or their copolymers) (for a review, see Vagaská and Bačáková 2009, Chocholatá *et al.* 2019). In general, bioinspired scaffolds usually provide high biocompatibility on the one hand; however, they lack satisfactory mechanical properties on the other hand (for a review, see Vagaská and Bačáková. 2009, Przekora 2019b). Similarly to metallic implants, the osteoinductivity of the scaffolds can be enhanced by loading with various biological factors. The family of FGFs, TGF β s, BMPs, IGFs and PDGFs has been reported to regulate differentiation and maturation of bone cells (for a review, see Ho-Shui-Ling *et al.* 2018). Moreover, loading the porous scaffolds with VEGF should also enable and support the formation of small blood vessels that are desirable for further nutrition of a newly formed tissue (for a review, see Hu and Olsen 2016).

Currently, bone tissue engineering is focused on autologous adult stem cells in combination with an appropriate scaffold aiming to mimic and to simulate the properties of the natural bone tissue (Dawson *et al.* 2008, Reichert *et al.* 2016, Przekora 2019b). The scaffold can serve as a conductive and inductive microenvironment to support the proliferation and differentiation of stem cells into osteoblasts and osteocytes, and potentially also to support small blood vessel formation (for a review, see Arvidson *et al.* 2011). Moreover, the isolated adult mesenchymal stem cells can be of autologous origin that increases the possibility for better acceptance of an implanted biomaterial by a patient's body.

1.2.2.1.3. Differentiation of ADSCs towards osteoblasts

ADSCs and bmMSCs are adult MSCs of mesodermal origin. To meet one of the criteria of MSCs, the cells by definition should undergo successful differentiation towards osteoblasts (Dominici *et al.* 2006). The osteogenic differentiation of MSCs is therefore considered as a relatively easy type of differentiation under *in vitro* conditions. Concerning the optimal source of MSCs, it seems that both ADSCs and bmMSCs are suitable for potential bone scaffold seeding. Available literature was reviewed by Liao and Chen (2014) and by Przekora (2019b). In their review articles, they reported a

slightly inferior osteogenic potential of ADSCs than of bmMSCs mainly under *in vitro* conditions. However, some other factors such as easier accessibility or higher proliferation rate can make ADSCs comparable to or even more suitable than bmMSCs (Liao and Chen 2014, Przekora 2019b). Nevertheless, the comparative studies were usually performed on ADSCs and bmMSCs from healthy donors. It usually does not fully reflect conditions in patients needing the implants. Thus, concerning the potential autologous use of MSCs for implant seeding, any systemic illness mainly in elderly patients can affect the results of successful osteogenic differentiation (Liao and Chen 2014).

In the human body, the osteogenic differentiation is guided from MSCs via osteoprogenitor cells towards preosteoblasts and further towards osteoblasts (Przekora 2019b). Each of these cell phases is characterized by specific levels of most characteristic markers (**Table 4**). Specifically, Runt-related transcription factor 2 (RUNX2), type I collagen, osteopontin and alkaline phosphatase (ALP) are considered as early or mid-term markers. The increase of the early and mid-term markers is typical for osteoprogenitor cells and for preosteoblasts, i.e. for the proliferation phase and for the ECM synthesis phase respectively (Przekora 2019b). On the contrary, the increase of osteocalcin and calcium deposition is a characteristic of mature osteoblasts, i.e. of the ECM mineralization phase (Przekora 2019b).

Under *in vitro* conditions, the osteogenic differentiation can be triggered by (i) additive biological factors contained in the culture media, by (ii) biomaterial properties or by (iii) mechanical stimulation. Similarly to differentiation towards VSMCs, these culture conditions can be applied separately or in various combinations to potentiate the synergic effects on differentiation.

The biological molecules such as ascorbic acid, β -glycerophosphate, and dexamethasone in various concentrations seem to be essential for proper osteogenic differentiation of MSCs (for a review, see Langenbach and Handschel 2013, Liao and Chen 2014, Shafaei and Kalarestaghi 2020). Variably, the culture medium can also be supplemented with vitamin D, L-glutamin, TGF β s, and BMPs (for a review, see Vater *et al.* 2011, Shafaei and Kalarestaghi 2020).

Table 4. The specific early, mid-term and late markers of osteogenic differentiation. (Id Boufker *et al.* 2010, Miron and Zhang 2012, Przekora 2019b). The presence and specific intensity for each marker during osteogenic differentiation is as follows: - almost no presence, + low intensity, ++ moderate intensity, +++ high intensity.

	MSCs	Osteoprogenitors	Mature osteoblasts	Late osteoblasts
RUNX2	-/+	++	+	-
RANKL	+++	++	++	+/-
Osterix	-/+	++	+	-
Type I collagen	+ /+++	+++	++	-
ALP	+	++/+++	+++ /++	+/-
Osteopontin	+ /++	+ /++	+++	+++
Osteocalcin	-	+	+++	-
Ca²⁺ deposition	-	+	+++	+++
Bone sialoproteins	-	+++	+++	+++

Concerning the ADSCs, it seems that FGFs, which has been a variable component of the ADSC growth medium, can influence subsequent osteogenic differentiation of these cells. FGF2 is crucial for mesodermal development and maintains cell stemness at the same time (Lim *et al.* 2016). It has been reported to promote initial expansion of ADSCs (Lim *et al.* 2016, Khan *et al.* 2017), but it limits their proliferation in later passages (Cheng *et al.* 2020). Nevertheless, it is still unclear, what is the role of FGF2 during osteogenic differentiation *in vitro*. Pre-treatment with FGF2 plays a beneficial role in subsequent osteogenic differentiation (Lim *et al.* 2016). During proper osteogenic differentiation, the FGF2 has been reported to have an inhibitory effect on expression of osteogenic markers (Hu *et al.* 2013, Song *et al.* 2013, Simann *et al.* 2017) that could corresponds to its supportive effect on the cell expansion. On the contrary, several studies reported stimulating effect of FGF2 on osteogenic differentiation (Pri-Chen *et al.* 1998, Fei *et al.* 2011, Byun *et al.* 2014). It therefore seems that the role of FGF2 within the osteogenic differentiation can be ambiguous depending on specific preconditioning, on specific concentration, and on other molecules concurrently used in the culture medium.

As mentioned above, chemical composition, surface properties and topographical structure of biomaterials are crucial for proper initial adhesion, proliferation and subsequent differentiation of stem cells (for a review, see Gao *et al.* 2017). Out of the

biomaterial properties, osteoinductivity is the most important providing the suitable microenvironment for stem cells to differentiate into osteoblasts followed by mineral and calcium deposition.

Last but not least, specific physical stimuli can enhance osteogenic differentiation of bmMSCs and ADSCs under *in vitro* or under *in vivo* conditions. Specifically, stimuli such as mechanical forces (i.e. cyclic stretch or tensile, compressive strain or shear stress), ultrasound treatment (i.e. low-intensity pulsed ultrasound stimulation), electrical stimulation (e.g. cells on piezoelectric materials), magnetic stimulation or heat treatment have been reported to have supportive influence on osteogenic differentiation (for a review, see Heng *et al.* 2004, Przekora 2019a, Shafaei and Kalarestaghi 2020).

2. Hypotheses and objectives

Hypotheses of the dissertation thesis:

(i) The characteristics of ADSCs depend on the region of the liposuction and on the negative pressure used during the liposuction procedure.

(ii) With an appropriate medium composition and newly developed biomaterials, ADSCs can serve as source cells for the reconstruction of a blood vessel wall.

(iii) With an appropriate medium composition and newly developed biomaterials, MSCs can serve as optimal source cells for bone tissue engineering.

Objectives of the dissertation thesis:

(i) To evaluate the cell yields, proliferation and phenotypic profile of ADSCs harvested from different liposuction regions and under different negative pressures. Subsequently, by comparing the obtained data to determine an optimal source and harvesting method of ADSCs for further tissue engineering applications.

(ii) To induce and optimize biochemical differentiation of ADSCs towards VSMCs by various growth factors. To study the interaction of the biochemically-differentiated ADSCs with newly fabricated biomaterials appropriate for vascular tissue engineering (i.e. PLLA foils modified by plasma-treatment and by grafting with PEG or dextran (Dex)).

(iii) To induce and optimize biochemical differentiation of ADSCs towards osteoblasts with an emphasis to the role of FGF2 within the differentiation. To study the interaction of biochemically-differentiated MSCs (i.e. ADSCs and bmMSCs) with newly fabricated biomaterials suitable for bone tissue engineering (i.e. DLC-layers doped with Cr or Ti).

3. Materials and Methods

The listing provides a general overview of the methods used in the experimental work. The detailed parameters and conditions of performed methods and experiments are described in the published articles.

Author's contribution: Tissue engineering is an interdisciplinary field based on the collaboration of material engineers and biologists. In external collaboration, material engineers have been usually responsible for preparation of suitable material and for subsequent evaluation of its physical and chemical properties. The role of the author of the thesis has been in evaluating the optimal harvesting conditions and the optimal source of ADSCs, and further in evaluating cell-material interactions under *in vitro* conditions. Hence, the methods listed in this chapter were performed by the author of the thesis unless otherwise specified.

3.1. Cell characterization for tissue engineering

3.1.1. Isolation of ADSCs and their characterization

The characterization of ADSCs was performed to study potential differences with respect to a negative pressure used during the liposuction procedure, and with respect to a harvesting site of the adipose tissue, and hence to find the optimal source of ADSCs for tissue engineering applications. Detailed conditions of the studied characteristics and compared parameters summarizing this project have been described in Trávníčková *et al.* 2020a.

Cell isolation

The samples of subcutaneous adipose tissue were obtained in collaboration with MUDr. Martin Molitor, Ph.D. (Hospital Na Bulovce in Prague). The isolation of ADSCs was performed according to the isolation protocol by Estes *et al.* (2010) with slight modifications. In brief, the lipoaspirate was washed with a phosphate-buffered saline (PBS; Sigma-Aldrich) and digested by a collagenase solution. The obtained cell suspension of SVF (i.e. the lowest layer after centrifugation) was washed, filtered, and seeded into culture flasks. The isolated cells were cultured in a proliferation medium consisting of Dulbecco's Modified Eagle Medium (DMEM; Gibco), supplemented with

10% (vol/vol) foetal bovine serum (FBS; Gibco), gentamicin, and recombinant human fibroblast growth factor basic (10 ng/mL; FGF2; GenScript). The primary cells, referred to as “passage 0”, were cultured until they reached 70%–80% confluence. Then the cells were passaged and characterized.

In total, samples from 15 donors were collected from the inner thigh region (n=3), from the outer thigh region (n=7), and from the abdomen region (n=9). Moreover, both low negative pressure (i.e. -200 mmHg) and high negative pressure (i.e. -700 mmHg) were used during liposuction in selected harvesting sites for each donor. **Fig. 6** visualizes the scheme of the experimental work.

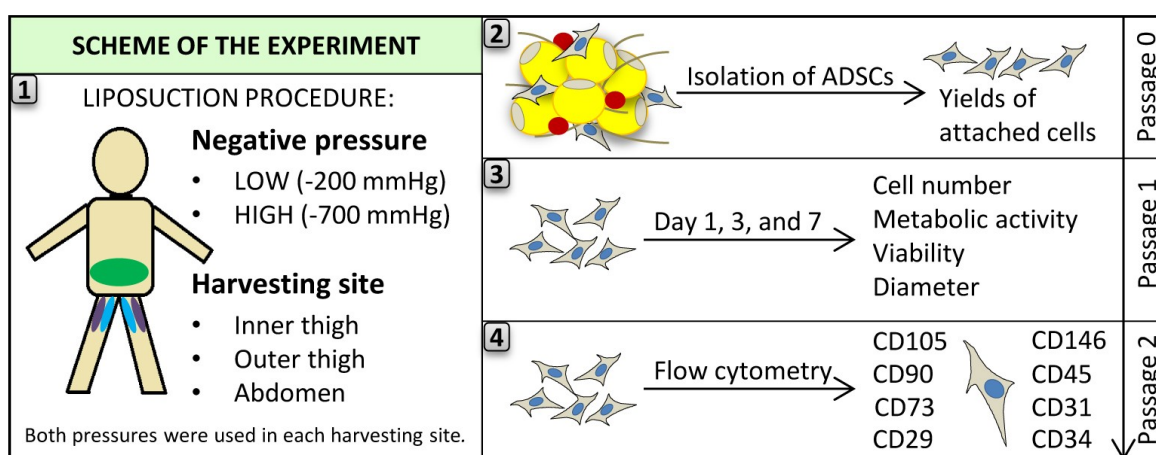


Fig. 6. Scheme of the experimental work characterizing the isolated ADSCs that were obtained under different harvesting conditions (Trávníčková et al. 2020a).

Yields of attached cells

After the isolation, the seeding density of original lipoaspirate/cm² was the same for all the samples that enabled later comparison. The yields of initially attached cells per 1 mL of lipoaspirate were calculated based on manual counting from phase-contrast microscopy photographs on day 1 after isolation.

Cryopreservation of cells

The abundant isolated cells that were not used for determining ADSC characteristics were cryopreserved for further experiments concerning differentiation and seeding on newly developed materials for vascular and bone tissue replacements. In brief, the cells were detached by trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Sigma-Aldrich). The effect of the trypsin-EDTA solution was subsequently inhibited by adding a medium with FBS. The cells were centrifuged and the supernatant was removed. Then, the cells were resuspended in a solution of 90% of FBS and 10% of

dimethylsulfoxide (DMSO; Sigma-Aldrich), were aliquoted into vials and were subjected to a gradual decrease of temperature in a freezer (temperature of -80°C). The cells were subsequently transferred into liquid nitrogen for long-term storage.

Cell counting and analyzing

In passage 1, the cells were seeded in the proliferation medium into tissue culture polystyrene plates and cultured for 7 days. On days 1, 3, and 7, Vi-CELL XR Cell Viability Analyzer was used to count the number, viability and diameter in suspension of the previously detached ADSCs. Subsequently, the cell population doubling time (DT) was calculated from cell numbers according to the following equation: $DT = t \times \ln(2) / (\ln(N) - \ln(N_0))$, where t represents the duration of culture, N represents the number of cells on day 3, and N_0 represents the number of cells on day 1.

Cell mitochondrial activity

In passage 1, the cells were seeded in the proliferation medium into tissue culture polystyrene plates and cultured for 7 days. The activity of mitochondrial enzymes was measured in order to estimate the cell proliferation activity. On days 3 and 7, a CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS; Promega Corporation) was performed according to the manufacturer's protocol. The principle of the MTS assay is based on a colorimetric change of the yellow tetrazolium salt to brown formazan. This change is brought about by the activity of mitochondrial enzymes and the absorbance of the solution at a wavelength of 490 nm is being measured by a spectrophotometer.

Flow cytometry analysis

In passage 2, a phenotypic analysis of the cells was performed by flow cytometry. The analysis was performed on living cells that were previously detached by trypsin-EDTA solution from a culture flask and were pre-treated with FITC-, Alexa488-, Alexa647-, or PE-conjugated monoclonal antibodies against CD105, CD90, CD73, CD29, CD146, CD45, CD34, and CD31. The percentage of cells positive for given CD markers was measured. Unstained cells were used as a negative control.

3.2. Soft tissue engineering

For the use of ADSCs in vascular tissue engineering, the optimal composition of the culture medium supporting differentiation of ADSCs towards VSMCs has been studied. Some of the results concerning optimization of medium composition have been

summarized in a study by Bačáková *et al.* 2018b. Subsequently, in external collaboration, newly-developed PLLA materials were produced and the use of ADSCs was studied for a potential construct of the *tunica media* of blood vessel wall. The ADSCs-material interactions were compared with VSMCs-material interactions. Detailed conditions of studied characteristics and compared parameters summarizing this project are described in Trávníčková *et al.* in press.

3.2.1. Optimization of the differentiation medium

Prior to cell seeding on biomaterials, the optimal composition of the differentiation medium had been studied to ensure proper differentiation of isolated ADSCs towards VSMCs.

First set of experiments aimed to investigate a potential effect of (i) DMEM supplemented with 10% (vol/vol) FBS, (ii) Smooth Muscle Cell Growth Medium-2 (SMGM2; Lonza) or (iii) Endothelial Cell Growth Medium 2 (EGM2; Lonza) on ADSCs differentiation towards VSMCs. Due to the weak differentiation response of ADSCs, second set of experiments focused on the additional use of medium supplements, specifically TGF β 1 and BMP4. The ADSCs were grown in the proliferation medium. On day 3 (i.e. when the cells reached 70% confluence), the medium was changed with DMEM supplemented by 2% (vol/vol) FBS, by 2.5 ng/mL TGF β 1 (Abcam) and variably by 2.5 ng/mL BMP4 (Sigma-Aldrich). The differentiation experiment was adjusted for 14 days and the medium without growth factors, i.e. DMEM supplemented by 2% (vol/vol) FBS, was used as a control. The medium was being replaced twice a week. Alternatively, the additional use of ascorbic acid in the differentiation medium has been applied for further studies.

The results concerning optimizing the composition of differentiation medium have been summarized in Bačáková *et al.* 2018b.

3.2.2. Biocompatibility of modified PLLA foils

Material preparation, characterization and subsequent cell seeding

Modified PLLA foils were prepared and characterized in external collaboration by Ing. Nikola Slepíčková Kasálková, Ph.D., Ing. Petr Slepíčka, Ph.D. and prof. Ing. Václav Švorčík, DrSc. (Department of Solid State Engineering, University of Chemistry and Technology, Prague). In brief, pristine PLLA circular foils were treated by Ar⁺ plasma for 240 s (the samples were labelled as PLLA240). Some of the plasma-treated samples were

then chemically grafted by immersing them into a solution of polyethylene glycol (PEG) or of dextran (Dex). The non-bonded PEG or Dex was removed by immersing the samples into distilled water.

The wettability of the samples was measured by water drop contact angle method during the ageing time of the samples (i.e. 50 days). The surface morphology and the roughness of samples were visualized by atomic force microscopy (AFM) and chemical composition of surface layer was measured by X-ray Photoelectron Spectroscopy (XPS).

Cell models and culture conditions

The human ADSCs and porcine VSMCs were used for seeding on modified PLLA foils considered for vascular tissue engineering applications. The origin, isolation and characterization of human ADSCs were described in chapter 3.1.1. The porcine VSMCs were isolated from aorta and characterized by Mgr. Elena Filova, PhD. (Institute of Physiology, Department of Biomaterials and Tissue Engineering). Prior to cell seeding, the materials were sterilized in 70% ethanol. The cells were seeded in the proliferation medium. As the cells reached 70% confluence, the proliferation medium was partially changed with the “non-differentiation” medium (i.e. DMEM supplemented with 10% (vol/vol) FBS) or with “differentiation” medium (DMEM supplemented with 2% (vol/vol) FBS containing TGF β 1, BMP4, and ascorbic acid (AA)) both in ADSCs and in VSMCs (**Fig. 7**). The experiment was carried out for 3 weeks. Detailed conditions of the experimental work summarizing this project have been described in Trávníčková *et al.* in press.

3.2.3. Methods common for soft tissue engineering

Metabolic activity of the cells

Conversion of resazurin (Sigma-Aldrich) was used to measure the metabolic activity of the cells that can be considered as an indirect indicator of cell proliferation. The assay is based on the colorimetric change of blue resazurin to pink resorufin while incubating the cells with resazurin solution. This change is brought about by the activity of mitochondrial enzymes of the viable cells and the fluorescence of the solution (Ex/Em = 530/590 nm) is being measured by a fluorescence reader.

(Immuno)fluorescence staining

(Immuno)fluorescence staining was used to visualize the cells during the process of adhesion, growth and differentiation on modified PLLA foils. Prior to all types of

(immune)fluorescence staining, the cells were fixed with 4% paraformaldehyde. The cells were then pre-treated in a solution of bovine serum albumin (BSA) and TritonX-100, and were subsequently incubated in Tween 20 to enable permeabilization of cell membrane and to avoid non-specific binding of antibodies to proteins. **Table 5** shows the listing of fluorescent dyes and antibodies used in this experimental work to visualize cell cytoplasm, cell nuclei, actin cytoskeleton and markers specific for VSMCs maturation.

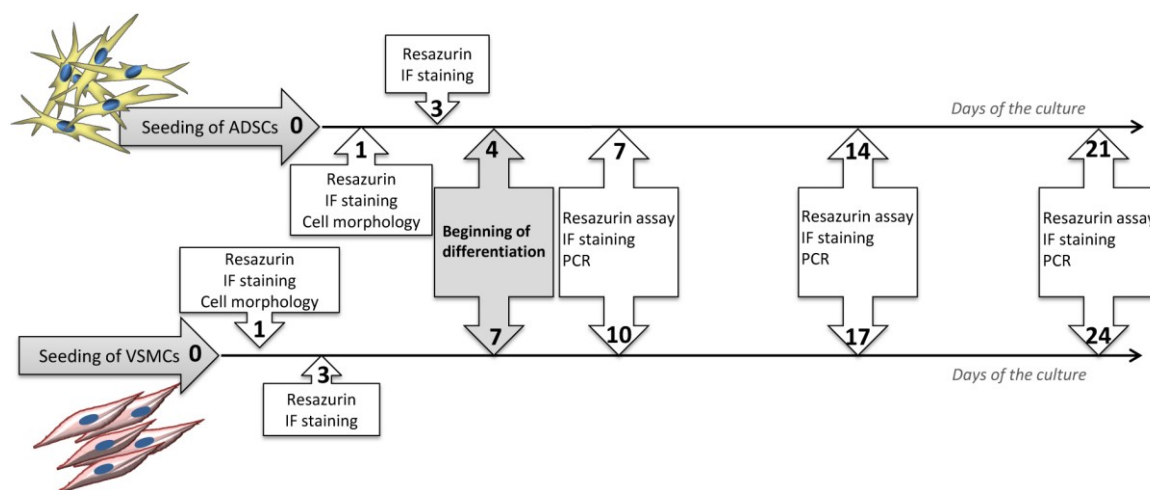


Fig. 7. Scheme of the experimental work studying the adhesion, proliferation and differentiation of ADSCs and VSMCs on newly developed PLLA foils that were variably modified by plasma-treatment, and subsequently by PEG or by Dex.

Image analysis – cell counting and evaluation of cell morphology

Image analysis was performed by ImageJ Software. Hoechst counterstaining of the cell nuclei was used to manually/automatically count the cells from multiple randomly selected microscopic fields in appropriate time intervals. Cell population DT was counted based on the cell numbers according to a similar equation as in section 3.1.1.

The cell morphology during adhesion was evaluated by the spreading area, the circularity, the aspect ratio, and the solidity of the ADSCs and VSMCs on day 1. The analyzed parameters were counted in ImageJ Software according to the following calculations; i.e. circularity: $4\pi \times \text{area}/\text{perimeter}^2$, aspect ratio: major axis/minor axis, solidity: area/convex area. The image analysis was performed in cooperation with Mgr. Antonín Sedlář (Institute of Physiology, Department of Biomaterials and Tissue Engineering).

Table 5. *Fluorescent dyes and antibodies used in the experimental work (catalogue numbers and specific dilutions have been listed in Trávníčková *et al.* in press).*

Type	Name	Producer
Fluorescent dyes	Texas Red C2-maleimide	Invitrogen
	Phalloidin-TRITC	Sigma-Aldrich
	Hoechst 33258	Sigma-Aldrich
Primary antibodies	Anti-vinculin (mouse)	Sigma-Aldrich
	Anti- α SMA (mouse)	Sigma Aldrich
	Anti-calponin (rabbit)	Abcam
	Anti-type I collagen (rabbit)	CosmoBio
	Anti-SM-MHC (mouse)	Santa Cruz Biotechnology
Secondary antibodies	Anti-rabbit IgG-Alexa Fluor 488	Thermo Fisher Scientific
	Anti-mouse IgG-Alexa Fluor 488	Thermo Fisher Scientific
	Anti-mouse IgG-Alexa Fluor 546	Thermo Fisher Scientific

RNA isolation and PCR

Total RNA isolation was performed using a Total RNA Purification Plus Micro Kit (Norgen Biotek) according to the manufacturer's protocol. The RNA concentration and purity was evaluated measuring the absorbance at 260 and 280 nm by spectrophotometer. Subsequent reverse transcription was performed using an Omniscript Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol with the use of random hexamers (New England Biolabs). The mRNA levels were quantified with hydrolysis probes specific to COL1, ACTA2, CNN1, SMTN, and MYH11. β 2 microglobulin (B2M) was used as a reference gene. Real-time quantitative PCR analysis was performed in cooperation with Mgr. Antonín Sedlár (Institute of Physiology, Department of Biomaterials and Tissue Engineering) and detailed specification of the probes and of the protocol has been described in Trávníčková *et al.* in press.

3.3. Hard tissue engineering

In this part, the optimization of osteogenic differentiation medium for ADSCs was studied. Moreover, special emphasis was placed on the suitability of FGF2 during osteogenic differentiation of ADSCs (Trávníčková and Bačáková 2018).

At the same time, the biocompatibility of newly developed metal-doped diamond-like carbon (DLC) layers was tested. These DLC layers were investigated with respect to their potential application as a coating for metal joint replacements hence the samples were seeded with multiple cell types involved/used in bone tissue engineering. Detailed conditions of studied characteristics and compared parameters summarizing this project are described in Jelínek *et al.* 2016a, Jelínek *et al.* 2017, Trávníčková *et al.* 2020b.

3.3.1. The impact of FGF2 during osteogenic differentiation

For the use of ADSCs in bone tissue engineering, the optimal composition of the culture medium supporting differentiation of ADSCs towards osteoblasts has been studied. Namely, the impact of FGF2 pre-treatment and its role during osteogenic differentiation was studied. Moreover, the potential benefit of various osteogenic supplements has been investigated.

Cell models and culture conditions

The ADSCs (previously isolated) were grown in the proliferation medium, i.e. DMEM supplemented with 10% (vol/vol) FBS and with 10 ng/mL FGF2. On day 3, the cells reached 70% confluence and the growth medium was changed with differentiation media of various compositions. The differentiation medium was supplemented with dexamethasone (DXM), β -glycerophosphate (BGCP), ascorbic acid (AA), variably also with L-glutamin (L-GLU) and vitamin D3 (vit. D3) (for detailed composition of media, see **Table 6**). The medium without osteogenic supplements was used as a control. The experiment was carried out for 27 days.

Detailed conditions of the experimental work summarizing this project have been described in Trávníčková and Bačáková 2018.

Table 6. Composition of the culture media (A-I) used in our experiment.

medium	A	B	C	D	E	F	G	H	I
DMEM + 10% FBS	+	+	+	+	+	+	+	+	+
FGF2 (5 or 10 ng/mL)	-	-	-	10 ng	10 ng	10 ng	5 ng	5 ng	5 ng
DXM (10 nM)	-	+	+	-	+	+	-	+	+
BGCP (10 mM)	-	+	+	-	+	+	-	+	+
AA (50 µg/ml)	-	+	+	-	+	+	-	+	+
L-GLU (2 mM)	-	-	+	-	-	+	-	-	+
vit. D3 (1 µM)	-	-	+	-	-	+	-	-	+

3.3.2. Biocompatibility of Cr-doped and Ti-doped DLC layers

Material preparation and characterization

The DLC layers were prepared and characterized in external collaboration by Ing. Tomáš Kocourek, Ph.D. and by prof. Ing. Miroslav Jelínek, DrSc. (Department of Optical and Biophysical Systems, Institute of Physics, Czech Academy of Sciences, Prague). In brief, the Cr-doped and Ti-doped DLC layers were prepared by a hybrid laser technology combining simultaneous pulsed laser deposition and magnetron sputtering on fused silica substrates. The layers were prepared containing different atomic percent (at. %) of metals. Specifically, Cr-doped layers contained 0.9, 1.8, 7.3 or 7.7 at. % Cr and Ti-doped layers contained 0.4, 2.1, 3.7, 6.6 or 12.8 at. % of Ti (for a detailed description of fabrication process and material characterization, see Jelínek *et al.* 2015, Jelínek *et al.* 2016a, Jelínek *et al.* 2016b, Jelínek *et al.* 2017). In all the experiments, the metal-doped materials were compared with (i) pure DLC, (ii) glass without any deposited DLC layer, and (iii) tissue culture polystyrene (PS).

Cell models and culture conditions

Cr-doped and Ti-doped DLC layers were fabricated as a coating improving physicomechanical properties mainly of bone and joint implants. The following experiments covering the biocompatibility and osteogenic potential of these layers were therefore performed either with osteoblast-like cell lines or with primary undifferentiated cells suitable for subsequent bone tissue engineering applications. Prior to every cell seeding, the tested samples were sterilized in 70% ethanol.

- Saos-2 cells (purchased from ATCC), a cell line originating from primary osteosarcoma, were used for initial seeding experiments to test potential cytotoxicity of Ti-doped samples in a 7-day culture. In brief, the cells were seeded and cultured in McCoy's 5A medium supplemented with 15% (vol/vol) FS. The cell adhesion was

evaluated by the cell spreading area according to immunofluorescence staining, proliferation was measured by cell counting, and osteogenic differentiation was observed by immunofluorescence staining (Jelínek *et al.* 2016a, Jelínek *et al.* 2017).

- Commercially available human bone marrow mesenchymal stem cells (bmMSCs) (ScienCell Research Laboratories) were used as a model of immature primary cells to observe the impact of Cr-doped DLC layers on cell adhesion, proliferation, and osteogenic differentiation. In brief, the cells were seeded in the proliferation medium (i.e. α -MEM medium), supplemented with 15% (vol/vol) FBS, L-glutamine (2mM) and gentamicin. On day 3, the proliferation medium was enriched with dexamethasone (10 nM), β -glycerolphosphate (20mM) and ascorbic acid (50 μ M) to initiate osteogenic differentiation of bmMSCs. The culture medium was changed twice a week and the proliferation and osteogenic differentiation was analyzed within 21 days of the culture (Trávníčková *et al.* 2020b).

- ADSCs (previously isolated) were used as a model of immature primary cells to investigate the impact of Ti-doped DLC layers on cell proliferation and osteogenic differentiation. In brief, the cells were seeded in the proliferation medium (i.e. DMEM), supplemented with 10% (vol/vol) FBS. The proliferation medium was also supplemented with gentamicin and with 10 ng/mL FGF2. On day 3, the proliferation medium was enriched with dexamethasone (10 nM), β -glycerolphosphate (20mM), L-glutamin (2mM), vitamin D3 (1 μ M), and ascorbic acid (50 μ M) to initiate osteogenic differentiation of the cells. The culture medium was changed twice a week and the proliferation and osteogenic differentiation was analyzed within 21 days of the culture (Trávníčková *et al.* in preparation).

3.3.3. Methods common for hard tissue engineering

Metabolic activity of the cells

Similarly to section 3.2.3., the proliferation of the cells was estimated based on the conversion of resazurin. The detailed procedure and incubation times are specified in (Trávníčková *et al.* 2020b).

(Immuno)fluorescence straining

The method was used to visualize the cell cytoplasm, cell nuclei, cytoskeleton of the cells, and focal adhesions during cell adhesion. Special emphasis was put on visualization of specific markers involved in a process of osteogenic differentiation.

Prior to staining, the cells on the materials were fixed with 70% ethanol (temperature of -20°C) or with 4% paraformaldehyde. Similarly to section 3.2.3., the cells were then pre-treated with BSA, TritonX-100, and Tween 20 to enable permeabilization of cell membrane and to avoid non-specific binding of antibodies to proteins. **Table 7** shows the listing of fluorescent dyes and antibodies used in this experimental work.

Table 7. *Fluorescent dyes and antibodies used in the experimental work (catalogue numbers and specific dilutions are listed in Jelínek et al. 2016a, Jelínek et al. 2017, Trávníčková et al. 2020b).*

Type	Name	Producer
Fluorescent dyes	Texas Red C2-maleimide	Invitrogen
	Phalloidin-TRITC	Sigma-Aldrich
	Hoechst 33258	Sigma-Aldrich
Primary antibodies	Anti-vinculin (mouse)	Sigma-Aldrich
	Anti-type I collagen (mouse, rabbit)	CosmoBio
	Anti-osteocalcin (rabbit)	Peninsula Laboratories
	Anti-osteopontin (rabbit)	Enzo life Sciences
Secondary antibodies	Anti-rabbit IgG-Alexa Fluor 488	Thermo Fisher Scientific
	Anti-rabbit IgG-Alexa Fluor 546	Thermo Fisher Scientific
	Anti-mouse IgG-Alexa Fluor 488	Thermo Fisher Scientific
	Anti-mouse IgG-Alexa Fluor 546	Thermo Fisher Scientific

Image analysis

Hoechst counterstaining of the cell nuclei was used to manually count the cell number from multiple randomly selected microscopic fields in appropriate time intervals within the cell culture.

Cell spreading area indicating the rate of cell adhesion to materials was measured by Atlas Tescan software. The measuring was performed on the photographs of cells stained with Texas Red C2-maleimide staining on day 1 after seeding.

ALP activity assay

In specific time intervals, the ALP activity of the cells was measured by incubating with a substrate solution, i.e. 0.1 mg/ml p-nitrophenyl phosphate in the substrate buffer (50 mM glycine, 1 mM MgCl₂, pH 10.5) (Sigma-Aldrich). Then the solution was added into 1M NaOH (ratio of 1:1). A calibration curve was prepared using 9-90 µM p-

nitrophenol (Sigma-Aldrich). The absorbance of the samples was measured at 405 nm. The results were then normalized to the cell metabolic activity previously measured by the resazurin assay. Exact procedure and specification of incubation time is given in (Trávníčková and Bačáková 2018, Trávníčková *et al.* 2020b).

Calcium colorimetric assay

To measure deposition of calcium, the cells were dried for 3 hours, and were then incubated on a shaker in 0.5 M HCl at 4°C overnight. Supernatants obtained after centrifugation of the cell lysates were analyzed using a Calcium Colorimetric Assay (MAK022-1KT, Sigma-Aldrich) according to the standard manufacturer's protocol. The results were then normalized to the cell metabolic activity on the same day.

PCR analysis

For an evaluation of the osteogenic differentiation, real-time quantitative PCR was used. Specifically, the changes in the expression of genes for RUNX2, type I collagen, ALP, and osteocalcin were studied.

Total RNA was extracted from the cells using Total RNA purification Micro Kit (NORGENE Biotek Corp) according to the manufacturer's protocol. The mRNA concentration was measured using NanoPhotometer™ S/N. The cDNA was synthesized with the ProtoScript®M-MuLV First Strand cDNA Synthesis kit (New England BioLabs). The PCR primers were purchased from Generi Biotech Ltd. Real-time PCR was performed using SYBR Green (Roche) in the iCycler detection system (iQ™ 5 Multicolor Real-Time PCR Detection System, Bio-Rad). Data were analyzed by the $2^{-\Delta\Delta C_t}$ method.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene. The data were related to the bmMSCs grown on the PS on day 3 before adding the differentiation medium. A list of primers and detailed description of PCR analysis setting is stated in Trávníčková *et al.* 2020b.

3.4. Statistical analysis

The data having normal distribution are expressed as mean + SD (Standard Deviation) or as mean + SEM (Standard Error of Mean). The data failing normal distribution are expressed in box plots or as the median value and the interquartile range (IQ range). Three parallels from each sample type were used unless otherwise specified in figure captions.

Comparison of 2 samples:

For the data having normal distribution, an unpaired two-sample *t*-test was used, $p \leq 0.05$ unless otherwise specified. For the data failing normal distribution, a Mann-Whitney Rank Sum test was used, $p \leq 0.05$ unless otherwise specified.

Multiple comparisons of 3 and more samples:

For the data having normal distribution, multiple comparisons among the studied samples were performed by One Way ANOVA, Student-Newman-Keuls test, $p \leq 0.05$ unless otherwise specified. For the data failing normal distribution, the multiple comparisons among the studied samples were performed by ANOVA on RANKs, Dunn's method, $p \leq 0.05$ unless otherwise specified.

The plots were generated in MS Excel 2010 (Microsoft), in SigmaPlot 10.0 software (Systat Software Inc.), in GraphPad Prism 8 (GraphPad Software, Inc.) or in R studio. Statistical analyses were performed using SigmaStat 3.5 software (Systat Software Inc.) or GraphPad Prism 8. The use of specific statistical software and detailed statistical analyses have been described within individual articles.

4. Results

The results summarize the main findings concerning (i) the optimal harvesting conditions of ADSCs for further use in tissue engineering applications, (ii) the use of ADSCs in combination with newly developed materials for vascular tissue engineering application and (iii) the use of ADSCs in combination with newly developed materials for bone tissue engineering application.

Results are based on 5 articles with IF, 2 reviews containing our results with IF, 1 book chapter containing our results, and 1 manuscript in preparation.

4.1. Cell characterization

4.1.1. Characteristics of ADSCs regarding to the harvesting site and to negative pressure used during a liposuction procedure

(i) Bačáková L, Zárubová J, Trávníčková M, Musílková J, Pajorová J, Slepíčka P, Slepíčková Kasálková N, Švorčík V, Kolská Z, Motarjemi H, Molitor M. ***Stem cells: their source, potency and use in regenerative therapies with focus on adipose-derived stem cells - a review.*** *Biotechnol Adv.* 2018;36(4):1111-1126. doi: 10.1016/j.biotechadv.2018.03.011. (Published review with the Results section)

(ii) Trávníčková M, Pajorová J, Zárubová J, Kročilová N, Molitor M, Bačáková L. ***The Influence of Negative Pressure and of the Harvesting Site on the Characteristics of Human Adipose Tissue-Derived Stromal Cells from Lipoaspirates.*** *Stem Cells Int.* 2020;2020:1016231. doi: 10.1155/2020/1016231. (Published primary article)

Author's contribution: (i) Martina Trávníčková contributed with the initial results of cell isolation, characterization and differentiation, and drafted appropriate parts of the manuscript concerning these experiments (Bačáková *et al.* 2018). (ii) In cooperation with other co-authors, Martina Trávníčková performed the isolation procedures and following characterization experiments (i.e. cell counting to estimate cell yields after isolation, measurements of cell proliferation, viability and diameter, and flow cytometry analysis). She analyzed the data (i.e. including the plots and statistical analyses) and drafted the manuscript. She was also responsible for drafting the revised and final version of the manuscript (Trávníčková *et al.* 2020a).

The use of ADSCs in regenerative medicine is complicated with high variability in their characteristics which can also be influenced by many factors during a liposuction procedure. This study was focused on two important factors of liposuction procedure (i.e. the anatomical harvesting site within the donor and the value of negative pressure used for liposuction). Two types of negative pressure (i.e. -200 mmHg and -700 mmHg) and three harvesting sites (i.e. inner thigh, outer thigh and abdomen) have been studied regarding to the optimal source of adult mesenchymal stem cells for tissue engineering applications/purposes. Due to a relatively small number of samples from the inner thigh region, further statistical comparison has been made between the outer thigh region and the abdomen region only. **Fig. 8A** shows the representative images of the process of adhesion and growth in ADSCs after isolation from the same patient. No influence of negative pressure on cell yields was observed in the case of the outer thigh region (**Fig. 8B**). However, in case of the abdomen region, the cell yields were higher under high negative pressure than under low negative pressure (**Fig. 8B**). Summarizing the data from all donors, we counted higher initial yields of attached cells from the outer thigh region than from the abdomen region (**Fig. 8C**). Interestingly, in subsequent passages, no significant influence of the used negative pressure on later cell characteristics was identified. Similarly, almost no differences between the ADSCs from the studied harvesting sites reached statistical significance. Specifically, flow cytometry revealed similar phenotype profile in all studied cell groups (**Table 8**). Moreover, similar results among the compared ADSCs were obtained for proliferation estimated by cell number counting on days 1 and 3 (**Fig. 9**) and the ADSCs were characterized by almost identical initial doubling time between days 1 and 3, i.e. from 24.99 (low abdomen region) to 28.65 hours (high inner thigh region). We only observed a tendency in the higher cell number of thigh-ADSCs than of abdomen-ADSCs if the thigh-ADSCs (i.e. outer thigh-ADSCs and inner thigh-ADSCs) were evaluated together (**Fig. 9**). Our results showed that although there can be potential differences in initial ADSCs yields depending on the harvesting site, later *in vitro* proliferation and phenotypic characteristics of ADSCs is almost unaffected by the harvesting site or by the negative pressure used during liposuction. These finding concerning similar proliferation potential of the isolated cells are important for tissue engineering purposes where a large quantity of multiplied cells are needed, e.g. for scaffold seeding. For tissue engineering purposes, guided differentiation is another important factor. Similar basic phenotypic characteristics of ADSCs independently of

harvesting site or of negative pressure predetermine the isolated cells to behave similarly in further differentiation experiments.

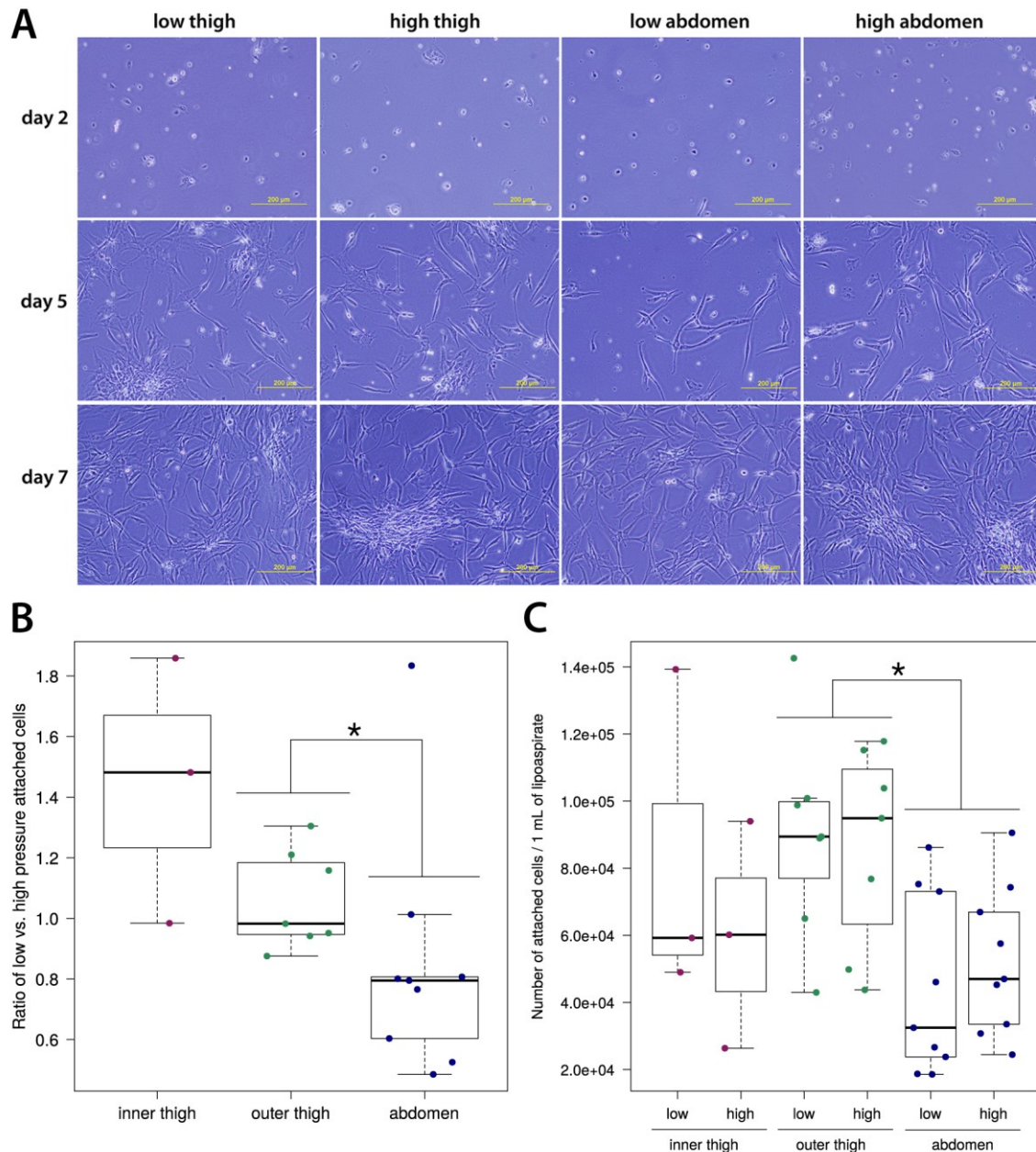


Fig. 8. (A) The process of attachment, spreading and growth in thigh ADSCs and in abdomen ADSCs from the same patient on specific days after the isolation procedure. The cells were isolated from the inner thigh region and from the abdomen region both under low negative pressure (-200 mmHg) and under high negative pressure (-700 mmHg). Scale bar 200 μ m. (B) Cell yields expressed as a ratio of low pressure cells to high pressure cells for each donor on day 1 after isolation. (C) Cell yields expressed as the number of attached cells per 1 mL of original lipoaspirate (Trávníčková et al. 2020a).

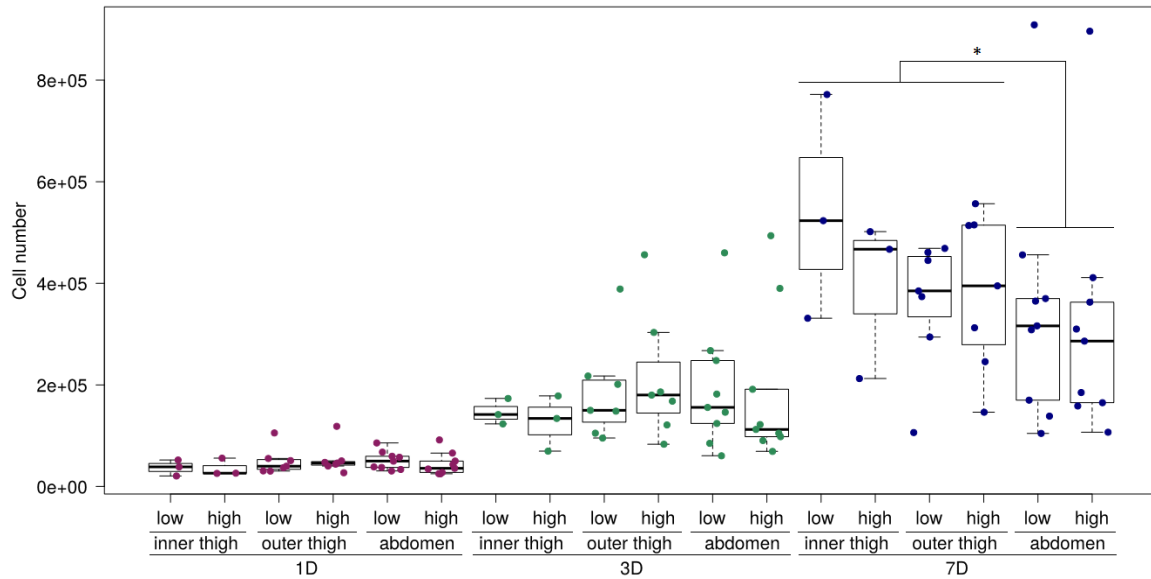


Fig. 9. The number of isolated ADSCs. The cells were automatically counted on days 1, 3 and 7 in passage 1. *The number of thigh ADSCs (i.e. inner thigh ADSCs and outer thigh ADSCs together) was significantly higher ($p < 0.05$) than the number of abdomen ADSCs (Trávníčková et al. 2020a).

Table 8. The percentage of ADSCs positive for specific CD surface markers* (Trávníčková et al. 2020a).

Group of cells	CD markers (% positive cells)							
	CD105		CD90		CD73		CD29	
	Median	IQ range	Median	IQ range	Median	IQ range	Median	IQ range
Low I thigh	99.9	(99.2-99.9)	99.5	(99.5-99.7)	99.9	(99.8-100)	99.8	(99.2-100)
High I thigh	99.9	(94.1-99.9)	99.6	(99.3-99.8)	99.9	(99.9-100)	99.8	(99.8-100)
Low O thigh	99.9	(98.3-100)	99.6	(99.2-99.9)	100	(99.9-100)	99.8	(99.8-100)
High O thigh	99.9	(96.2-99.9)	99.6	(99.2-99.9)	100	(99.9-100)	99.9	(99.8-100)
Low abdomen	99.5	(82.3-99.9)	99.4	(97.5-99.8)	99.8	(99.6-99.9)	99.6	(90.5-99.8)
High abdomen	98.9	(89.1-99.8)	99.5	(97.3-99.8)	99.8	(99.6-99.9)	99.6	(95.1-99.8)
Group of cells	CD markers (% positive cells)							
	CD146		CD45		CD31		CD34	
	Median	IQ range	Median	IQ range	Median	IQ range	Median	IQ range
Low I thigh	3.9	(2.9-4.5)	4.3	(3.9-4.7)	0.5	(0.3-1.0)	0.4	(0.3-0.7)
High I thigh	6.0	(2.5-7.4)	3.3	(2.9-4.0)	0.8	(0.4-1.0)	0.8	(0.4-1.7)
Low O thigh	3.9	(1.3-5.2)	1.8	(1.5-6.9)	0.5	(0.2-0.7)	1.1	(0.4-6.3)
High O thigh	5.4	(2.7-24.6)	1.8	(1.1-12.6)	0.6	(0.1-2.4)	0.9	(0.3-6.1)
Low abdomen	10.9	(3.4-35.4)	5.2	(4.5-7.5)	0.3	(0.2-0.5)	1.0	(0.5-1.6)
High abdomen	4.7	(2.6-28.5)	4.1	(3.1-5.4)	0.4	(0.2-1.0)	0.9	(0.5-1.7)

*No significant differences were observed between the harvesting sites, i.e. inner (I) thigh, outer (O) thigh and abdomen region or between low negative pressure (-200 mmHg; Low) and high negative pressure (-700 mmHg; High).

4.2. Soft tissue engineering

4.2.1. Differentiation of ADSCs towards VSMCs (optimization of differentiation media)

Bačáková L, Trávníčková M, Filová E, Matějka R, Štěpanovská J, Musílková J, Zárubová J, Molitor M. Vascular Smooth Muscle Cells (VSMCs) in Blood Vessel Tissue Engineering: The Use of Differentiated Cells or Stem Cells as VSMC Precursors. In: Sakuma K, editor. Muscle Cell and Tissue Current Status of Research Field. Rijeka, Croatia: IntechOpen; 2018. p. 289-308. doi: 10.5772/intechopen.77108. (Published book chapter with the Results section)

Author's contribution: Martina Trávníčková contributed with the results concerning the optimization of the differentiation medium and with manuscript drafting.

Differentiation of ADSCs towards VSMCs can be biochemically induced; however, the differentiation protocols are not uniform and specific composition of the medium and concentration of supplements vary greatly. Thus, our aim of studying various compositions of culture media was to find the optimal differentiation medium prior to seeding the newly developed materials with cells. Firstly, three types of culture media were tested, i.e. (i) DMEM, (ii) SMGM2 and (iii) EGM2. Even though SMGM2 and EGM2 contain several supplements such as EGF, VEGF, FGF2, insulin, heparin, ascorbic acid or hydrocortisone, the beneficial effect of these media on ADSCs differentiation towards VSMCs was not observed in our study. We therefore focused on the addition of TGF β 1 and/or BMP4 into a differentiation medium. The addition of TGF β 1 and BMP4 into SMGM2 or into EGM2 caused rapid proliferation followed by detachment of ADSCs from culture plates. Thus, for further differentiation experiments, the supplements were added into DMEM supplemented with 2% (vol/vol) FBS. Within 3 days, TGF β 1 alone or in combination with BMP4 triggered formation of α SMA filaments and development of calponin in majority of the ADSCs detected by immunofluorescence staining (**Fig. 10**). Moreover, the number of calponin-positive cells visually increased by day 9 (**Fig. 10**). On day 9, α SMA filaments in cells treated with supplements were also well developed in comparison with the negative control (i.e. DMEM+FBS) (**Fig. 11A**).

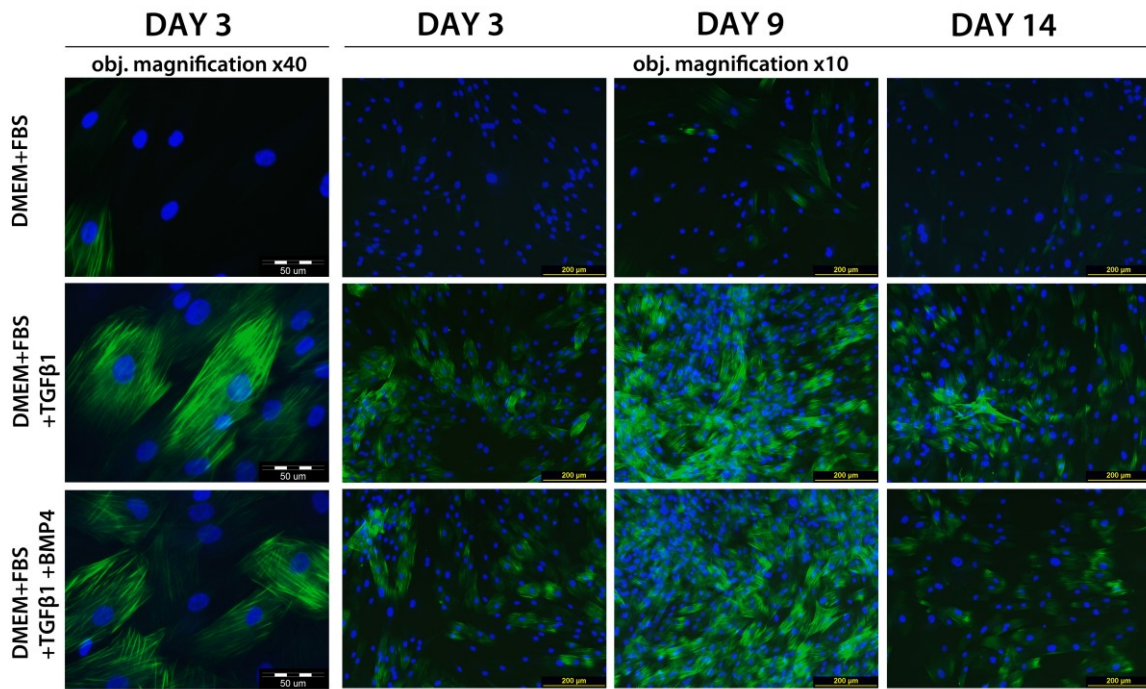


Fig. 10. The immunofluorescence staining of calponin (green) and cell nuclei (blue) in ADSCs treated with different medium composition. Olympus IX 71 microscope, objective magnification $\times 40$ (scale bar $50\ \mu\text{m}$) or $\times 10$ (scale bar $200\ \mu\text{m}$).

Interestingly, depending on the specific day of the culture, it was possible to observe different cell morphology and calponin distribution within some of the differentiated cells (i.e. polygonal cells and general distribution of calponin on day 3, but spindle-shaped cells and calponin concentrated at cell borders on day 14) (**Fig. 11B**). Moreover, several SM-MHC-positive cells were observed in later time intervals (**Fig. 11C**). Addition of TGFβ1 and BMP4 also influenced the proliferation of the ADSCs. Specifically, TGFβ1 alone increased the proliferation, but the addition of TGFβ1 and BMP4 caused similar or even lower proliferation rate than DMEM without these supplements (**Fig. 12**). Next experiment ensured the beneficial effect of ascorbic acid on synthesis of type I collagen (i.e. intracellular and extracellular) that is an important ECM protein of blood vessel walls. Based on these results, further differentiation experiments were carried out using the combination of TGFβ1 ($2.5\ \text{ng/mL}$), BMP4 ($2.5\ \text{ng/mL}$) and ascorbic acid ($50\ \mu\text{g/mL}$) in DMEM supplemented with 2% FBS.

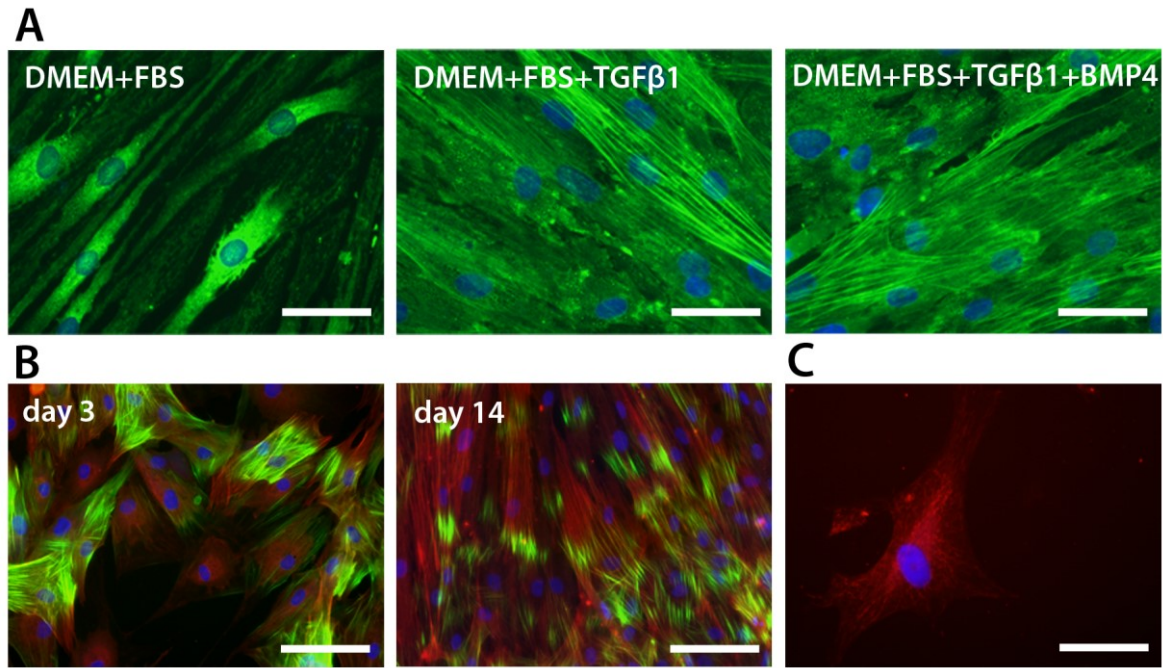


Fig. 11. (A) The immunofluorescence staining of α SMA (green) and cell nuclei (blue) in ADSCs treated with different medium composition on day 9, objective magnification $\times 40$ (scale bar $50\ \mu\text{m}$). (B) The immunofluorescence staining of α SMA (red), calponin (green), and cell nuclei (blue) in ADSCs treated with DMEM+FBS+TGF β 1+BMP4 on days 3 and 14, objective magnification $\times 20$ (scale bar $100\ \mu\text{m}$). (C) The immunofluorescence staining of SM-MHC (red) and cell nuclei (blue) in ADSCs treated with DMEM+FBS+TGF β 1+BMP4 on day 17, objective magnification $\times 40$ (scale bar $50\ \mu\text{m}$). Olympus IX 71 microscope was used for all microphotographs.

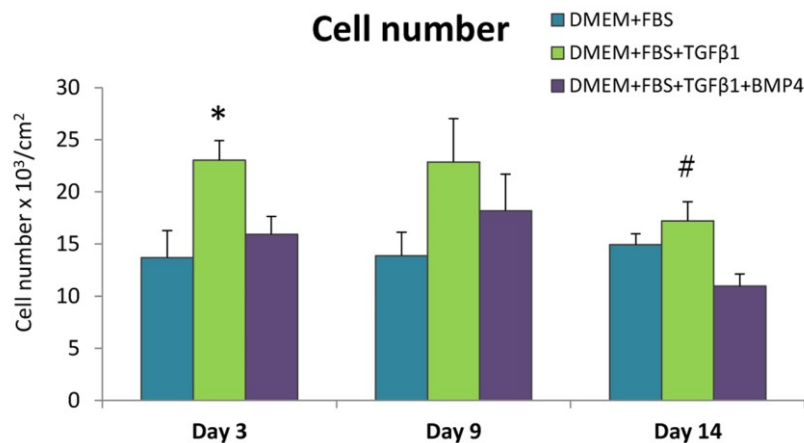


Fig. 12. The proliferation of ADSCs evaluated by cell counting on days 3, 9, and 14 after adding the differentiation media. The data are expressed as mean + SEM. ANOVA, Student-Newman-Keuls method. * means a significantly higher value ($p < 0.05$) than on all other samples on the same day of the culture. # means a significantly higher value ($p < 0.05$) than in DMEM+FBS+TGF β 1+BMP4 on the same day of the culture.

4.2.2. Differentiation of ADSCs towards VSMCs on variously modified PLLA foils for vascular tissue engineering

Trávníčková M, Slepíčková Kasálková N, Sedlář A, Molitor M, Musílková J, Slepíčka P, Švorčík V, Bačáková L. Differentiation of adipose tissue-derived stem cells towards vascular smooth muscle cells on modified poly (L-lactide) foils. (Article accepted to Biomedical Materials on 14th August 2020; currently in press)

Author's contribution: Martina Trávníčková designed the concept of the biological study. She performed or participated in all biological experiments (i.e. cell proliferation measurements, fluorescence microscopy, and PCR analysis), analyzed the data (i.e. including the plots and statistical analyses) and drafted the manuscript. She was also responsible for drafting the revised and final version of the manuscript.

PLLA is a biocompatible and slowly degradable polymer with various applications in tissue engineering (e.g. for blood vessel replacements or artificial skin grafts). However, its relatively high hydrophobicity can limit the cell adhesion and the later implant integration into the surrounding tissue. The aim of our study was to modify PLLA foils for further vascular tissue engineering applications. To ameliorate cell adhesion and later proliferation, the PLLA foils were modified by plasma treatment and alternatively subsequently grafted with PEG or Dex (i.e. molecules having beneficial role in vascular tissue engineering due to their anti-fouling, non-thrombogenic and low-immunogenic properties). The cell-material interactions were then tested with ADSCs and VSMCs either in a non-differentiation medium (i.e. medium without specific supplements) or in a differentiation medium (i.e. previously verified composition of the medium supporting differentiation towards VSMCs). Our results showed similar cell morphology within the initial adhesion (**Fig. 13**) and similar proliferation (**Fig. 14A**) of ADSCs on all PLLA samples when cultured in the non-differentiation medium. By contrast, culturing ADSCs in the differentiation medium showed significant differences in proliferation, estimated by measuring the metabolic activity, on the tested PLLA materials (**Fig. 14A**). Specifically, the highest cell numbers and proliferation was measured on plasma-treated PLLA resulting in similar values as the reference PS. The lowest values were obtained for pristine PLLA. Interestingly, these differences in cell growth were similar to those obtained in cultures of VSMCs. On pristine PLLA, the

VSMCs showed round morphology (**Fig. 13**) which indicated a worse cell adhesion to this sample, and this sample was characterized by the worst VSMCs proliferation in both types of the culture media (**Fig. 14B**).

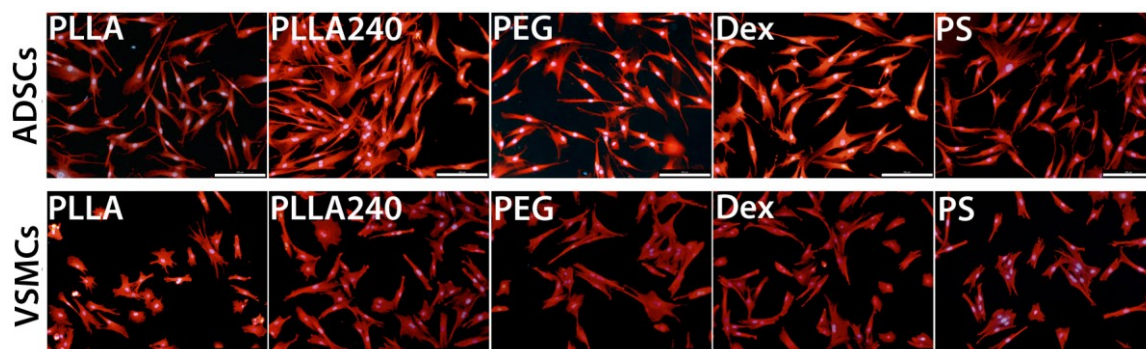


Fig. 13. The morphology of ADSCs and VSMCs on day 1 after seeding on pristine PLLA, on plasma-treated PLLA (PLLA240), on plasma-treated PLLA grafted with PEG (PEG), on plasma-treated PLLA grafted with Dextran (Dex), and on the control tissue culture polystyrene (PS). The cells were visualized by Texas Red C2-maleimide (red) and the cell nuclei were counterstained with Hoechst 33258 (blue). IX71 Olympus microscope, DP71 digital camera. Objective magnification $\times 10$, scale bar $200\ \mu\text{m}$.

Concerning the ADSCs differentiation, the main influence on the expression of specific markers was caused by the medium composition. Specifically, early, mid-term and/or late markers of differentiation (i.e. type I collagen, αSMA , calponin, smoothelin, and SM-MHC) were detected by PCR and/or by immunofluorescence staining (**Fig. 15**) in ADSCs cultured in the differentiation medium. The differentiation of ADSCs was successful on all tested samples and no significant differences in the rate of ADSCs differentiation among the samples were observed. Moreover, the differentiated ADSCs retained the early and mid-term markers even in later time intervals of the culture.

In general, our study proved very good biocompatibility of the newly modified PLLA foils verified by two different cell types (i.e. ADSCs and VSMCs) in a three-week culture. The cells formed the most stable monolayer on plasma-treated PLLA and PS control, whereas on pristine PLLA and PLLA grafted with PEG and Dex, a slight later cell detachment was observed. Although the cell behaviour on the tested materials slightly differed depending on a specific cell type and on specific culture conditions, modified PLLA seems to be suitable for vascular tissue engineering purposes.

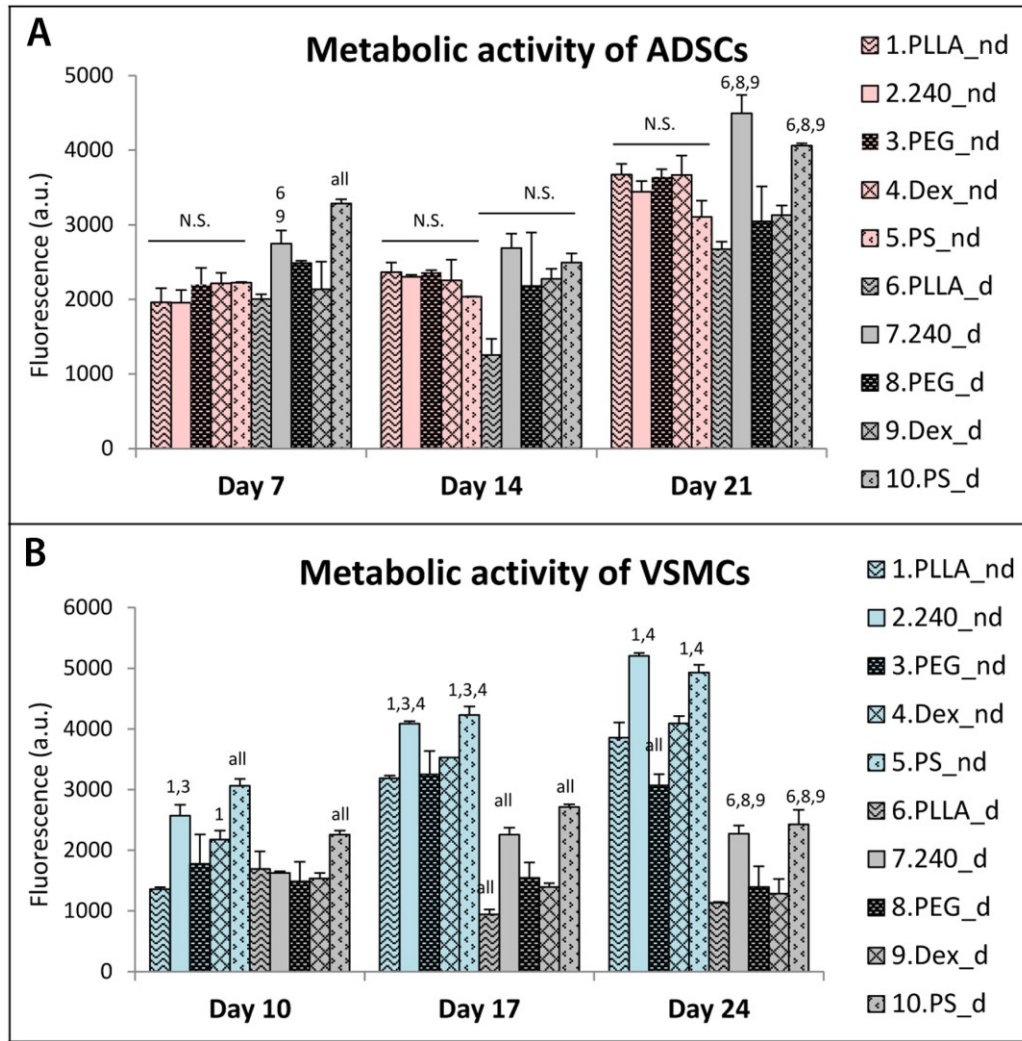


Fig. 14. The metabolic activity of the ADSCs (A) and VSMCs (B) measured by the resazurin conversion assay. The studied samples were as follows; pristine PLLA, plasma-treated PLLA (240), plasma-treated PLLA grafted with PEG (PEG), plasma-treated PLLA grafted with Dextran (Dex), and the control tissue culture polystyrene (PS). The cells were cultivated either in the non-differentiation medium (nd) or in the differentiation medium (d). Mean $\pm$ SD, One Way ANOVA, Student-Newman-Keuls test. The statistical comparison among the samples was made on cells cultured in the same medium type and on the same day of the culture. Statistically significant differences ($p \leq 0.05$) are marked above the columns by the numbers of tested groups of samples. All: statistically significant differences in comparison with all other groups of samples.

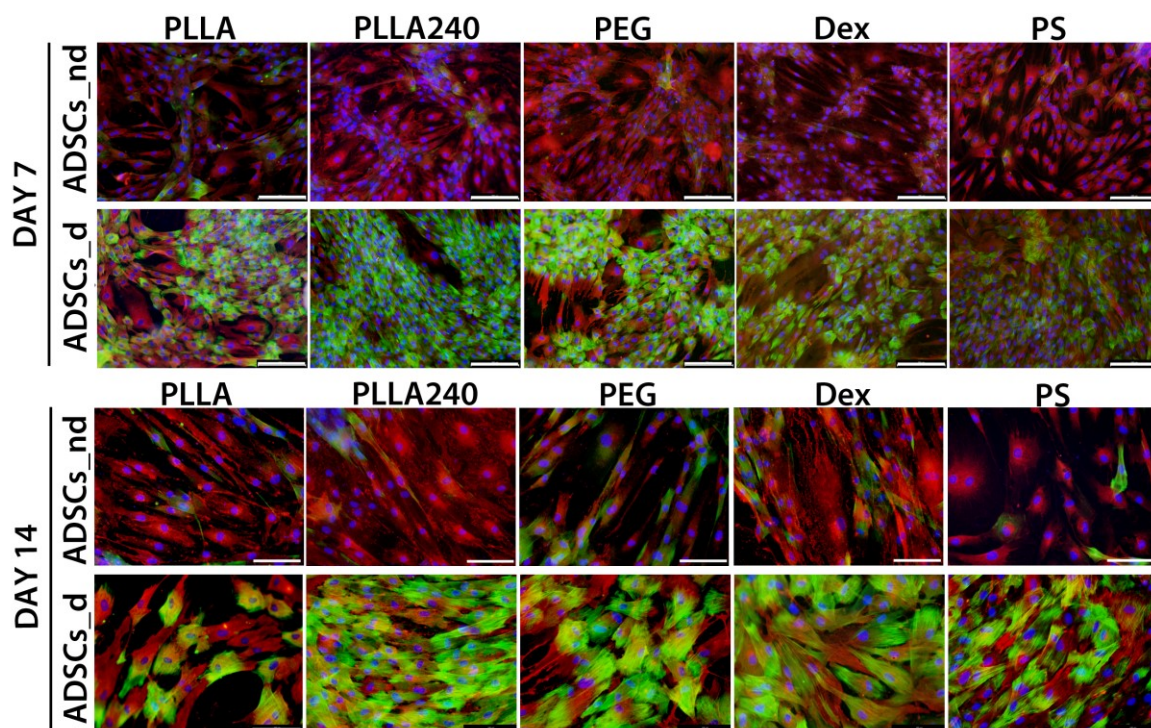


Fig. 15. Immunofluorescence staining of α SMA (red) and calponin (green) in ADSCs on day 7 and on day 14 of the culture on pristine PLLA (PLLA), plasma-treated PLLA (PLLA240), plasma-treated PLLA grafted with PEG (PEG), plasma-treated PLLA grafted with Dextran (Dex), and the control tissue culture polystyrene (PS). ADSCs_nd were cultured in the non-differentiation medium. ADSCs_d were cultured in the differentiation medium. The cell nuclei were counterstained with Hoechst 33258 (blue). IX71 Olympus microscope, DP71 digital camera, objective magnification x10 and scale bar 200 μ m (day 7), magnification x20 and scale bar 100 μ m (day 14).

4.3. Hard tissue engineering

4.3.1. The influence of FGF2 on ADSCs differentiation towards osteoblasts

Trávníčková M, Bačáková L. Application of adult mesenchymal stem cells in bone and vascular tissue engineering. Physiol Res. 2018;67(6):831-850. doi: 10.33549/physiolres.933820. (Published review with the Results section)

Author's contribution: Martina Trávníčková designed the concept of the biological study. She performed all biological experiments (i. e. cell proliferation measurements, fluorescence microscopy, PCR analysis, and assays confirming osteogenic differentiation), analyzed the data (i.e. including the plots and statistical analyses) and drafted the manuscript. She was also responsible for drafting the revised and final version of the manuscript.

In this study, the impact of FGF2 on ADSCs proliferation and osteogenic differentiation was evaluated. The cells were treated with osteogenic medium either with 10 ng/mL or 5 ng/mL of FGF2, or without FGF2. A basal medium was used as a negative control. Our results showed that the proliferation of ADSCs, estimated by measuring the metabolic activity, was higher in the presence of osteogenic supplements than in the culture medium without supplements, especially in the case of media without any FGF2 (**Fig. 16**). The lowest proliferation within the cell culture was observed in ADSCs treated with 5 ng/mL of FGF2 (**Fig. 16**). Surprisingly, the proliferation rate depended more on the addition of osteogenic supplements than on the addition of FGF2 itself.

A strong beneficial effect of supplements (i.e. dexamethasone, β -glycerophosphate and ascorbic acid) on osteogenic differentiation of ADSCs was proved by immunofluorescence staining, Ca deposition assay, and PCR analysis. The role of FGF2 was not uniform and different results were obtained for different studied markers. Specifically, the gene expression of ALP showed increasing tendency within the cell culture in all cells treated with osteogenic media. ALP activity, measured by a colorimetric assay, was significantly higher in the osteogenic medium without any FGF2. In contrast, osteocalcin (**Fig. 17**) and calcium deposition (**Fig. 18**), considered as the late markers of osteogenic differentiation, were more apparent in the cells treated with

osteogenic media additionally supplemented with 5 or 10 ng/mL FGF2. Specifically, on day 20, the highest gene expression of osteocalcin was detected in cells treated with osteogenic supplements and 5 ng/mL of FGF2 which was in accordance with the positive staining for osteocalcin on the same day of the culture (**Fig. 17**) and with the highest calcium deposition on day 27 (**Fig. 18**). Within the cell culture, we did not observe any beneficial effect of a richer osteogenic medium (i.e. with L-GLU and vitamin D3) on osteogenic differentiation.

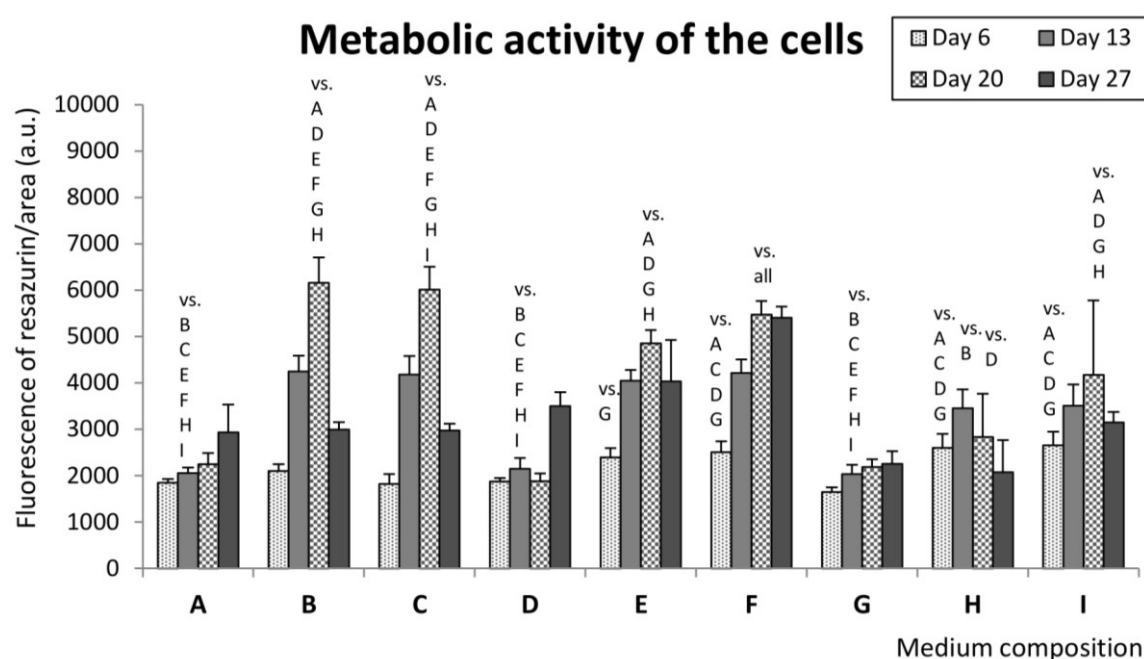


Fig. 16. The metabolic activity of ADSCs cultured in media of different compositions (A-I) on days 6, 13, 20, and 27, measured by resazurin assay. A-C: without FGF2; D-F: supplemented with 10 ng/ml of FGF2; G-I: supplemented with 5 ng/ml of FGF2. A, D, G: without osteogenic supplements; B, E, H: supplemented with dexamethasone, β -glycerophosphate, ascorbic acid; C, F, I: also with L-glutamin and vitamin D3. The data is expressed as mean + SD. ANOVA, Student-Newman-Keuls method. The statistical comparison was made on cells on the same day of the culture. The statistical significance ($p < 0.05$) is specified above the columns (in comparison with samples on the same day of the culture (Trávníčková and Bačáková 2018)).

Our study proved the ability of isolated ADSCs to differentiate towards osteoblasts in osteogenic media both with and without FGF2. Although the results obtained by measuring various osteogenic markers were not completely uniform, several methods showed a beneficial effect of lowering the concentration of FGF2 during differentiation.

Specifically, the ADSCs were cultured and pre-treated by the culture medium containing 10 ng/mL of FGF2; however, the late osteogenic markers were preferentially present in the osteogenic medium containing 5 ng/mL of FGF2 rather than 10 ng/mL of FGF2. Nevertheless, even the ADSCs differentiated in the osteogenic medium without FGF2 developed a satisfactory osteogenic response.

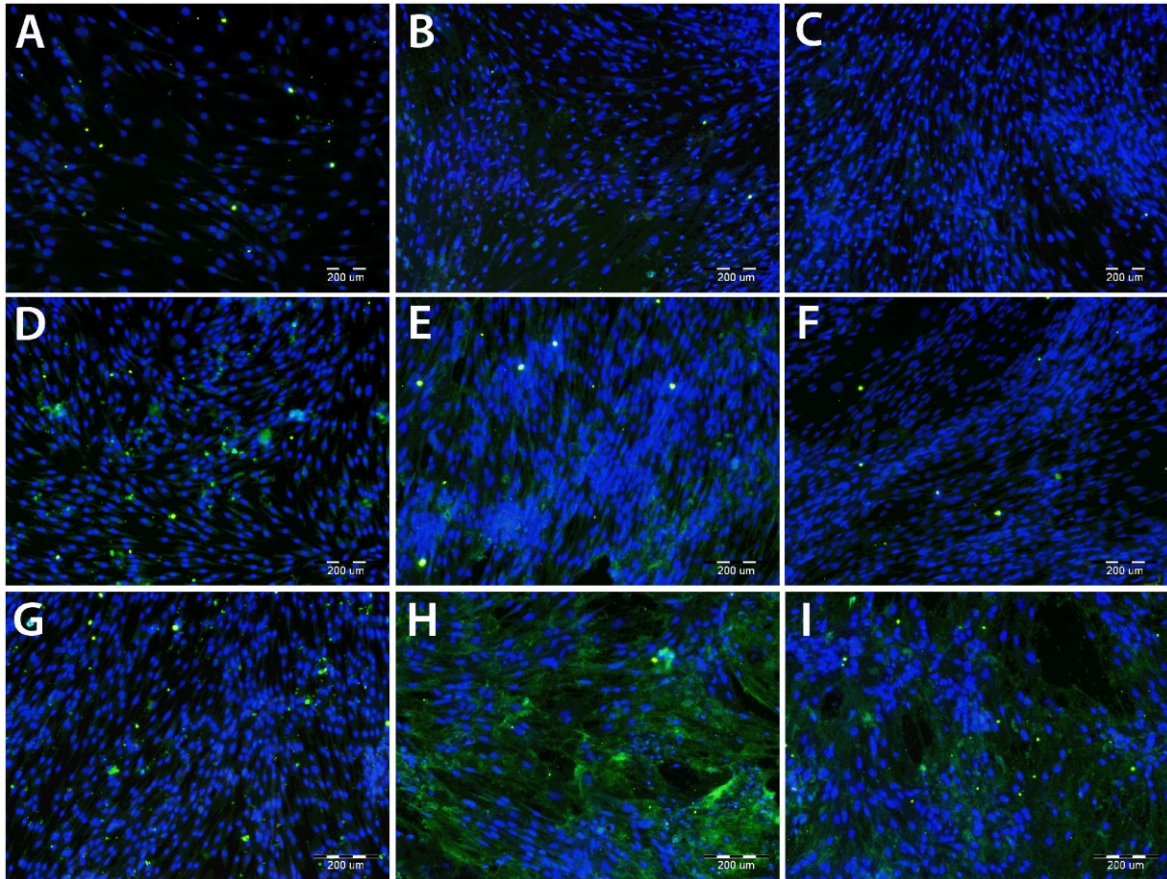


Fig. 17. The immuno-fluorescence staining of osteocalcin (green) and cell nuclei (blue) in ADSCs cultured in media of different compositions (A-I) on day 20. A-C without FGF2; D-F supplemented with 10 ng/ml of FGF2; G-I supplemented with 5 ng/ml of FGF2. A, D, G without osteogenic supplements; B, E, H supplemented with dexamethasone, β -glycerophosphate, ascorbic acid; C, F, I also with L-glutamin and vitamin D3. Olympus IX 71 microscope, objective magnification $\times 10$, scale bar 200 μ m (Trávníčková and Bačáková 2018).

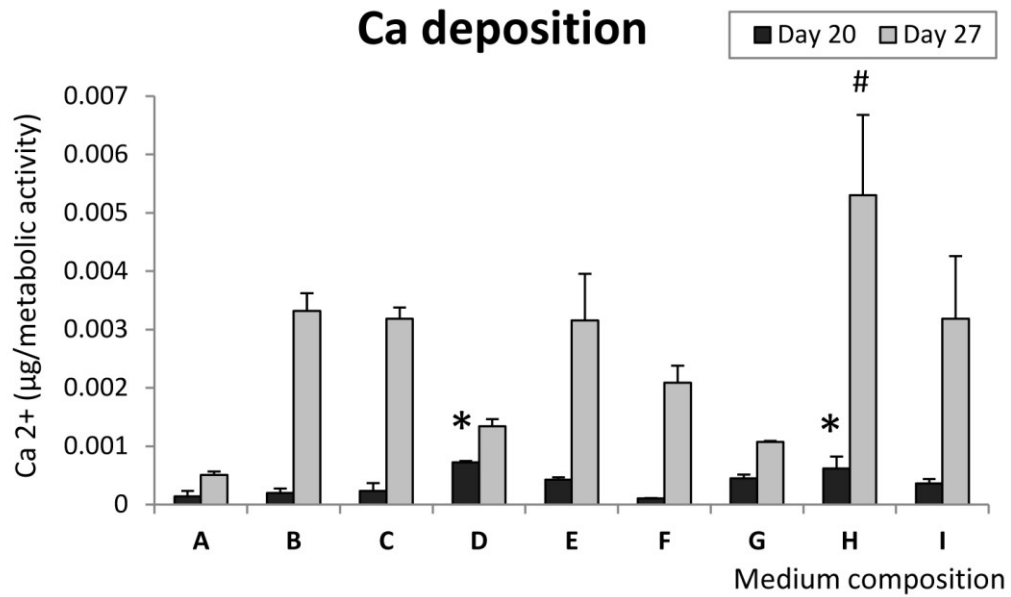


Fig. 18. Deposition of calcium in ADSCs cultured in media of different compositions (A-I) on days 20 and 27. A-C: without FGF2; D-F: supplemented with 10 ng/ml of FGF2; G-I: supplemented with 5 ng/ml of FGF2. A, D, G: without osteogenic supplements; B, E, H: supplemented with dexamethasone, β -glycerophosphate, ascorbic acid; C, F, I: also with L-glutamin and vitamin D3. The data is expressed as mean + SD. ANOVA on Ranks, Tukey method. The statistical comparison was made on cells on the same day of the culture. The statistical significance ($p < 0.05$) is specified above the column (* means significantly higher values ($p < 0.05$) than on A and F, # means a significantly higher value ($p < 0.05$) than on A, D, G).

4.3.2. Biocompatibility of Cr-doped and Ti-doped DLC layers for bone tissue engineering

(i) Jelínek M, Zemek J, Kocourek T, Remsa J, Mikšovský J, Písařík P, Jurek K, Tolde Z, Trávníčková M, Vandrovcová M, Filová E. **Dual laser deposition of Ti: DLC composite for implants.** *Laser Phys.* 2016;26(10):105605. doi: 0.1088/1054-660X/26/10/105605. (Published primary article)

(ii) Jelínek M, Zemek J, Remsa J, Mikšovský J, Kocourek T, Písařík P, Trávníčková M, Filová E, Bačáková L. **Hybrid laser technology and doped biomaterials.** *Appl Surf Sci.* 2017;417:73-83. doi:10.1016/j.apsusc.2017.03.103. (Published primary article)

(iii) Trávníčková M, Vandrovcová M, Filová E, Steinerová M, Racková J, Kocourek T, Bártová J, Suchý T, Žaloudková M, Jelínek M, Bačáková L. **Effect of diamond-like carbon doped with chromium on cell differentiation, immune activation and apoptosis.** *Eur Cell Mater.* 2020;40:276-302. doi: 10.22203/eCM.v040a17. (Published primary article)

(iv) Trávníčková et al. **The interaction of ADSCs and bmMSCs with Ti-doped DLC layers** (Manuscript in preparation)

Author's contribution: (i) and (ii) Martina Trávníčková contributed with biological evaluation of newly developed implant layers and drafted appropriate parts of the manuscript concerning experiments of the cell-material interactions (Jelínek *et al.* 2016a, Jelínek *et al.* 2017). (iii) She performed the experiments, analyzed data and drafted appropriate parts of the manuscript concerning proliferation and osteogenic differentiation of bmMSCs on Cr-doped DLC layers (Trávníčková *et al.* 2020b). (iv) She performed the experiments, analyzed data and drafted appropriate parts of the manuscript concerning proliferation and osteogenic differentiation of ADSCs on Ti-doped DLC layers (Trávníčková *et al.* manuscript in preparation).

Various metallic implants are widely used for joint replacements or for internal fixation for fractures. Moreover, metallic components have important applications in dental medicine or in cardiovascular surgery. However, long-term use of such implants often leads to decrease in wear or corrosion resistance. Thus, newly developed treatments

of implant surface or additional coatings (e.g. DLC) are being studied to ameliorate osseointegration of an implant and to enhance its wear and corrosion resistance at the same time. Pure DLC is characterized by weak adhesion to an underlay material. Our study was therefore focused on additive dopants. Ti and Cr in various concentrations were used as the additional dopants of DLC layer to strengthen adhesion of DLC layer to biomedical alloys. Subsequently, the influence of these elements and their concentration was studied regarding the biocompatibility and differentiation of MSCs towards osteoblasts.

4.3.2.1. Cr-doped DLC layers

DLC layers were doped with Cr in a concentration of 0.9, 1.8, 7.3 or 7.7 at. %. Experiments using bmMSCs proved very good biocompatibility of Cr-doped DLC layers within 21 day *in vitro* culture. Specifically, initial cell numbers and metabolic activity did almost not differ among DLC samples and reached similar values as control Glass and PS samples. Moreover, in later time intervals, the metabolic activity gradually increased on all samples and was even higher on DLC samples than on control PS (**Fig. 19**).

Concerning the cell morphology, the bmMSCs were well spread and had a similar cell spreading area on all samples in initial time intervals. Nevertheless, we observed more apparent assembly of vinculin containing focal adhesions on samples with higher Cr content than on pure DLC, glass or PS.

Subsequently, the bmMSCs on samples were exposed to an osteogenic culture medium and the effect on osteogenic differentiation has been studied. In general, the bmMSCs developed osteogenic response on all Cr-doped DLC samples as well as on control glass and PS. The exact differences among the samples depended on the day of the culture and on the studied osteogenic markers. Taken together all performed analyses, DLC layers doped with 0.9 and 7.7 at. % of Cr resulted in slightly higher osteogenic response in bmMSCs than other samples. Specifically, we observed earlier onset of gene expression of RUNX2 and type I collagen on sample containing 7.7 at. % of Cr. These results were also in accordance with a higher intensity of immunofluorescence staining of type I collagen on day 7 (**Fig. 20**). The sample containing 0.9 at. % of Cr was characterized by an earlier onset of osteocalcin gene expression and by the highest intensity of immunofluorescence staining of type I collagen (**Fig. 20**) and osteocalcin on day 14.

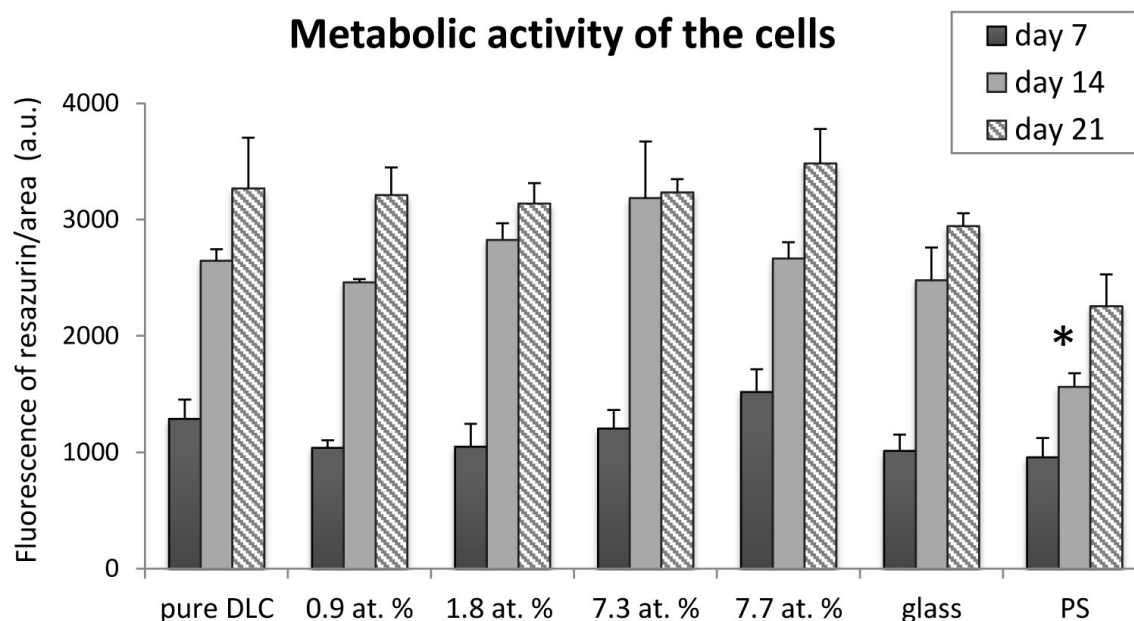


Fig. 19. The metabolic activity of bmMSCs on pure DLC, on DLC doped with Cr (0.9, 1.8, 7.3 or 7.7 at. % of Cr), on glass and PS on days 7, 14, and 21. The data is expressed as mean + SEM. ANOVA, Student-Newman-Keuls method. The statistical comparison was made on cells on the same day of the culture. The statistical significance ($p < 0.05$) is specified above the column (* means a lower value than on pure DLC, 1.8, 7.3 and 7.7 at. %). (Trávníčková et al. 2020b)

The ALP activity, measured by a colourimetric assay, showed increasing tendency with the maximum on day 14 on all samples. Additionally, the values were slightly higher for pure DLC and DLC containing 0.9 at. % of Cr than for other tested samples; however, they did not reach significant difference (**Fig. 21**).

Our study confirmed suitable cell adhesion to Cr-doped DLC layers which was followed by bmMSCs proliferation on the tested materials. The cell adhesion and proliferation on materials reached similar values as on PS that is routinely used as a reference material in cell cultures. Cr-doped DLC layers can therefore support integration of implant into the surrounding tissue. Moreover, these layers can enable osteogenic differentiation of MSCs when treated with an appropriate osteogenic medium. Based on the subsequent analyses concerning potential immune activation or tendency to apoptosis/necrosis, the sample containing 0.9 at. % of Cr showed a sufficient support for the cell adhesion, proliferation and osteogenic differentiation with a minimal immune activation or minimal increase in apoptosis at the same time.

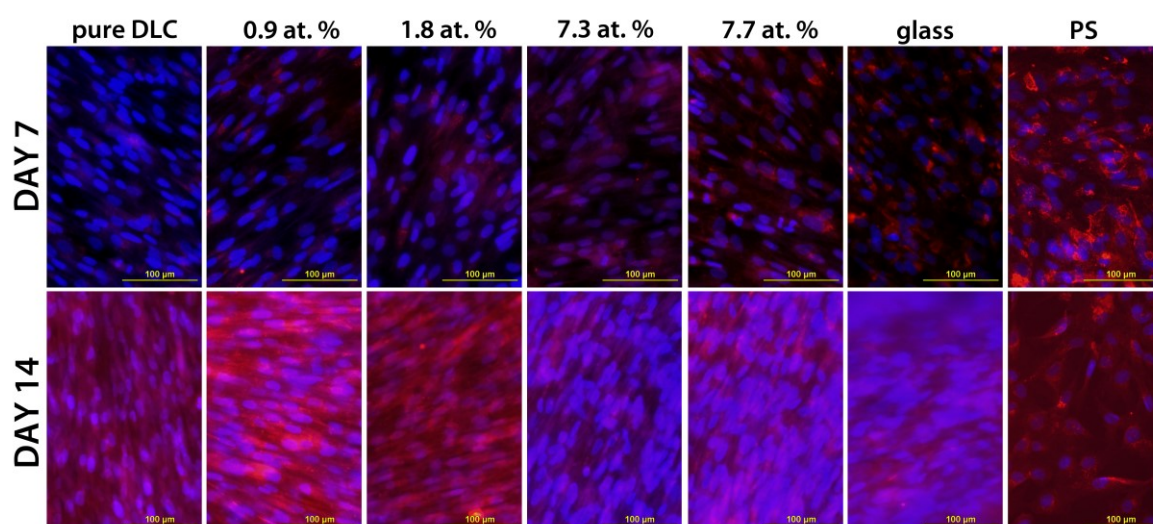


Fig. 20. The immunofluorescence staining of type I collagen (red) in bmMSCs cultured on pure DLC, on DLC doped with Cr (0.9, 1.8, 7.3 or 7.7 at. % of Cr), on glass and PS on days 7 and 14. Cell nuclei are counterstained with Hoechst 33258 (blue). Olympus IX71 microscope, IX71 digital camera, objective magnification $\times 20$, scale bar 100 μm .

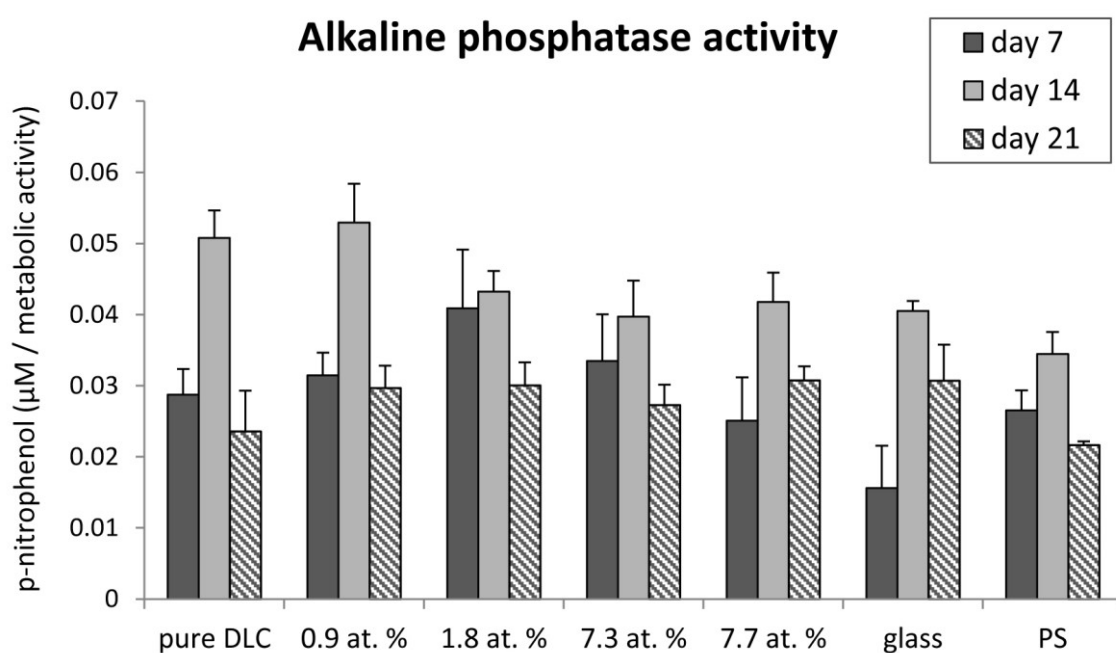


Fig. 21. The activity of alkaline phosphatase in bmMSCs on pure DLC, on DLC doped with Cr (0.9, 1.8, 7.3 or 7.7 at. % of Cr), on glass and PS on days 7, 14, and 21. The data is expressed as mean + SEM. ANOVA, Student-Newman-Keuls method. The statistical comparison was made on cells on the same day of the culture. No statistical difference ($p < 0.05$) was observed. (Trávníčková et al. 2020b)

4.3.2.2. Ti-doped DLC layers

DLC layers were doped with Ti in concentrations of 0.4, 2.1, 3.7, 6.6 or 12.8 at. %. Initial studies were carried out using Saos-2 osteoblast-like cells to observe the impact on cell adhesion, on proliferation and on osteogenic differentiation in a 7-day culture. On day 1 after seeding, pure DLC and glass samples developed a lower cell spreading area than Ti-doped DLC layers and PS (**Fig. 22**). In addition, Saos-2 showed polygonal morphology on all samples with more pronounced focal adhesion formation on samples with higher Ti content (**Fig. 23**).

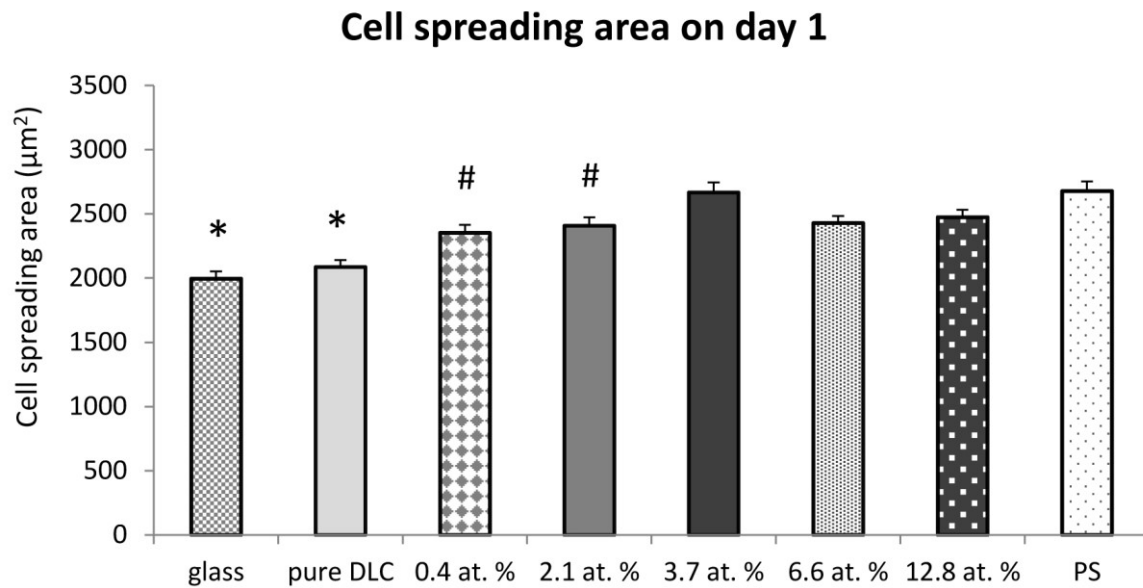


Fig. 22. The cell spreading area of Saos-2 cells on glass, on pure DLC, on DLC doped with Ti (0.4, 2.1, 3.7, 6.6, 12.8 at. % of Ti), and on polystyrene (PS) on day 1. The data is expressed as mean + SEM. ANOVA, Dunn's method. The statistical significance ($p < 0.05$) is specified above the columns (* means lower values than on 0.4, 2.1, 3.7, 6.6, 12.8 at. %, and PS; # means lower values than on 3.7 at. % and PS).

Interestingly, we observed tendency of slightly decreasing average cell numbers with increasing Ti content on day 7. However, the cell numbers on all Ti-doped DLC layers were at a similar level as the cell number on PS. These results were also proved by measuring the metabolic activity, where the values were even higher for glass, pure DLC, and Ti-doped DLC than for PS (**Fig. 24**). On all tested samples, Saos-2 developed type I collagen, osteopontin, and osteocalcin which was detected by immunofluorescence staining. The results of the initial studies confirmed appropriate biocompatibility of

prepared pure DLC and Ti-doped DLC layers and enabled following advanced longer term experiments involving primary mesenchymal stem cells.

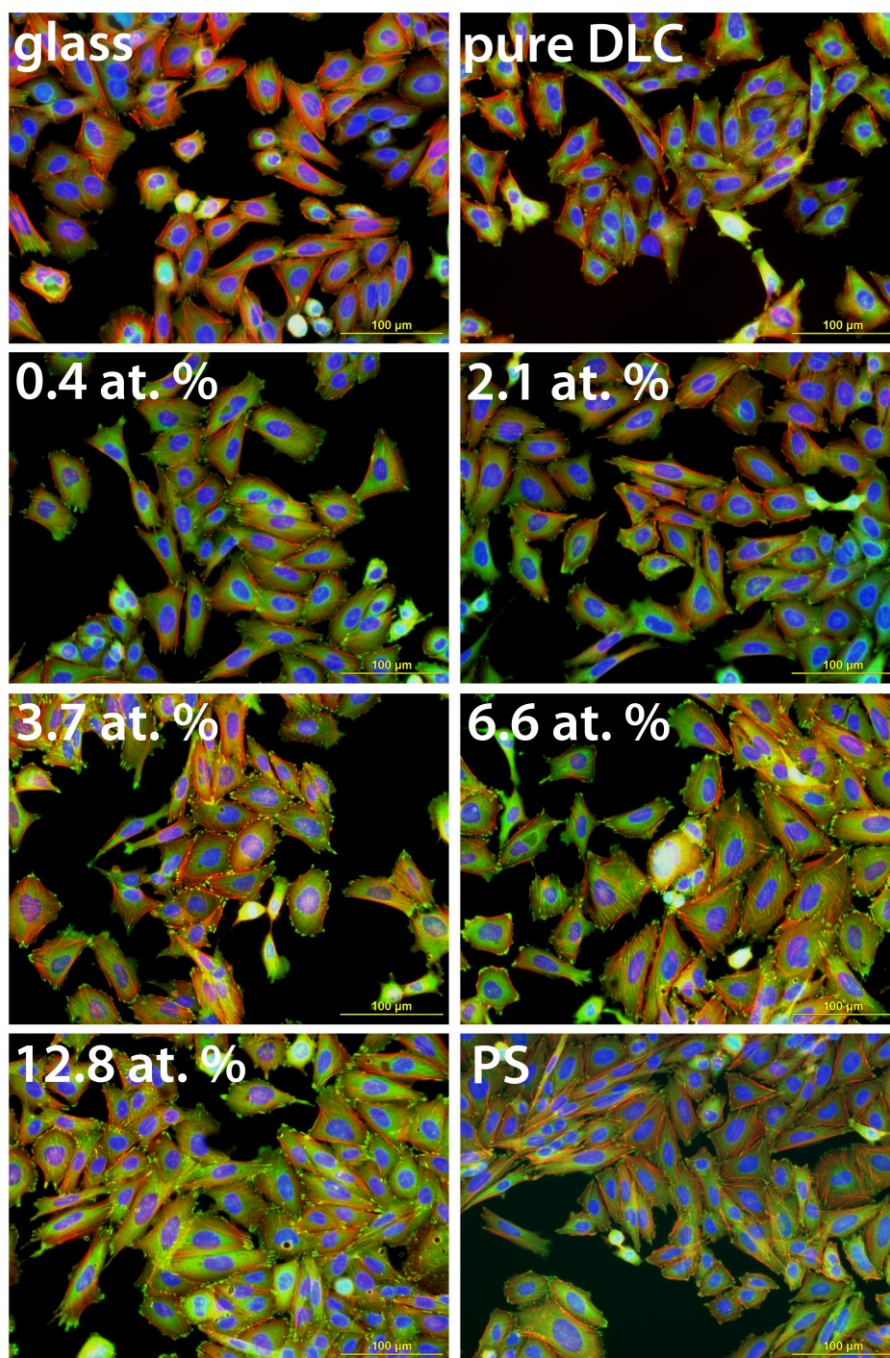


Fig. 23. The (immuno)fluorescence staining of vinculin (green) and staining for F-actin (red) in Saos-2 cells on glass, on pure DLC, on DLC doped with Ti (0.4, 2.1, 3.7, 6.6, 12.8 at. % of Ti), and on polystyrene (PS) on day 3. Cell nuclei are counterstained with Hoechst 33258 (blue). The focal adhesion plaques are present at cell borders mainly on the samples with higher Ti content (i.e. from 3.7 to 12.8 at. %). Olympus IX71 microscope, IX71 digital camera, objective magnification $\times 20$, scale bar 100 μm .

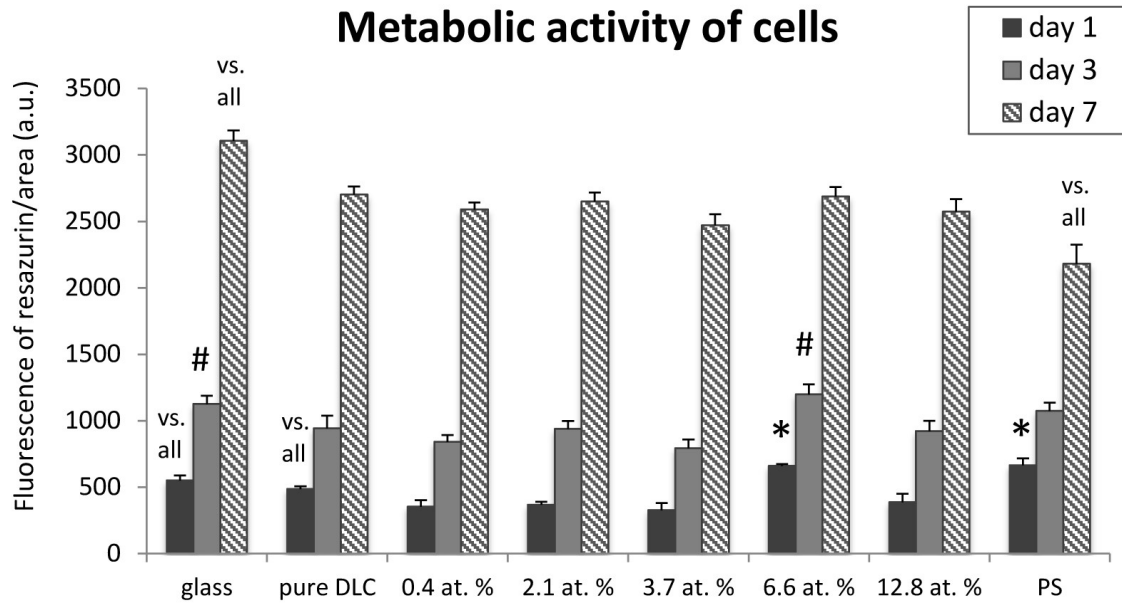


Fig. 24. The metabolic activity of Saos-2 cells on glass, on pure DLC, on DLC doped with Ti (0.4, 2.1, 3.7, 6.6, 12.8 at. % of Ti), and on polystyrene (PS) on days 1, 3 and 7. The data is expressed as mean + SEM. ANOVA, Student-Newman-Keuls method. The statistical comparison among the samples was made on cells on the same day of the culture. The statistical significance ($p < 0.05$) is specified above the column (* means higher values than on 0.4, 2.1, 3.7 and 12.8 at. %; # means higher values than on 0.4 and 3.7 at. %; “all” means values significantly different from all other samples on the specific day of the culture).

In the second part of our work, cell-material interactions of ADSCs with Ti-doped DLC layers were studied. The cells were grown in a three-week culture. The ADSCs showed polygonal morphology with similar cell spreading area on all samples. Nevertheless, similarly to Saos-2, most apparent assemblies of focal adhesions were observed on samples with a higher Ti content in comparison with DLC layers doped with a lower Ti content, pure DLC, glass or PS samples. Concerning the average cell numbers and metabolic activity of ADSCs, the measured values were almost identical among the all studied samples in the specific time intervals (**Fig. 25**). Only some variables in later time intervals were obtained in the case of pure DLC because this layer was partially prone to peeling. After initial adhesion, the ADSCs were exposed to an osteogenic differentiation medium which supported synthesis of type I collagen (i.e. both the intracellular and the extracellular one) within the cell culture (**Fig. 26**). The level of calcium deposition, which was measured as a late marker of osteogenic differentiation, revealed higher values on Ti-doped DLC layers with higher Ti content on day 14 than on

glass, on pure DLC, on 0.9 at.% of Ti and on PS (**Fig. 27**). Similarly on day 21, all Ti-doped DLC layers reached higher values than on pure DLC and glass samples. Nevertheless, the highest value on this day of the culture was obtained for PS sample (**Fig. 27**).

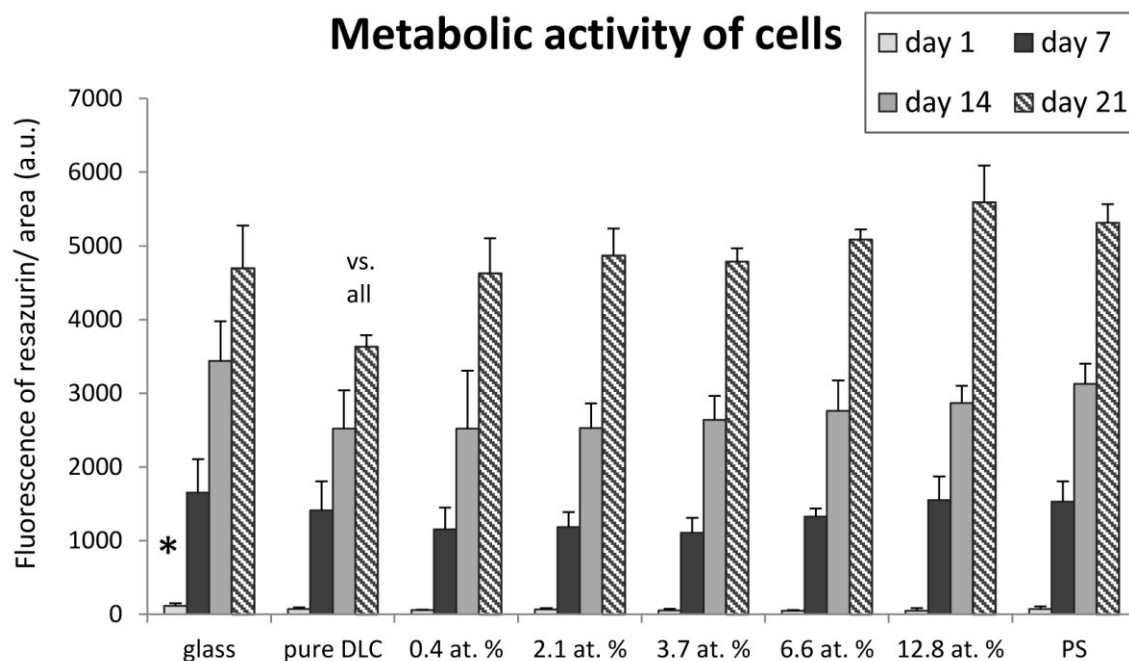


Fig. 25. The metabolic activity of ADSCs on glass, on pure DLC, on DLC doped with Ti (0.4, 2.1, 3.7, 6.6, 12.8 at. % of Ti), and on polystyrene (PS) on days 1, 7, 14 and 21. The data is expressed as mean + SD. ANOVA, Student-Newman-Keuls method. The statistical comparison among the samples was made on cells on the same day of the culture. The statistical significance ($p < 0.05$) is specified above the column (* means a higher value than on 6.6 and 12.8 at. %; “all” means a different value from all other samples on the specific day of the culture).

Although the behaviour of ADSCs can be different from the Saos-2 behaviour as the ADSCs are primary immature cells, we observed similar tendency in cell proliferation on tested samples. Specifically, the proliferation of both cell types did not almost differ among the DLC samples. Moreover, the cell numbers and the metabolic activity measurements reached at least similar levels as control PS. In addition, both cell types developed more apparent focal adhesion plaques on DLC samples doped with higher Ti content. All the samples supported cell osteogenic differentiation which was biochemically induced by the composition of the differentiation medium. An unstable cell-material response was obtained in the case of pure DLC samples, because this layer

was prone to occasional peeling within the cell culture. The Ti-doped DLC layers proved very good biocompatibility for both tested cell types and subsequent suitability in bone tissue engineering applications. Together with their superior physical and chemical properties, they seem to be promising material coatings.

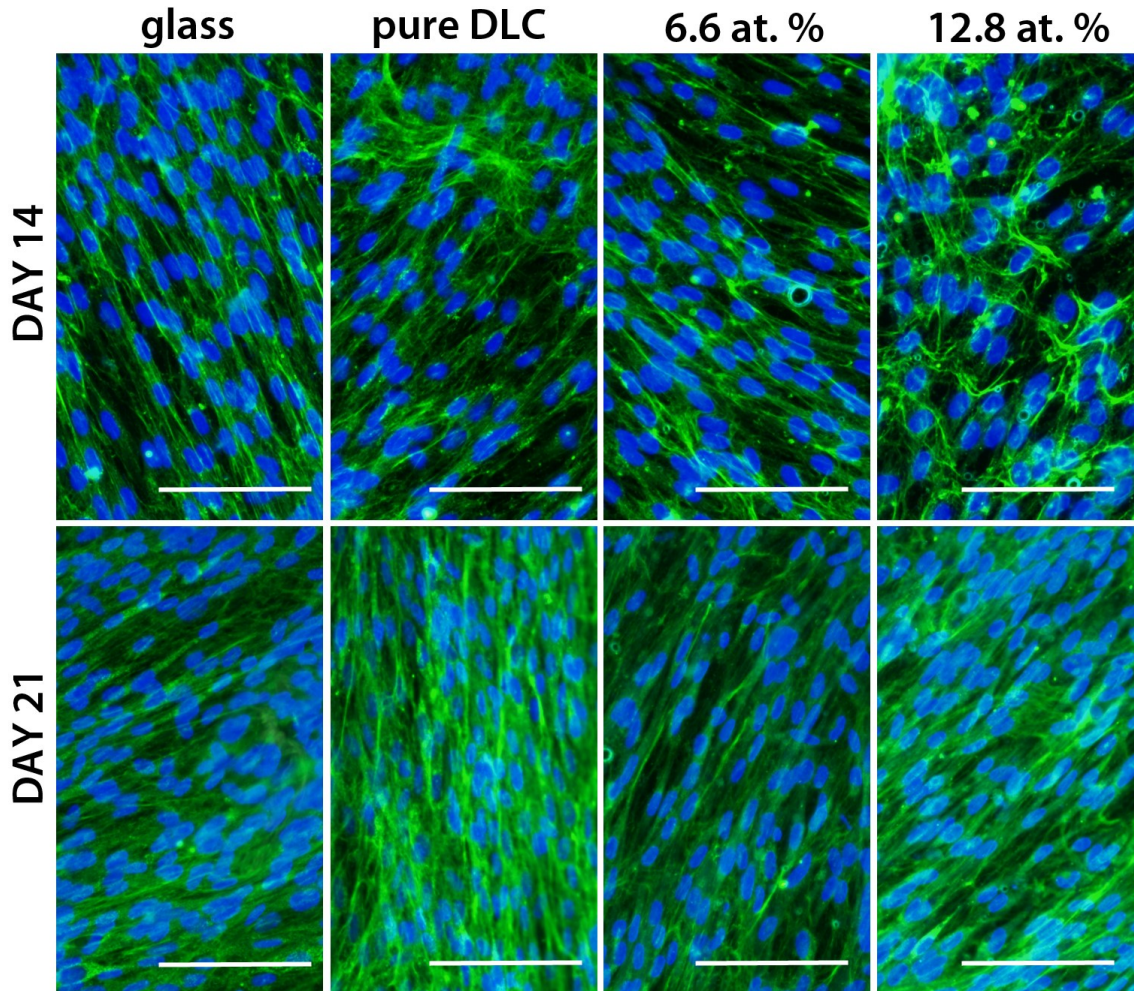


Fig. 26. The immunofluorescence staining of type I collagen (green) in ADSCs on glass, on pure DLC, and on DLC doped with Ti (6.6 and 12.8 at. % of Ti) on days 14 and 21. Cell nuclei are counterstained with Hoechst 33258 (blue). Representative images were selected. Olympus IX71 microscope, IX71 digital camera, objective magnification $\times 20$, scale bar 100 μm .

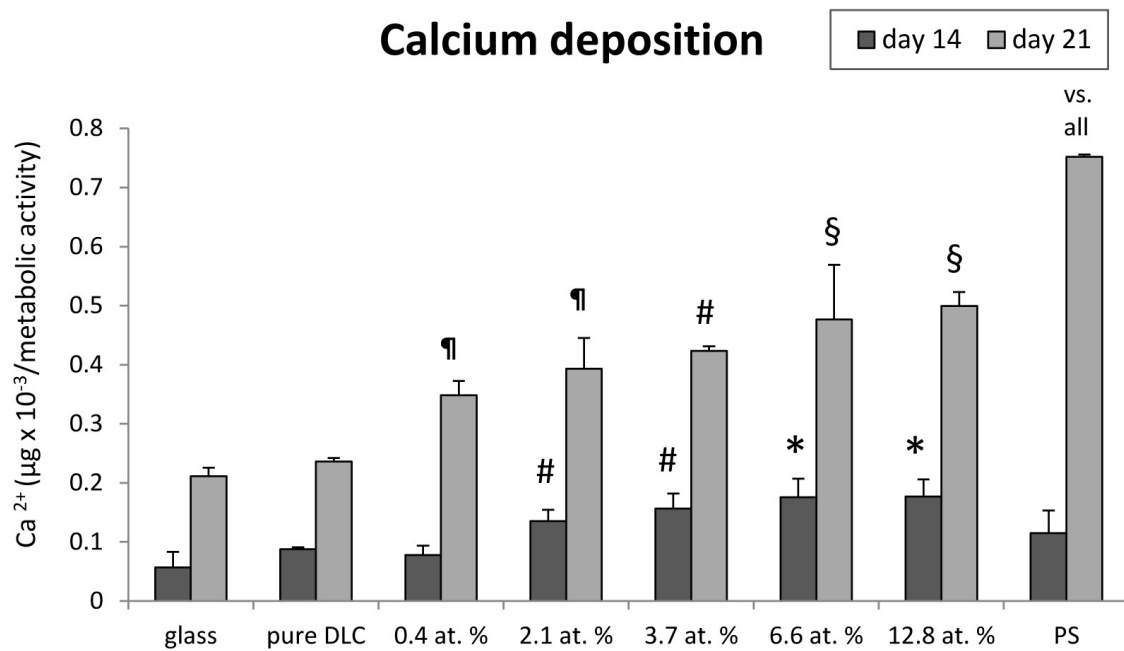


Fig. 27. The calcium deposition in ADSCs on glass, on pure DLC, on DLC doped with Ti (0.4, 2.1, 3.7, 6.6, 12.8 at. % of Ti), and on polystyrene (PS) on days 14 and 21. The data is expressed as mean + SD. ANOVA, Student-Newman-Keuls method. The statistical comparison among the samples was made on cells on the same day of the culture. The statistical significance ($p < 0.05$) is specified above the column (§ means higher values than on glass, pure DLC, 0.4, 2.1 and 3.7 at. %; * means higher values than on glass, pure DLC, 0.4 at. % and PS; # means higher values than glass, pure DLC and 0.4 at. %; ¶ means higher values than on glass and pure DLC; “all” means a significantly different value from all other samples on the specific day of the culture).

5. Discussion

Optimal source of autologous or allogeneic stem cells is very important for fabricated tissue-engineered replacements. Such replacements containing an autologous cellular component can be more easily accepted by a patient's body. ADSCs are very promising adult mesenchymal stem cells suitable for a wide range of tissue engineering applications.

This dissertation thesis aimed to confirm the suitability of ADSCs for use in vascular and bone tissue engineering. The study was drafted into 3 main units: (i) the study of factors potentially influencing further characteristics of isolated ADSCs, (ii) the study of interaction of ADSCs with newly fabricated biomaterials for vascular tissue engineering purposes, (iii) the study of interaction of ADSCs with newly developed biomaterials for bone tissue engineering purposes. The Discussion section provides general discussion combined of all the three studied units. The detailed discussion for each topic is provided in the published articles.

(i) ADSCs are isolated from the adipose tissue. Methods by which the adipose tissue is harvested, i.e. excision or liposuction, play important role in later cell characteristics (Iyyanki *et al.* 2015). Even within the liposuction procedure, many factors such as a diameter of cannula, harvesting negative pressure or the use of local anaesthetics influence the quality and quantity of harvested cells (for a review, see Fontes *et al.* 2018). Moreover, there are important factors on the side of a patient (e.g. harvesting site, age, BMI, other diseases, medication) influencing the characteristics of isolated cells (for a review, see Varghese *et al.* 2017). Numerous studies that were focused on these influencing factors predominantly studied characteristics of all nucleated cells, adipocytes or preadipocytes right after the harvesting procedure. Our set of experiments was focused on the influence of the negative pressure during liposuction and of the harvesting site on the further characteristics of isolated and adhered ADSCs. Further characteristics of ADSCs were evaluated in passage 1 and 2, because it is usually important to obtain a large quantity of cells under *in vitro* conditions for tissue engineering purposes.

Our study showed a potential influence of harvesting site on the initial cell attachment, as we observed a higher number of attached cells per 1 mL of lipoaspirate for the outer thigh region than for the abdomen region. Our findings are in accordance with the studies by Dai Prè *et al.* (2020) and by Tsekouras *et al.* (2017), in which higher yields

of ADSCs from SVF cells were obtained from the thigh region than from the abdomen region. Different number of attached ADSCs could be caused by a different frequency of ADSCs in the isolated SVF cells. Specifically, Jurgens *et al.* reported higher ADSC frequency in adipose tissue from the abdomen area than from the hip/thigh region; however, the number of all nucleated cells or the number of viable cells did not differ depending on a harvesting site (Jurgens *et al.* 2008). Similarly, Fraser *et al.* reported no significant difference in the yields of nucleated cells and of preadipocytes; however the abdomen-adipocyte yield was 1.7-fold higher than the hip-adipocyte yield (Fraser *et al.* 2007). By contrast, other study reported different numbers of all SVF cells depending on the harvesting sites, i.e. higher SVF yields from the abdomen region than from the flank or axilla region; however, the ADSCs yields did not differ significantly (Iyyanki *et al.* 2015). The large variability in the reported results concerning cell yields can probably stem from the different target cell populations being studied or from different harvesting and isolation procedures. Clinicians are usually focused on cell yields of adipocytes, preadipocytes, SVF cells or all nucleated cells right after isolation that can be used for the purpose of immediate plastic reconstructive surgery. However, tissue engineering focuses more on the ADSCs, especially on the plastic-adherent ADSCs that can be further expanded and/or differentiated under *in vitro* conditions. The study by Dai Prè *et al.* also suggested that some of the significant differences among harvesting sites may arise after a non-enzymatic isolation procedure, even though can remain non-significant after an enzymatic digestion by collagenase (Dai Prè *et al.* 2020).

Interestingly, negative pressure has also been reported to influence initial cell yields. Lower negative pressure resulted in higher SVF yields (Mojallal *et al.* 2008), higher yields and higher viability of adipocytes (Cheriyann *et al.* 2014). Nevertheless, these studies were slightly limited by 3 donors only. In contrast, Charles-de-Sá *et al.* reported no significant influence of different apparatuses and of different levels of negative pressure on the percentage and on the viability of adipocytes and of mesenchymal stem cells (Charles-de-Sá *et al.* 2015). In our study, we did not observe significant difference in yields of attached ADSCs from the outer thigh region obtained under two different negative pressures; however, we observed slightly higher cell yields of high-pressure ADSCs than of low-pressure ADSCs from the abdomen region. The discrepancies in the role of negative pressure on cell yields and viability among the above mentioned studies can be probably caused by different studied cell populations. Adipocytes have a bigger size than ADSCs, thus the negative pressure techniques may be

harmful for adipocytes with the minimal negative effect on ADSCs yields or viability at the same time (Fraser *et al.* 2007).

For tissue engineering purposes, further characteristics of ADSCs under *in vitro* conditions such as proliferation, cell viability, CFU, specific surface markers or ability to differentiate towards different cell types are subjects of interest. Our results showed similar cell numbers, metabolic activity and viability in ADSCs from both harvesting sites (i.e. from the outer thigh region and from the abdomen region). Similar viability of thigh-ADSCs and abdomen-ADSCs was also observed by Tsekouras *et al.* (2017). Only minimal influence of harvesting site on later cell numbers were observed in our study, i.e. higher cell numbers of thigh-ADSCs (inner + outer thigh evaluated together) than of abdomen-ADSCs on day 7. Our results about initial similar growth kinetics in ADSCs originating from different harvesting sites are in accordance with other researchers (Jurgens *et al.* 2008, Iwen *et al.* 2014). Furthermore, differences in later cell numbers may correspond with the study by Dai Prè *et al.* in which the thigh-ADSCs showed higher replicative rate than abdomen-ADSCs up to passage 10 when the significant differences disappeared (Dai Prè *et al.* 2020). Additional explanation for higher cell numbers in thigh-ADSCs than in abdomen-ADSCs can be more numerous and larger CFU (Fraser *et al.* 2007, Dai Prè *et al.* 2020). Concerning the influence of negative pressure, Chen *et al.* reported higher proliferation rate in SVF cells obtained under lower negative pressure than in SVF cells obtained under higher negative pressure (Chen *et al.* 2017). Nevertheless, these differences disappeared in passage 3 (Chen *et al.* 2017). Our results could also support the theory that the differences become less noticeable within *in vitro* passaging, as we did not notice any influence of negative pressure on cell numbers, metabolic activity or cell viability. Moreover, our results were obtained for ADSCs only, not for all SVF cells, and slightly different results could therefore be obtained.

Our study showed that harvesting site or negative pressure do not have almost any influence on the presence of several CD surface markers of isolated ADSCs (i.e. CD105, CD90, CD73, CD29, CD146, CD45, CD34, CD31). The obtained results are in accordance with other studies, where no influence of harvesting site (Jurgens *et al.* 2008, Iwen *et al.* 2014, Tsekouras *et al.* 2017) or of negative pressure (Chen *et al.* 2017) on the presence of selected CD markers was reported. Although it seems that the presence of CD surface markers is relatively constant and uniform, the results can also be influenced by the selected panel of studied CD markers in each study, and some differences in other CD markers cannot be excluded. Interestingly, even though no influence on

immunophenotypic characteristics was observed, some of these studies showed significant differences in cell yields or in cell growth depending on harvesting site (Tsekouras *et al.* 2017) or on negative pressure (Chen *et al.* 2017). These differences could potentially be caused by donor-to-donor and cell-to-cell heterogeneity, manifested by the presence of fast-growing and slow-growing clones (McLeod and Mauck 2017) with the minimal effect on the basic panel of CD markers. A wide range of donor-to-donor variability of the obtained data was also observed in our study and can be potentially considered as one of the study limitations and would benefit from a higher samples size.

(ii) ADSCs play an important role in cell-based therapies as well as in potential tissue engineering applications. Although the cell yields and the cell growth showed to have donor-to-donor variability in our experiments, ADSCs were harvestable from all obtained lipoaspirate samples, which enabled their further proliferation under *in vitro* conditions. Such cell multiplication provides sufficient quantities for subsequent material seeding. ADSCs are considered as a promising source of autologous stem cells for vascular tissue engineering applications (Zhang *et al.* 2017, Hielscher *et al.* 2018).

Our set of experiments aimed to evaluate the suitability of ADSCs in interaction with newly modified PLLA foils for potential blood vessel wall reconstructions. PLLA foils were modified by plasma-treatment and variably by PEG or Dex. ADSCs seeded on these foils were biochemically differentiated towards VSMCs, and mature VSMCs were used as positive control to the differentiated ADSCs. The ADSCs adhered and grew similarly on all tested samples when cultured in the non-differentiation medium. However, ADSCs cultured in the differentiation medium on the same materials displayed higher cell numbers and metabolic activity on PLLA240 and on PS than on the other samples. Moreover, similar cell behaviour as in the case of differentiated ADSCs was observed for VSMCs both with the non-differentiation medium and with the differentiation medium. Additionally, the VSMCs cultured in the differentiation medium reached lower cell numbers and a lower metabolic activity than the VSMCs cultured in the non-differentiation medium. It is obvious that cell-material interactions were influenced by the specific cell type used but the influence of culture media on cell-material interaction within one cell type was also observed.

Plasma-treatment is known to improve cell-material interactions by increasing the surface roughness and lowering the hydrophobicity of the material surface (for a review,

see Bačáková *et al.* 2011, Recek 2019). By these mechanisms, the plasma-treatment enables a higher adsorption of specific proteins present in the culture medium which subsequently support the adhesion and further proliferation of cells (for a review, see Recek 2019). Similarly, our results showed that plasma-treatment of pristine PLLA improved the adhesion and spreading of VSMCs. However, the cell spreading of ADSCs was well-developed on all PLLA samples and was not dependent on PLLA modifications. Cell type-specific adhesion to material surfaces has been previously reported with fibroblasts, endothelial cells, keratinocytes, osteoblast-like cells or VSMCs (Lišková *et al.* 2019, Černochová *et al.* 2020). Furthermore, the differences in cell adhesion to pristine PLLA or to plasma-treated PLLA can be highly cell type-specific even among mesenchymal stem cells. Argentati *et al.* reported spheroid structures of ADSCs on pristine PLLA and well-spread cells on plasma-treated PLLA. In contrast, Wharton's jelly mesenchymal stem cells formed spheroid structures on both tested materials, and bmMSCs were well spread and maintained their fibroblast-like morphology on both tested materials (Argentati *et al.* 2018). Concerning our results, VSMCs seemed to be more sensitive to the material surface properties than ADSCs. These differences could stem from the fact that VSMCs are mature cells, whereas ADSCs are immature mesenchymal stem cells. Both cell types differ in the presence of surface adhesion molecules and cytoskeleton proteins (for a review, see Moiseeva 2001, Argentati *et al.* 2019). Moreover, VSMCs are characterized by the presence of contractile proteins (i.e. α SMA, calponin, caldesmon, SM-MHC) that could also play a role in the cell adhesion to specific materials. Additionally, similar behaviour on the tested materials as in the case of VSMCs (i.e. different cell growth and latter cell detachment on pristine PLLA, PEG and Dex) was observed in ADSCs cultured in the differentiation medium. We could suppose that the similar cell behaviour was influenced by the triggered synthesis of the contractile proteins in differentiated ADSCs in a similar manner as in the mature VSMCs, as proved by immunofluorescence staining and PCR analysis. Our study therefore emphasized the importance of detailed testing of considered materials with more cell types, as the obtained results can differ depending not only on the specific cell type but also on the specific composition of culture medium within one cell type.

As mentioned above, our newly modified PLLA foils also supported biochemical differentiation of ADSCs towards VSMCs. The differentiation, which was previously optimized, was observed on all tested samples with no significant differences among the samples. Similarly to other studies, the mRNA expression and synthesis of α SMA,

calponin, smoothelin, and SM-MHC was initiated by the addition of TGF β 1 and BMP4 (for a review, see Zhang *et al.* 2017). We observed continual expression of the several markers within a 3-week culture. However, the late markers of successful differentiation towards VSMCs on our materials were only marginally detected by immunofluorescence staining. We can suppose that this could be caused by the static culture conditions. Specifically, the complete deposition of ECM and the synthesis of late differentiation markers of VSMCs can be reached by a synergic effect of biochemical and mechanical stimulation (Wang *et al.* 2010, Helms *et al.* 2020). In addition, some cryopreservation methods of ADSCs were reported to alter several signalling pathways which can result in a lower differentiation towards VSMCs (Zhang *et al.* 2018).

Last but not least, hemocompatibility is one of the very important aspects for artificial vascular grafts, and it needs to be evaluated prior to clinical applications (Weber *et al.* 2018). According to the literature, it seems that plasma treatment of grafts can introduce new functional groups on a material surface and hence inhibit the platelet adhesion and activation (Wang *et al.* 2006, Junkar *et al.* 2011). Further grafting with PEG and Dex in our study was designed as these molecules are known to possess superior hemocompatibility especially by their anti-thrombogenic properties (Hahn *et al.* 2007, Alexandre *et al.* 2015). For further studies, hemocompatibility analyses of our newly fabricated materials could provide an interesting insight of how the PLLA modifications can resist the adhesion of platelets or the activation of leukocytes.

(iii) Due to the mesenchymal origin, ADSCs have also been studied for bone tissue engineering applications. Although they were reported to develop a later and a lower response to osteogenic stimuli than bmMSCs in most of the donors (Mohamad-Ahmed *et al.* 2018), the easier harvesting procedure and subsequent multiplication of ADSCs still make them equivalent alternative to bmMSCs (Liao and Chen 2014, Przekora 2019b).

One of our experiments was focused on the optimization of osteogenic medium for ADSCs. Specifically, the role of FGF2 in the pre-treatment and during the differentiation was the subject of interest for us. A previous study reported that the presence of 1 ng/mL or 10 ng/mL of FGF2 in the culture medium supports ADSCs proliferation and cell migration (Khan *et al.* 2017, Cheng *et al.* 2020), but can have an opposite effect within prolonged *in vitro* expansion (Cheng *et al.* 2020). Additionally, 5 ng/mL or 10 ng/mL of FGF2 can promote the proliferation of ADSCs from diabetic patients up to the values of

ADSCs from healthy donors (Nawrocka *et al.* 2017). This promotion is realised by the reduction of cellular oxidative stress (Nawrocka *et al.* 2017).

The role of FGF2 during osteogenic differentiation and bone regeneration is very important; however it seems that it can cause osteogenesis promotion as well as osteogenesis inhibition (for a review, see Charoenlarp *et al.* 2017). Most of the studies reported positive effect of FGF2 on osteogenic differentiation (Martin *et al.* 1997, Pri-Chen *et al.* 1998, Ito *et al.* 2008, Fei *et al.* 2011, Byun *et al.* 2014, Lim *et al.* 2016). However, several studies reported a negative effect of FGF2 on osteogenic differentiation (Lai *et al.* 2011, Hu *et al.* 2013, Song *et al.* 2013, Simann *et al.* 2017). Some of the obtained different results can be explained by the different concentrations of FGF2 being studied (from 1 ng/mL up to 25 ng/mL). It seems that the studies reporting promotion of osteogenic differentiation preferentially used concentrations of 1 to 3 ng/mL of FGF2. Nevertheless, there are many other factors potentially influencing the results, as some of the studies were conducted under *in vitro* conditions but some of them were conducted under *in vivo* conditions. Additionally, some of the studies were focused on the differentiation of mesenchymal stem cells whereas some others tested osteoblasts. Last but not least, the variable cell pre-treatments or subsequent differences in culture media composition used in the studies hamper general conclusions about the FGF2 usage during osteogenic differentiation.

In our experiment, ADSCs were standardly cultured in DMEM supplemented with 10% FBS and 10 ng/mL FGF2. For proper osteogenic differentiation induced by osteogenic supplements (i.e. DXM, BGCP, AA, L-GLU, vit. D3), the concentration of FGF2 was left unchanged, lowered to 5 ng/mL or FGF2 was excluded from the differentiation medium. Our results showed that DXM, BGCP and AA strongly supported the differentiation of ADSCs towards osteoblasts. The additional positive effect of L-GLU and vit. D3 was not observed. The osteogenic differentiation was detected by several methods and was successful in sufficient rate in all groups of cells treated with osteogenic supplements. Although the results concerning the role of FGF2 concentration within the differentiation were not completely uniform, we can conclude that the results obtained from several methods suggested the beneficial role of lowering FGF2 concentration to 5 ng/mL during differentiation. Logically, the higher levels of late osteogenic markers in ADSCs treated with osteogenic supplements and 5 ng/mL of FGF2 seem to correspond with the lowest proliferation rate observed in these cells. These findings are also supported by the literature, because the terminal differentiation of cells

is usually accompanied by a reduced proliferation or by a cell cycle arrest realised by differentiation-inducing transcription factors (for a review, see Ruijtenberg and van den Heuvel 2016). Furthermore, we can also hypothesize about the increased proliferation of ADSCs treated with osteogenic supplements and with 10 ng/mL of FGF2 or without FGF2. As these cells showed sufficient osteogenic response especially in later time intervals, we can consider the higher initial proliferation as a potential sign of proliferation phase in the beginning of osteogenic commitment (i.e. at the stage of pre-osteoblasts) (Infante and Rodríguez 2018). Our results also support the previously mentioned studies reporting higher or earlier osteogenic response with lower concentration of FGF2. These findings also correspond with the fact that FGF2 is one of the major regulators of a molecular pathway involving early stage differentiation (i.e. from a mesenchymal stem cell to an osteogenic precursor) (Valenti *et al.* 2016).

The second set of experiments concerning bone tissue engineering was focused on the newly fabricated DLC layers doped with Cr or Ti in various concentrations. Such layers doped with the elements were designed to prevent later corrosion, ion release and wear of the surface layer in metal implants used mainly for joint replacements. These properties are more pronounced for Cr-doped DLC layers. Specifically, the Cr-doped DLC layers displayed greater hardness and resistance under mechanical load than Ti-doped DLC layers (Jelínek *et al.* 2017). Nevertheless, the use of Cr can potentially alter biological functions of cells depending on its specific form, i.e. trivalent form or hexavalent form. The trivalent form in common daily intake is considered to be relatively harmless for a human body. In contrast, hexavalent form can alter cell behaviour by various mechanisms of cytotoxicity, carcinogenicity and allergenicity (Dayan and Paine 2001). According to previous analysis, the prepared Cr-doped DLC layers preferentially contain trivalent form (Jelínek *et al.* 2016b) and can therefore be considered as relatively harmless with the respect to the Cr content. Due to the potential cytotoxicity of Cr, an alternative method to increase the mechanical resistance of DLC layer by doping with Ti was developed. However, doping with Ti is less efficient than doping with Cr regarding mechanical properties of the coating layer (Jelínek *et al.* 2017). Although the Cr- and Ti-doped DLC layers differ in physicochemical and mechanical properties, our study proved a similar biocompatibility of both these layers in terms of cell adhesion, growth and osteogenic differentiation.

Specifically, our experiments firstly focused on initial biocompatibility of Cr- and Ti-doped DLC layers. Moreover, subsequent detailed experiments using mesenchymal

stem cells (i.e. ADSCs or bmMSCs) provided the opportunity to study osteoinductive properties of the layers doped with Cr or Ti, eventually depending on the concentration of these elements.

DLC layers are generally known to possess superior tribological, mechanical and biocompatibility properties (Roy and Lee 2007). The biocompatibility was proved both under *in vitro* and under *in vivo* conditions (Alanazi *et al.* 2000, Allen *et al.* 2001, Liao *et al.* 2016). Our results proved very good biocompatibility of pure DLC and also of DLC doped with Cr or Ti. The biocompatibility was tested with three cell types (i.e. Saos-2, bmMSCs, and ADSCs). The rate of the biocompatibility was at the same level with conventionally used PS or even better than PS both in Saos-2 cell line and in bmMSCs and ADSCs representing primary cells. The rate of cell adhesion to materials and later cell proliferation and differentiation under *in vitro* conditions can indicate further efficiency of *in vivo* osseointegration of implants. Tight cell adhesion *via* focal adhesions and fast cell spreading *in vitro* is desirable as the insufficient *in vivo* osseointegration can lead to the formation of fibrous tissue, relative movement under functional loading and eventually to the loss of an implant (Shah *et al.* 2019). Our experiments showed that all three cell types performed good adhesion to the tested materials. ADSCs and bmMSCs did not prove any significant differences in cell spreading area among the tested Cr-doped DLC layers (bmMSCs) or Ti-doped DLC layers (ADSCs). However, we observed more apparent focal adhesion assemblies on DLC layers with higher Cr or Ti content. Similar results were obtained in a previous study by Filová *et al.* (2017) where Saos-2 cells showed greater spreading area on DLC layers doped with higher Cr content. These observations are also in accordance with our current study where Saos-2 cells had the most apparent focal adhesions on DLC layers doped with higher Ti concentration (i.e. 3.7, 6.6 and 12.8 at. % of Ti). Moreover, Saos-2 cells showed a larger cell spreading area on Ti-doped samples than on glass and pure DLC. Such differences could stem from a different surface roughness of the materials. Specifically, optimal nanoroughness of the material surface promotes protein adsorption followed by improved cell-material interactions represented mainly by a better cell adhesion (Webster *et al.* 2000, Feller *et al.* 2015). Slightly increasing nano- and microroughness with a Cr- or Ti-depended manner was also observed in our layers prepared by pulsed-laser deposition (Jelínek *et al.* 2016a, Jelínek *et al.* 2017, Filová *et al.* 2017).

Apart from the slight initial differences in cell adhesion, all the Cr-doped and Ti-doped DLC layers supported subsequent cell proliferation, which was at the same level

with glass and even higher than on PS especially in later time intervals. We did not observe any influence of Cr or of Ti concentration on cell proliferation estimated by metabolic activity or cell numbers. Thus, we can assume that concentration of Ti or Cr in DLC layer was not a key determinant regarding the cell proliferation. The effect of various Ti concentrations on cell growth was also investigated in a study by Vandrovcová *et al.* (2015). Hydrocarbon polymer films were doped with Ti up to 20 at. %. Lower concentrations of Ti (mainly 5 at. %) were shown to promote initial cell adhesion and osteogenic differentiation, whereas higher concentrations of Ti (mainly 15 at. %) promoted cell growth on day 7 (Vandrovcová *et al.* 2015). This study suggests that there probably can be some initial differences in cell growth depending on Ti concentration; however a different cell type (i.e. MG-63 osteoblast-like cells) was investigated. Moreover, the study by Vandrovcová *et al.* (2015) was designed as a relatively short-term study (i.e. 7-day culture) focusing on initial differences whereas our study was designed for 21-days. Thus, as the cells in our study reached confluence on all the tested DLC layers, the later growth did not have to significantly differ.

The presence of Cr and Ti in DLC layers can potentially influence osteogenic differentiation of osteoblast-like cells or of mesenchymal stem cells. This influence can be caused by the presence of the elements itself, but can be more complex due to the changes of physicochemical properties of the layers based on the Cr/Ti concentration. For example, in the study by Stiehler *et al.* (2008), discs coated with Cr showed lower cell spreading area and inferior cell proliferation in comparison with discs coated with Ti or tantalum (Ta). However, these Cr-coated discs performed better than Ti- or Ta-coated discs in the initial osteogenic differentiation, i.e. they increased all studied bone-related genes either in a growth medium or in a differentiation medium (Stiehler *et al.* 2008). Our experiments focused on the osteogenic differentiation of bmMSCs on Cr-doped DLC layers and of ADSCs on Ti-doped DLC layers. Concerning the Cr-doped DLC layers, the osteogenic differentiation of bmMSCs induced by the composition of the culture medium was sufficient on all samples. The differences in osteogenic differentiation among the samples varied depending on time intervals and on the specific detection methods. Based on the various analyses, it seems that the sample containing 0.9 at. % of Cr can act as the material supporting early onset of osteogenic differentiation, having optimal physicochemical properties at the same time. Moreover, this sample did not increase the percentage of necrotic cells or apoptotic cells and did not evoke immune activation under *in vitro* conditions (Trávníčková *et al.* 2020b). Concerning the Ti-doped DLC layers, the

osteogenic differentiation of ADSCs was also proved by immunofluorescence staining and by ALP and Ca detection assays on all prepared materials. Interestingly, significant differences were obtained for Ca deposition where the values increased in Ti-dependent manner. Specifically, the Ca deposition was higher on Ti-doped DLC layers, mainly with higher Ti concentrations, than on glass and pure DLC both on days 14 and 21. Titanium is generally well-tolerated by human body and non-toxic even in high doses (Chen and Thouas 2015). However, some studies suggested that Ti particles in higher concentrations can have an inductive effect on cell apoptosis and inhibitory effect on osteogenic differentiation of MSCs, especially when the wear debris in the form of particles is considered (Wang *et al.* 2003, Haleem-Smith *et al.* 2012, Gu *et al.* 2017). Further studies such as the study concerning potential immune activation or the influence of Ti-doped DLC layers on cell apoptosis are therefore needed to ensure the optimal concentration of Ti prior to *in vivo* studies.

6. Conclusion and further perspectives

Our studies demonstrated the possibilities of wide use of MSCs, mainly of ADSCs, in tissue engineering.

In the field of ADSCs harvesting, we showed that neither the harvesting site nor the negative pressure used within the liposuction procedure plays a crucial role for subsequent ADSCs multiplication and characteristics under *in vitro* conditions. ADSCs can be therefore expanded in large quantities and can be used for seeding of biomaterials. Nevertheless, it is necessary to mention that there are other factors on a patient side, which can significantly influence the characteristics of isolated ADSCs. The other factors on a patient side need to be taken into account for further ADSCs harvesting.

ADSCs were successfully differentiated towards VSMCs by the composition of the culture medium. Moreover, we proved very good biocompatibility of newly designed PLLA foils, which were modified by plasma treatment and by grafting with PEG or Dex. These foils supported biochemical differentiation of ADSCs towards VSMCs and could therefore be used for subsequent seeding with ECs to create bi-layered artificial vascular grafts or vascular patches. Subsequent experiments would also benefit from the exposure of seeded PLLA foils to dynamic culture conditions such as flow bioreactor or cyclic stretching of a substrate material. Moreover, experiments involving hemocompatibility testing would bring an interesting insight to how the materials can resist the adhesion and activation of blood elements.

ADSCs and bmMSCs also showed to be a very good source for bone tissue engineering. Partial studies were focused on the role of FGF2 during osteogenic differentiation of ADSCs. Our results indicated a potential beneficial role of pre-treating the cells with FGF2 but with subsequent lowering the FGF2 concentration within the differentiation itself. Following experiments focused on the interaction of ADSCs and bmMSCs with newly developed Cr-doped and Ti-doped layers. Specifically, the potential of the cells to differentiate towards osteoblasts on Cr-doped and Ti-doped DLC layers ensured the biocompatibility and suitability of these layers for implant coatings. Further experiments will probably aim to perform *ex vivo* or *in vivo* tests with the conventionally used implants coated with metal-doped DLC layers.

7. References

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8. Publications

8.1. Publications related to the dissertation thesis

8.1.1. Publications with impact factor (IF)

Trávníčková M, Slepíčková Kasálková N, Sedlář A, Molitor M, Musílková J, Slepíčka P, Švorčík V, Bačáková L. Differentiation of adipose tissue-derived stem cells towards vascular smooth muscle cells on modified poly (L-lactide) foils.

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IF = 3.174 (2019)

Trávníčková M, Vandrovcová M, Filová E, Steinerová M, Racková J, Kocourek T, Bártová J, Suchý T, Žaloudková M, Jelínek M, Bačáková L. Effect of diamond-like carbon doped with chromium on cell differentiation, immune activation and apoptosis. Eur Cell Mater. 2020;40:276-302. doi: 10.22203/eCM.v040a17.

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9. Appendices

Appendix I

Trávníčková M, Slepíčková Kasálková N, Sedlář A, Molitor M, Musílková J, Slepíčka P, Švorčík V, Bačáková L. Differentiation of adipose tissue-derived stem cells towards vascular smooth muscle cells on modified poly (L-lactide) foils.

Appendix II

Trávníčková M, Vandrovcová M, Filová E, Steinerová M, Racková J, Kocourek T, Bártová J, Suchý T, Žaloudková M, Jelínek M, Bačáková L. Effect of diamond-like carbon doped with chromium on cell differentiation, immune activation and apoptosis. *Eur Cell Mater.* 2020;40:276-302.

Appendix III

Trávníčková M, Pajorová J, Zárubová J, Kročilová N, Molitor M, Bačáková L. The Influence of Negative Pressure and of the Harvesting Site on the Characteristics of Human Adipose Tissue-Derived Stromal Cells from Lipoaspirates. *Stem Cells Int.* 2020;2020:1016231.

Appendix IV

Trávníčková M, Bačáková L. Application of adult mesenchymal stem cells in bone and vascular tissue engineering. *Physiol Res.* 2018;67(6):831-850.

Appendix V

Báčáková L, Zárubová J, Trávníčková M, Musílková J, Pajorová J, Slepíčka P, Slepíčková Kasálková N, Švorčík V, Kolská Z, Motarjemi H, Molitor M. Stem cells: their source, potency and use in regenerative therapies with focus on adipose-derived stem cells - a review. *Biotechnol Adv.* 2018;36(4):1111-1126.

Appendix VI

Jelínek M, Zemek J, Remsa J, Mikšovský J, Kocourek T, Písařík P, Trávníčková M, Filová E, Bačáková L. Hybrid laser technology and doped biomaterials. *Applied Surface Science.* 2017;417:73-83.

Appendix VII

Jelínek M, Zemek J, Kocourek T, Remsa J, Mikšovský J, Písařík P, Jurek K, Tolde Z, Trávníčková M, Vandrovcová M, Filová E. Dual laser deposition of Ti: DLC composite for implants. *Laser Phys.* 2016;26(10):105605.

Appendix VIII

Báčáková L, Trávníčková M, Filová E, Matějka R, Štěpanovská J, Musílková J, Zárubová J, Molitor M. Vascular Smooth Muscle Cells (VSMCs) in Blood Vessel Tissue Engineering: The Use of Differentiated Cells or Stem Cells as VSMC Precursors. In: Sakuma K, editor. *Muscle Cell and Tissue Current Status of Research Field.* Rijeka, Croatia: IntechOpen; 2018. p. 289-308.