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Developmental and activation pathways underlying mesenchymal stromal cell recruitment and differentiation in the context of neoplasias

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List of abbreviations

ATP

adenosine triphosphate..... 27, 31

BM

bone-marrow..... 20

CAF

cancer associated fibroblasts16, 19, 20, 22, 25

CAR

chimeric antigen receptor 14

CCL5

syn.

Rantes / C-C motiv ligand 53, 4, 5, 6, 8, 16, 18, 19, 20, 21, 22, 23, 25, 29, 30, 33, 34, 46, 47, 48, 49, 50, 51, 53, 54, 55, 56, 57, 58, 59, 60, 61, 63, 64, 65, 71, 72, 73, 74, 77, 79, 82, 83, 84, 85, 87

CD

cluster of differentiation 15

CAR

chimeric antigen receptor 14

CoCl2

cobalt chloride..... 26, 27, 44, 50

ECM

extra-cellular matrix 19, 27

EGFR

epithelial growth factor..... 19

EMT

epithelial-to-mesenchymal transition 20, 24, 27, 71, 78, 86, 89, 93

EPO

erythropoietin..... 27

FGF

fibroblast growth factor..... 26

hBMSC

human bone marrow stem cells 15

Her-2

human epidermal growth factor receptor 2) 19, 81

HGF

hepatocyte growth factor..... 15

HIF-1

hypoxia induced factor16, 26, 77, 87, 88, 92

HLA

human leucocyte antigen 15

IFN-γ

Interferon gamma..... 21

IFNγ

Interferon gamma..... 21

IL

interleukin..... 18, 21, 45, 92

LPS

Abstract

Solid tumors are a leading cause of mortality in adults across the world. Treatment options are often limited trough toxicity in non-target tissues. Mesenchymal stem cells (MSCs) present a tropism toward the inflammatory microenvironments present within solid tumors. Once there, they condition the tumor environment and differentiate into components of the tumor stroma. Our group had previously demonstrated the effectiveness of genetically engineered stem cells containing a therapeutic transgene, using the CCL5 promoter as a delivery vehicle for the treatment of solid tumors. CCL5/Rantes is a proinflammatory cytokine produced by a variety of cells including MSCs within tumor microenvironments. The CCL5 gene promoter can be activated by many factors including proinflammatory stimuli but varies with the cell type studied. The tumor-associated stimuli that activate the CCL5 promoter in MSCs was poorly understood. The aim of this thesis was to better understand the mechanisms of activation of CCL5 in stem cells, specifically in response to various signals present in the tumor microenvironment. A construct containing the CCL5 promoter driving a Gaussia luciferase reporter gene was created using Gateway cloning and a vector platform designed in house for the efficient stable integration of complex constructs into target cells. The CCL5 reporter construct was used to verify that the CCL5 promoter is activated by TNFα, but not significantly by IFN-γ; however, the combination of TNFα and IFN-γ showed a more than additive activity than the individual components. Neither hypoxia nor TGF-β, whether alone or in combination with other stimuli, activated the CCL5 promoter in the *in vitro* setting. We then developed synthetic variants of the promoter. 3AB, a synthetic promoter containing three tandem NFkB sequences taken from the immediate upstream region of the human CCL5 promoter [(R)AB region] and previously shown to be important for the functional activity of the promoter was generated. The 3AB synthetic promoter responded more effectively to TNFα and IFN-γ, with higher fold induction. It also showed a response to hypoxia that was increased in conjunction with TNFα and IFN-γ. TGF-β was not effective in the activation of 3AB. A second synthetic promoter MegaRantes, in which the R(AB) element in the native promoter was exchanged for the triplicate 3AB element, showed the same approximate pattern of activation as native CCL5, suggesting upstream regulating elements counteract any advantage derived from the 3AB modification. The results presented provide more complete information as to CCL5 is activation in MSCs and suggest that the synthetic construct 3AB, through its more specific activation, may represent an attractive candidate promoter for the delivery of therapeutic transgenes in the context of MSC-based tumor therapy.

Zusammenfassung

Solide Tumore repräsentieren weltweit eine der führenden Todesursachen bei Erwachsenen. Oft werden Therapieoptionen aufgrund von Toxizität limitiert. Mesenchymale Stammzellen (MSC) erweisen eine ausgeprägten Tropismus gegenüber inflammatorischen Umgebungen auf, wie sie in soliden Tumoren vorhanden sind. Hier differenzieren sie dann in Komponenten des Tumorstromas. Diese Eigenschaft macht sie zu potentiellen therapeutisch nutzbaren Vehikeln. In Vorarbeiten konnte unsere Gruppe die Effektivität von genetisch modifizierten mesenchymalen Stammzellen aufzeigen. Hierbei wurden MSCs mit einem therapeutisches Transgen welches den CCL5 Promotor enthielt, als Transport -Vehikel für Therapien bei soliden Tumoren nutzbar gemacht.

CCL5 ist ein Pro-Inflammatorisches Zytokin, das von verschiedenen Zellarten produziert wird, unter anderem MSCs im Tumor Mikromilieu. Welche Faktoren CCL5 stimulieren ist abhängig von der Zellart und Kontext; pro-inflammatorische Moleküle führen nicht in jeden Setting zu einer Stimulation und insbesondere in Bezug auf MSCs ist die Stimulation nur zum Teil erforscht.

Das Ziel dieser Dissertation ist das bessere Verständnis über die Mechanismen der Aktivierung von CCL5 in MSC, insbesondere in Bezug auf Signale, die im Tumor Micromilieu vorhanden sind. Wir entwickelten ein CCL5 Gaussia Luciferase Reporter Konstrukt, sowie eine Plattform für stabile Integration von Konstrukten in das genetische Material von Zielzellen. Damit konnte demonstriert werden, dass der native CCL5 Promoter von TNFα aktiviert wird, aber nicht signifikant von IFN-γ. Die Kombination dieser Substanzen zeigte eine deutlich höhere Aktivierung als die Summe der einzelnen Zytokine, somit einen synergistischen Effekt. Auf der anderen Seite konnte weder die Stimulation durch Hypoxie, noch die mit Stimulation mit TGF-β, sei es alleine oder in Kombination mit anderen Faktoren, eine signifikante Aktivierung des CCL5 Promotors in vitro erreichen.

In einer zweiten Phase wurden synthetische Varianten des Promotors entwickelt. Eine davon, genannt 3AB, bestand aus 3 Tandem Sequenzen der [(R)AB Region], die als NFkB Bindestelle für die funktionelle Aktivierung von CCL5 ausschlaggebend ist. Der 3AB Promotor wies eine effektivere und spezifischere Antwort zu TNFα und IFN-γ, mit höherer Vervielfachung der Induktion auf. Es zeigte zudem eine Response zum Stimulus mit Hypoxie, insbesondere additiv zu TNFα und IFN-γ. Eine Aktivierung durch TGF-β konnte nicht nachgewiesen werden.

Ein zweiter synthetischer Promotor, genannt MegaRantes, in dem das 3AB Element in den nativen CCL5 Backbone integriert wurde, zeigte den gleichen Aktivierungsmuster wie CCL5. Dies suggeriert einen Einfluss von regulierenden Elementen weiter upstream.

Diese Ergebnisse ergänzen unser Verständnis über die Aktivierung des Promotors in mesenchymalen Stammzellen. Im speziellen tragen diese dazu bei, dass der 3AB Promotor aufgrund seiner vergleichsweise spezifischen Aktivierung als attraktiver Kandidat für die Expression von therapeutischen Transgenen in Zukunft berücksichtigt werden sollte.

1. Introduction

1.1. Context of cancer epidemiology and need for novel therapies

In Germany almost 250 000 people are diagnosed each year with one of the four most prevalent types of solid malignant tumor – breast, colon, lung or prostate. If one considers all types of cancers - excluding non-melanoma skin cancers – that figure increases to around 475,000 new diagnoses every year[1]. While systemic cancer therapy has made striking advances since the first chemotherapy agents were used more than fifty years ago, toxicity resulting from action of these agents in non-target tissues has remained one of the major issues in oncology. Traditional chemotherapy also presents problems pertaining to their efficient delivery to tumors, due to the heterogenic blood supply, as well as half-life and posology affecting patient quality of life – weekly hospital visits and side effects are only some of the negative aspects[1]. These issues highlight the importance of developing more tumor-specific therapies with fewer side effects. Cellular therapies, such as chimeric antigen receptor (CAR) T cell therapy have decisive advantages in this regard and have acquired a prominent role in the treatment of many hematological malignancies[2–4]. For solid tumors, however, there has been limited success with such therapies[5]. The majority of diagnosed malignancies are solid tumors[1]. This demonstrated the great need for targeted therapies for this group of diseases.

In light of this, mesenchymal stem cells offer an attractive candidate for novel therapy approaches, as they are naturally recruited into solid tumors through inflammation to help facilitate tissue repair[6,7,8] Once within the tumor, they differentiate into tissues that comprise tumor stroma, vasculature and scaffolding, which are essential to tumor growth and their ability to metastasize[9].

2. Background

2.1. Mesenchymal Stem Cells

Mesenchymal Stem cells (MSC) are defined as multipotent cells originating from the embryonic mesoderm and capable of replication as undifferentiated cells (self-renewal) and differentiation. They were first discovered in bone marrow stroma, but have since been found to exist tissues throughout the organism, and play an essential role in tissue regeneration[10]. MSC present multilineage differentiation and can differentiate into diverse mesenchymal tissues, including bone, cartilage, fat, tendon, muscle, and marrow stroma.[11,12].

In adults, MSC reside and may be obtained from various tissues, including brain, spleen, liver, kidney, lung, bone marrow, muscle, thymus and pancreas, distributed often in the peri-vascular compartment[13]. The main source of MSC for most in vitro studies is the bone marrow, referred to here as hBMSC. While MSCs are able to proliferate *in vitro*, they cannot proliferate beyond approximately 50 population doublings, a limit that is not encountered *in vivo*[14].

2.1.1. Criteria for defining MSC

While it is possible to characterize MSC according to minimal criteria in respect to surface protein expression there is no unique marker that that is distinctive to them. As discussed, they can be isolated from a multitude of tissues in the body and therefore also present a certain degree of variation as a population. To define MSC the following criteria must be fulfilled: the cells must adhere to plastic in culture and be able to differentiate into osteoblasts, chondrocytes or adipocytes in vitro. Further, they must express the surface markers CD105, CD73 and CD 90. Additionally, they may not express CD45, CD34, CD14, CD11b, CD79α, CD19 or HLA-DR in order to be considered MSC[15,16].

2.1.2. Physiological function of MSC

MSC reservoirs in different organs contribute to tissue maintenance, cell turnover and regeneration of tissues. Importantly, they may also differentiate into tissue other than that of the organ of origin[12]. MSC are thought to be mobilized in response to tissue damage, whether local or distant, and migrate to the affected area to participate in the healing process[17]. MSCs migrate towards areas with high concentrations of inflammatory mediators, therefore exhibit preferential tropism towards injury[7] or sites of tumor development[6].

In addition to their differentiation potential, MSC have been shown to have immune modulatory activity. which is believed to be accomplished by secretion anti-inflammatory mediators and inhibitory molecules such as TNFα, hepatocyte growth factor (HGF), prostaglandins, PD-L1[18].

2.1.3. Tropism towards inflammatory milieu and TME

MSC integrate into the tumor and differentiate into tumor stromal cells[19]. The molecular model for MSC homing to tumors is based on what is known about leukocyte homing. Upon chemotactic stimuli, MSC are mobilized from the bone marrow, and enter the circulation. They identify the target region and adhere to local endothelium, interacting with it in order to extravasate, and insert themselves into the desired site. Some of the cytokines and chemokines regulating these multi-step processes include TNFα, CXCL12 (which is expressed in a HIF-1 – dependent manner[20]) and CXCR4[21].

2.1.4. Role in tumors

MSCs play a pivotal role in the formation of the cancer scaffolding, or stroma. This has been demonstrated for multiple entities, including breast, pancreatic, ovarian and prostate cancers[22]. Once there, they convert into components of vasculature, extracellular matrix and preferentially into cancer associated fibroblasts (CAF) and fibrocytes, influenced by the tumor microenvironment [23,24,25]

Upon activation in the tumor micromilieu MSC produce cytokines and growth factors stimulated by factors present in the TME that can act on cancer cell proliferation, VEGF and EGF that facilitate angiogenesis and moderate infiltration of immune cells[26]. Mesenchymal stem cells in tumors are also driven by the tumor environment to induce expression of CCL5 that has been shown to enhance cancer cell motility, invasion and metastasis. *In vitro* experiments have demonstrated that the presence of stem cell lines in co-culture with cancer cells relevantly increased invasive properties[27,28]. This connection highlights the active contribution of MSC in the formation of the tumor, and provides a potential approach for the targeting of therapeutic strategies[29,30].

2.1.5. Rationale for the use of hBMSC for the delivery of therapeutic transgenes

Bone marrow-derived mesenchymal stem cells possess a series of characteristics that make them suitable candidates for the delivery of therapeutic transgenes. They are comparatively easy to harvest from donors and to expand in culture. This is widely used in autologous and allogeneic stem cell transplantation. As described above, their natural tropism towards tumors is a decisive factor. They not only migrate towards tumors, but also integrate into the stroma, a principle that underlies what has been referred to as the “trojan horse” approach. Furthermore, they are also efficiently attracted to tumor metastases. Lastly, as MSC do not express MHC, HLA or CD40. CD80 molecules on their surface[31], stem cells are not immunogenic, so the infusion of donor stem cells does not elicit anaphylaxis[12,32]. This characteristic has been key in the development of MSC as therapeutical approaches.

2.2. The tumor micromilieu

A solid tumor is formed of cancer cells and other structural and functional components such as blood and lymphatic vasculature, diverse types of other "benign cells", including connective tissue cells and, immune and inflammatory infiltrate. In conjunction with the cytokine landscape produced by this diverse group of cells within a complex extracellular matrix, all of these components comprise the tumor stroma[33]. The tumor micromilieu (TME) helps define neoplasic capabilities[34].

The crosstalk that occurs between the tumor and it's environment represents an important area of research[35]. Cancer cells have tumor defining capacities obtained through genetic mutations and acquired through their interaction with the local environment, including cellular and humoral elements. Such cancer defining characteristics include independence from growth signals and insensitivity to anti-growth signals, evasion of apoptosis, the ability to replicate indefinitely, and crucial to malignancy, tissue invasion and metastasis. They can also interact with the organism in such a way as to misuse its physiological mechanisms, thus promoting angiogenesis, evasion of the immune system, and inflammation[34].

One of the prominent features of TME is a proinflammatory micromilieu. Inflammation mediates the interactions between the components of the stroma and the tumor itself, and influences tumorigenesis and its survival[36].

2.2.1. Cancer as a chronic wound

Solid tumors have been described as resembling a chronic wound. In contrast to the physiological progression of wound recovery, this process is uninterrupted in tumors, and has an essential role in their survival and acquisition of malignant capabilities[37,38] The inflammatory reaction plays an important role here, engaging immune cells that produce chemical signals that recruit stem cell populations toward the area[39]. It is not possible to speak of stroma without mentioning inflammation and vice versa.[36]

2.2.2. Inflammation

The link between cancer and inflammation had been suspected for over a hundred years, and has recently entered the foreground of cancer research.[40] It's role in furthering neoplasm development depends on the nature of the inflammation, whether it's acute or chronic, and on what moment in the development one examines the process. Extensive literature on the subject suggests a correlation between degree of inflammation and poor patient outcomes in diverse solid cancers.[41,42,43]

The connection between inflammation and cancer is not restricted to a small subset of tumors. Many studies implicate chronic inflammatory processes in the onset of malignant tumors[44–49] . Inflammation is present in the tumor microenvironment and can promote tumor survival. It includes the infiltration of leucocytes, which, in concert with other cellular components of the stroma, produce cytokines that

regulate tissue remodeling[36].

2.2.3. Proinflammartory cytokines

Cytokines are small (~5-20kDa) protein molecules secreted by a variety of cells that mediate intercellular communication. They can act in autocrine, paracrine or endocrine manner and are viewed as immunomodulating agents - there are both pro-inflammatory cytokines and anti-inflammatory cytokines, and the action of an individual cytokine upon its target is highly context dependent[50].

Functionally, they can be classified as those that enhance cellular immune responses and those which favor antibody responses. Another classification includes dividing them into the subgroups chemokines, interferons (IFN), interleukins (IL), lymphokines and tumor necrosis factors (TNF). Cytokines are produced by a broad range of cells, including immune cells like macrophages, B lymphocytes, T lymphocytes and mast cells, as well as endothelial cells, fibroblasts, and various stromal cells; a given cytokine may be produced by more than one type of cell [51]

Chemokines, the largest of the subgroups of cytokines, act as chemoattractants for immune cells. Chemokines are named according to the location of cysteine residues near their amino terminus, that is, whether they are sequential (CC) or have a single amino acid between them (CXC), or three amino acids separating the C-C pair [52,53] Chemokines exert their biologic effects via G protein-coupled receptors with seven transmembrane domains, each type may bind one or multiple chemokines within a chemokine subfamily. The nomenclature system names each ligand and receptor pair according to their subfamily (C, CC, CXC, CX3C), followed by the letter L or R, respectively, and a number attributed in the chronologic order in which it was identified. Both ligands and receptors present redundancy, i.e., they may bind multiple receptors and vice-versa.[54]

2.2.4. Nuclear Factor Kappa B

Nuclear Factor Kappa B, NFκB for short, plays an important role in the pathogenesis and progression of malignancy, as it plays a pivotal part in inflammation and inflammatory diseases[55–59]. One of its target genes is CCL5[60,61]. NFκB is a family of five related proteins that work by forming homo- and heterodimers. Upon stimulation with pro-inflammatory stimuli, there is a release of NFκB proteins, followed by shuttling to the nucleusand their binding to a 10bp consensus DNA sequence in target genes that then effect a wide variety of responses related to inflammation, immunity and cellular stress response[111. 65 113,114. 66 67,68].

A variety of external ligands can activate NFκB. Some of the most well studied include members of the tumor necrosis factor (TNF), toll-like-receptor, antigen-receptor and interleukin-1 families[69,70]. The pathway may also be activated stress-related changes in the intracellular environment, such as DNA damage caused by radiation[71,72], reactive oxygen species[73,74] or intracellular pathogens[75,76].

NFκB shows a constitutive basal activity in most tumor cells[77–79] Mutations in many known oncogenes can lead to overexpression of NFκB : Her-2[80], EGFR[81], raf mutations[82], as examples of a few of the most clinically relevant mutations. This process has been linked with some of the events that lead to the malignant transformation of cells.

The activation pathways of NFκB include , the canonical and non-canonical pathways [66,83] The canonical pathway is involved in CCL5 activation and is described below. The five proteins of the NFκB family can be subdivided into two groups: p50 and p52, and p65/RelA, -B and –C[67]. NFκB dimers are present in the cytoplasm bound to inhibitory proteins[84]. Upon stimulation with pro-inflammatory stimuli, there is a release of NFκB proteins and their facilitated shuttling to the nucleus [67,68], [85,86]. NFκB proteins then form hetero- or homodimers that bind to specific kB sites in the promoter regions of target genes. Certain dimer combinations have different affinities. For example, typical combinations include p50-p65(Rel-A) and p50-p50[87]. Different dimers tend to bind preferentially to different binding site sequences[68,88] and each dimer regulates selected target promoters[89]. Thus, these transcription factors regulate functional gene programs.

2.2.5. Tumor stroma

The stroma is a product of the host-tumor interaction. Solid tumors require the surrounding structure of non-malignant cells to grow and invade surrounding tissues. Different tumors present varying proportions of stroma. Some, such as is seen in ductal breast carcinomas or pancreatic adenocarcinomas, may have up to 90% of their mass comprised of these non-cancerous cells, while in others, such as some lymphomas, it represents only a minimal fraction if their total volume[33].

The stroma is indispensable for any tumor larger than 2-3mm, because their rapid growth requires the sprouting of new capillaries – angiogenesis – to nourish the rapidly growing cells[90]. Other vital elements of the stroma include (myo-)fibrobalsts, endothelium, immune cells and neuroendocrine cells.[33]

The stroma provides a nutritional support milieu and a vital barrier to the tumor. Malignant cells can coerce the surrounding healthy cells to produce the growth factors and cytokines that help drive continuous growth and perpetuate a proinflammatory micromilieu [45,91]. In that way, the stroma can be seen as a functional constituent of the tumor that enables and fosters malignancy.

2.2.6. Cancer Associated Fibroblasts

Cancer-associated fibroblasts (CAFs) are a key component of the tumor microenvironment and stroma[92]. CAF play a significant role in tumor maintenance, progression and metastasis through remodeling of the extracellular matrix (ECM), promoting angiogenesis and cross-talk with inflammatory cells[93]. CAF presence and signaling is essential for vital tumor functions – so much so, that their increased presence is associated with poor patient outcomes[94] and more aggressive tumors

Under physiological conditions when tissue damage occurs, fibroblasts – whether resident at the site or recruited – become activated, proliferate, and differentiate into myofibroblasts. Fibroblasts are recruited by a variety of stimuli, particularly inflammation through processes such as chronic infection, autoimmune phenomena or persistent tissue injury in general – including cancer[95]. Upon activation, these cells produce extracellular matrix, particularly collagen, which in the context of a wound in an inflammation-driven physiological reaction, is meant to drive tissue repair[96]. CAFs present characteristics of myofibroblasts and express some of the same proteins. However, they differ radically in that their activation is permanent[97]

The presence of CAF within a tumor requires their recruitment and differentiation. Activated myofibroblasts may originate from quiescent fibroblasts, but they can also originate from cells of epithelial origin (which undergo epithelial-to-mesenchymal transition (EMT)), fibrocytes and stem cells[98]. The data suggest that at least 20% of CAFs originate from bone marrow (BM) and derive from mesenchymal stem cells[99]. MSCs are recruited into the developing tumor and then differentiate into a CAF-like phenotype through the influence of cytokines of the tumor microenvironment (including TGF-β). In thisprocess CAFs are induced to express CCL5[99,100]. This process will be discussed in more detail in 2.3.3. Myofibroblasts arising from bone-marrow MSC become a major component of cancer-induced stromal cells in the later stage of tumor development[22,99].

2.3. CCL5 / RANTES

2.3.1. Structure and physiological function

The C-C chemokine ligand 5 (CCL5), also known as RANTES (Regulated upon Activation, Normal T cell Expressed, and Secreted), and its receptor, C-C chemokine receptor type 5 (CCR5) belong to a large family of pro-inflammatory cytokines and their receptors. In this thesis, the terms CCL5 and Rantes will be used interchangeably. CCL5 is a member of the CC subfamily, with two adjacent N-terminal cysteine residues. CCL5 does not ligate exclusively to CCR5; it activates CCR1 and CCR3. Conversely, CCR5 can be activated by other ligands, among which CCL3, CCL4, CCL5, CCL8 and CCL14 [101].

Physiologically, CCL5 is expressed by a variety of cells, including components of the immune system, such as T-lymphocytes and macrophages, but also platelets, synovial fibroblasts, or epithelial cells. It is produced in states of inflammation and functions as a chemoattractant for T cells, dendritic cells, eosinophils, NK cells, mast cells and basophils, and as a trigger factor for histamine release from basophils[102103,104,105.] CCL5 transcription is known to be upregulated by pro-inflammatory Cytokines such as TNFα, IL-1β, IFN-γ and stimulation with bacterial Lipopolysaccharide (LPS) 1. TNFα and Interferon$_{\gamma}$, in particular, have been used experimentally to drive CCL5 promoter stimulation in many cellular settings. CCL5 has been implicated as a component of inflammatory reaction in a variety of conditions such as viral respiratory infections, asthma[106] and atopy[107], atherosclerosis[108], chronic liver disease[109,110], infections of the central nervous system[111], diabetic nephropaty[112] and rheumatic diseases such as lupus[113] and arthritis[114] .

The transcriptional regulation of CCL5 differs among the cell types that express the gene. Which factor leads to CCL5 activation in which tissue, is variable: in mesangial cells, fibroblasts, monocytes, astrocytes, for example, the transcription is stimulated by inflammatory cytokines, including TNFα and IFN-γ, TNFα alone, LPS and IL-1β, depending on the specific cell type[190.193.]

2.3.2. Functional organization of transcription factor binding sites within the CCL5 Promoter: modules

CCL5 expression is regulated by a series of transcription factors activated in the inflammatory milieu. It's regulation is both cell-type and context-dependent. CCL5 is activated by pro-inflammatory cytokines such as TNFα and IFNγ in endothelial cells and epithelial cells while in astrocytes the gene is activated by stimulation with ILβ. In T cells the gene is strongly upregulated during the functional maturation of effector function. In macrophages, these factors have no effect on expression of the gene, but it is activated by LPS stimulation.

The activation of CCL5 and similar genes depends on the specific cell apparatus in terms of ligand receptors and the overall ability to recognize specific stimuli. Yet on another level, transcriptional regulation takes place not only in the presence of binding sites for any given transcription factor, but also on their organization[115,116]. That is to say, the response of a promoter cannot be predicted simply

by knowing its sequence, because the promoter integrates the simultaneous effects of diverse signals and downstream transcription factors[117].

A group of regulatory elements within a promoter that in concert exert a transcriptional function, have been together termed a promoter module[115,118–120]. In the particular case of CCL5, transcription is controlled by six general regulatory elements within the promoter, termed R(A) through (G)[118]. Depending on the tissue and transcription factors involved, these regions may be functional and for specific transcription factors[121]. As one example: The cytokine IFNβ is a well-known immunoregulator, a property which has led to its use in the treatment of inflammatory conditions such as multiple sclerosis[199–202]. IFNβ can also activate CCL5 through activation of NFκB in certain tissues such as microglia [203–205]. The same can be said of LPS, it can be a potent activator of CCL5in microglia cells, but not in astrocytes[206]. Same substances, same promoter, different reaction depending on context and cell type.

2.3.3. Role of CCL5 in Cancer

CCL5 is present in high concentrations in the tumor microenvironment of several different tumors, ranging from lymphomas to GI tract tumors, to breast and prostate cancers[48,122–128]. Higher concentrations of CCL5 are generally associated with more aggressive tumor phenotypes[129].

Within the TME, CCL5 is produced by the cancer cells themselves and also by recruited MSC[130]. hBMSC have been shown to activate their CCL5 promoter after recruitment to the tumor microenvironment[131–134] while undergoing differentiation into components of the tumor stroma, in particular cancer-associated fibroblasts (CAF)[24,25,135]. This production of CCL5 in turn, influences the cancer cells themselves greatly increasing their metastatic potential and growth by leading to the production of matrix metalloproteases[27,136,137] and inducing the recruitment of inflammatory cells into the tumor[138–140].

2.3.4. NFκB binding sites in the CCL5 promoter

The expression of CCL5 is influenced by NFκB [7,61,141], that acts a central component of pro-inflammatory gene induction in cells of the immune system, as well as other tissues (section 2.2.)[189]. In different cell types, the transcription of CCL5 is more or less NFκB dependent.

The R(A)(B) regions of the CCL5 are involved in the activation of the promoter through the NFκB pathway in a variety of tissues[170.197]. R(A), located on position −73 to −61 of the promoter (relative to the site of transcription initiation) has been found to bind the molecules p50-p65, downstream heterodimers of NFκB, while R(B), located on position −57 to −34, binds the homodimers p50-p50[194,198]. The inflammatory cytokines IFN-γ and TNFα can activate the NFκB signaling pathway through p65 and in this way induce transcription of CCL5[142,106].

Both TNFα and IFN-γ can stimulate the transcription of CCL5[143]. TNFα works through the canonical NFκB pathway to activate CCL5, leading to the binding of homo/hereodimers on r(A) and r(B)[110,144]. IFN-γ leads to CCL5 transcription both through NFκB interaction and NFκB independent actions. Interferon Regulatory Factors (IRF) bind a site on the CCL5 Promoter located further upstream from the R(A) and R(B) regions stimulating transcription [145,146]. The IRFs can also lead to the activation of CCL5 through p65 translocation[147]. In this way, TNFα and IFN-γ can act synergistically to stimulate transcription of CCL5[142, 110, 148, 149, 150] .

2.4. Transforming Growth Factor ß

Transforming growth factor-β (TGF-β) is a member of a superfamily of growth factors that play a fundamental role in cell growth, differentiation, and development in diverse tissues by influencing cellular adhesion, migration and apoptosis[151,152]. Members of the TGF-β family are involved in embryonic development and in the physiological epithelial-to-mesenchymal transition (EMT)[153154]. TGF-β also has an impact on innate immunity[155,156]. The pathological re-expression of those pathways is intimately related to tumor pathogenesis, growth and metastasis[157,158].

2.4.1. Ligands and downstream effectors

The TGF- superfamily comprises 33 structurally related proteins. They exert their action over the TGF-β receptors type I and II, which form heterodimers. The effector proteins of the TGF-β pathway are SMADs that function as downstream intracellular mediators of the TGF-β Family. They are divided into stimulatory R-Smads (Smads 2. 3, 4, 5 and 8) and I-Smads, which have inhibitory actions (Smad-6 and -7) [159–161].

Upon activation of the transmembrane TGF receptor, phosphorylated Smad -2 and -3 partner with Smad-4, that are then translocated into the nucleus where they directly bind promoter DNA sequences leading to transcription of target genes. Inhibitory Smads, antagonize these actions upon activation leading to a negative feedback loop[162]. The expression of the target genes is then modulated by the

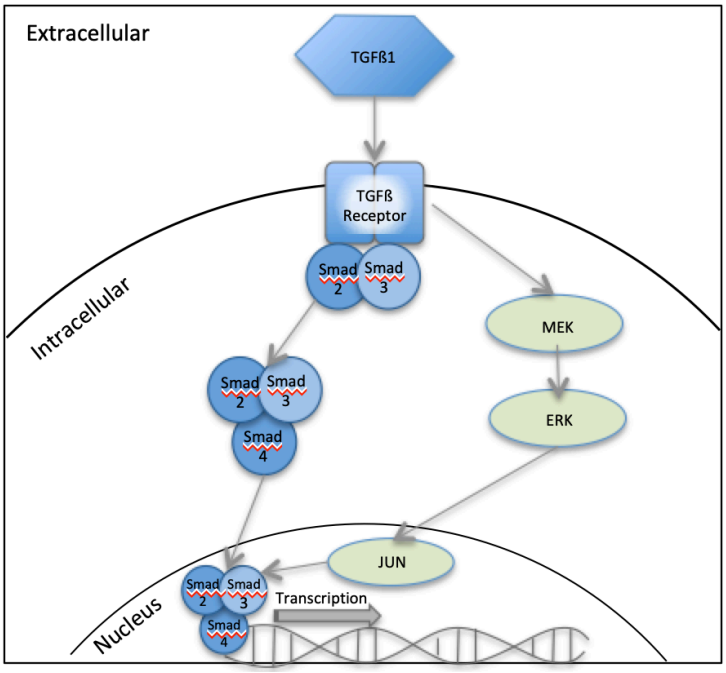

Figure 15: Canonical TGF-ß Signaling and non-canonical pathway

concomitant binding of other transcription factors[163]. Additionally, there are non-canonical pathways that involve activation of MAPK/MEK/Ras pathways[164,165].

TGF-β in the TME

Many tumor types show high concentrations of TGF-β in their micromilieu. This important factor is produced by fibroblasts and MSC. Because of its immunosuppressive properties, TGF-β decreases the anti-tumor activity of some immune cell populations promoting immune evasion[166]. In the context of malignancy, TGF-β stimulation may cause the production of additional growth factors, cytokines and metalloproteases that in turn promote carcinoma growth and metastatic potential by enhancing the activation of transcription factors that play a role in the loss of cell-cell adhesion, adhesion to the surrounding matrix and cell motility[1.167,168]. Current data shows that inhibition of the TGF-β axis correlates with decelerated cancer progression *in vitro*[169,170].

There appears to be extensive crosstalk between the TGF-β pathway and CCL5. Both in vitro and in vivo data has shown a positive correlation of the levels of both cytokines that points towards a direct effect of CCL5 on TGF-β-mediated immune escape[171,172]. How TGF-β may influence the expression of CCL5 in hBMSC is not clear. In particular, the recruitment and differentiation of MSC into CAF is directly dependent on its action[29,99]. This leads us to believe that TGF-β may play a role in the expression of CCL5 by hBMSC.

2.5. Hypoxia

The TME of most solid tumors is either permanently, or at least transiently, affected by hypoxia[173]. Hypoxia is defined as a level of O_2 deprivation that compromises tissue function[174]. Evidently, that depends on the tissue. Different organs in the human body present with varying levels of O_2 saturation usually ranging between 4% and 10% in adults, while diverse tumors have been show to present no higher than 1% to 2% O_2 saturation[175]. Broadly speaking, hypoxia in cancer may result from different factors, including insufficient O_2 concentration in blood, hematological issues causing ineffective transport of oxygen, compromised tissue perfusion or hindered diffusion of said oxygen[176]. In mammalian cells, low levels of oxygen, whether in an acute setting or on a long term, provoke adaptations in order for the cell to maintain its metabolism.

Hypoxia also leads to the transcription of up to 100-200 genes including gene products that allow for increased endothelial cell proliferation and angiogenesis with the intent of generating new vessels to supply the presumably lacking blood[177]. This occurs via the induction of vascular endothelial growth factor, VEGF[178], in concert with other factors like fibroblast growth factor (FGF) and platelet derived growth factor (PDGF) and involves degradation of the surrounding extracellular matrix and migration of endothelial cell precursors that is also facilitated by hypoxia through the induction of MMPs[179].

2.5.1. Hypoxia inducible factor (HIF) pathway

The hypoxia inducible factors (HIFs) are the principal intermediary of hypoxic effects, and are necessary for the initiation of signaling pathways that regulate proliferation, angiogenesis and apoptosis, both in adult organisms and in embryonic development[180,181]. HIF-1 and -2 proteins contain a helix-loop-helix structure[182]. Classically, under normoxic conditions, HIF- 1a and HIF-2 activity is regulated by hydroxylation through oxygen-dependent prolyl hydroxylases (PHD) resulting in their degradation, a process mediated by von Hippel-Lindau (pVHL) tumor-suppressor protein[183]. Under hypoxia, the PHD hydroxylation is limited leading to a HIF-α accumulation. HIFs are then stabilized and the a and b subunits dimerize with ARNTs and translocate to the nucleus[184].

HIFα can also be upregulated by other signaling pathways through oxygen-independent mechanisms via growth factors, deregulated oncogenes, and/or tumor suppressors[185]. Biologically, this is interpreted as a strategy to provide enough blood and oxygen to developing tissues[177].

2.5.1.1. Stabilization of HIF trough exposure to cobalt chloride

Treatment with cobalt chloride is often used in laboratory settings to simulate hypoxia [186]. This use was discovered through the fact that CoCl2 was used to ameliorate some forms of anemia as it induces the production of erythropoietin [187]. This effect, in turn, is owed to the fact that CoCl2 can, as a metal ion with a 2+ charge, substitute Iron in the prolyl hydroxylase enzyme, rendering it inactive and hindering the hydroxylation of HIF [188]. Thus, it has the same effect as true hypoxia, on a molecular level. This has

also has been shown to translate to the functional consequences of HIF: target genes like EPO, Glut-1. and VEGF. In animal models treatment results in protective effects against the hazardous effects of hypoxia upon short-term stimulation while causing tissue damage upon prolonged exposure to CoCl2 [189].

2.5.2. Hypoxia in the context malignancy

In tumors, O2 supply is disturbed by insufficient vasculature and poor diffusion, a consequence of their rapid growth thatis often not accompanied by angiogenesis[176]. Cancer cells alter their biology in order to cope with such conditions, not only resisting hypoxia, but also using it in their favor. Hypoxia has been shown to correlate with poor survival in diverse types of tumors, including cervix, head and neck carcinomas and sarcomas[190]. One of the first changes observable in cells subjected to low oxygen concentrations is a metabolic shift. As ATP generation through oxidative phosphorylation becomes impossible, cells shift their metabolism to attain more energy from anaerobic, allowing them to produce decreased amounts of harmful reactive oxygen species (ROS)[191,192].glycolysis[193]. This has been first described in the context of rat tumors by O2. Warburg in 1924, originating the eponym Warburg effect[194].

Concomitantly many tumors and their metastases exhibit an augmented level of HIF-1a and this correlates with aberrant p53 expression leading to increased cell survival [195]. HIF-1a induces angiogenesisin any tissue suffering from chronically low oxygen levels. In tumors, the formation of new vessels is disorganized and the vessels themselves may be abnormal and leaky. Moreover, the angiogenic process involves HIF inducing the production of metalloproteases, which degrade ECM around pre-existing vessels so as to facilitate sprouting of new ones. Both of these are contributing factors for metastatization.[196].

Hypoxia also contributes to the formation of the tumor stroma by the induction of transcription of a large series of genes and transcription factors involved in EMT and by maintenance of a pro-inflammatory TME.[197,198] [199]. Furthermore, it can promote formation of tumor stroma by inducing the production of collagen in fibroblasts[200], as well as by the secretion of TGF-β which acts in the maturation of new vessels.

2.6. Engineered mesenchymal stem cells as therapeutic vehicles for transgene delivery: existing approaches and limitations

The natural tropism of MSC toward solid tumors makes them promising potential candidates as vehicles for the delivery of therapies deep into tumor environments by engineering MSC to deliver therapeutic transgenes. The general approach of utilizing MSCs as therapy vehicles has been extensively explored in *in vitro* and *in vivo* models of tumor biology and in a series of phase I/II clinical studies. A variety of therapeutic transgenes (TT) in this setting have been studied, including interferons, interleukins[201] and other immune-stimulatory cytokines[202] used to promote tumor apoptosis, or enhance the immune response against the malignant cells, inhibit angiogenesis in the tumor and more, with varying degrees of success[1,203–210]. In this way, it is possible to use the engineered MSC as a form of "Trojan horse" for the selective delivery of the therapy transgenes [211].

Our group initially engineered MSCs to express the suicide gene thymidine-kinase (TK), an enzyme encoded by herpes viruses, that when expressed by MSC in the tumor stroma allows selective therapy after application of the prodrug ganciclovir. The approach was initially evaluated in a series of preclinical models and the approach has subsequently progressed to in phase 1/phase 2 trials in humans for gastrointestinal cancers[212,213]. The engineered MSC are infused, they home to the tumor environment and integrate into the tumor stroma where they express TK. Another example of a therapy gene used in MSC-based cancer therapy makes use of the sodium-iodine symporter (NIS) gene as a TT[214]. After integration of NIS-containg MSC into the tumor, radioiodine can be used against the malignancy, as has been used for well-differentiated thyroid carcinomas, which naturally express NIS, for decades[215].

A major issue in these approaches is the selectiveness of tissue targeting – the objective being optimally efficient expression of the transgene in the tumor environment with minimal non-target organ toxicity. Engineered MSC do not necessarily migrate only towards tumor tissues, but have been reported in the studies mentioned above, as well as others, to be found also in tissues such as skin, digestive tract, lymphatic organs and particularly, transient entrapment in the lungs, potentially leading to organ damage[216]. It is optimal, then, that the transgene be selectively expressed within the tumor environment and not in non-target organs.

2.7. Rationale and objective of this thesis

The limitations of the use of hBMSC as delivery vehicles have been in toxicity profiles seen in phase I/II studies[217]. Potential issues derive in part from non-target migration - MSC migrate to inflamed tissues other than the tumor, or for example, areas surrounding irradiated or infected tissue[218,219]. Expression of the transgene in a non-tumor context, could lead to off target damage to heathy tissue[220,221].

In this thesis, we chose to approach the problem of non-targeted expression of therapeutic transgenes under the lens of the activation of the promoter driving the transgene in engineered hBMSCs. The focus is the promoter of pro-inflammatory cytokine CCL5/Rantes, which is known to be upregulated in the process of hBMSC differentiation into carcinoma associated fibroblasts (CAFs)[212]. It has been successfully used as a promoter for driving the expression of TT in preclinical and clinical studies as stated. The focus of this thesis was to better understand the expression dynamics in the context of MSCs in response to tumor relevant signals.

The objective of this thesis is to better understand the components of the tumor micromilieu that promote activation of CCL5 promoter and differentiation of MSC and the mechanisms through which they do so, and secondarily to modify the promoter in such manner as to have a more context-specific activation, avoiding potential toxicity.

3. Hypotheses and strategies employed to investigate activation of CCL5 in the context of human MSCs

At the time of this study, engineered versions of autologous MSCs using the CCL5 gene promoter to drive the suicide gene thymidine kinase were being used in a phase I/II clinical trial for gastrointestinal cancers[213,222,223]. An important challenge in the treatment of solid tumors with engineered MSCs remains potential side effects through their migration into other organs or focal inflammatory regions potentially leading to tissue damage. The central goal of this thesis was to better understand the biology surrounding the activation of the CCL5 promoter in MSCs in the context of the tumor environment.

Questions relating to the specificity of the activation of the CCL5 promoter in hBMSC, and specifically on the potential effects of:

(i) Inflammatory cytokines IFN-γ and TNFα
(ii) Exposure to the hypoxic stimuli present in solid tumors
(iii) Exposure to TGF-β

To better characterize the dynamics of CCL5 activation by MSC in the context of the tumor milieu, the following approaches were used:

- The generation of a flexible cloning and expression platform for engineering constructs - validated in different cell types and lines for proof-of-concept.
- Optimization of stable integration of the constructs into target cell genomes
- Reporter constructs containing *Gaussia* luciferase coupled to the promoter of interest stably introduced into primary human MSC to quantify activation of the promoter when exposed to different tumor-relevant substances and conditions.
- Stimulation by stimuli present in the majority of solid tumors: these include the inflammatory cytokines TNFα and IFNγ, hypoxia and Transforming Growth Factor-β (TGF-β).

4. Materials

4.1. Cell lines	Source
Hek293, human embryonic kidney	ATCC CRL-1573, Manassas, Virginia (USA)
hbMSCAp172_1 (MSC)	Apceth GmbH & Co.

4.2. Bacteria	Source
One Shot® Mach1TM T1 Phage-Resistant Chemically Competent *E. coli*	Invitrogen, Thermo Fisher Scientific, Waltham (USA)

4.3. Medium/Supplement	Source
Antibiotics	
Blasticydin	invivoGen #ant-bl
Doxycycline	Santa Cruz #sc-204734A
Kanamycin	Roth #T832.1
Puromycin	InvivoGen #ant-pr-1
Streptomycin/Penicillin	PAN #P06-07100
Medium	
DMEM	
Hek293 Medium	90% DMEM, 9% PFS 0.9 % PS
MSC Medium	90% DMEM, 9% PFS, 1.7% TK, 0.9 % PS
Chemicals	
Trypsin/EDTA Solution (T/E)	PAN-Biotech GmbH, Aidenbach (Germany)
Dimethyl sulfoxide (DMSO)	Merck, Darmstadt, (Germany)
PBS	
Additional substances	
Thrombocyte concentrate	Donation of Klinikum Schwabing, Munich

4.4. Enzymes (not including restriction enzymes)	Source
Gateway BP Clonase II	Invitrogen, Carlsbad
Gateway LR Clonase II Plus	Invitrogen, Carlsbad
Phusion DNA Polymerase	NEB, Frankfurt
Restriction enzymes	NEB, Frankfurt
T4 DNA Ligase with 10mM ATP	NEB, Frankfurt
Taq DNA polymerase	NEB, Frankfurt

4.5. Restriction enzymes
AgeI, ApaI, AvrII, BamHI, BglII, BsaHI, BsrGI, BstBI, Eco53kI, EcoRv, FspI, KpnI, NcoI, Nde1. NheI, NotI, PstI, SacII, SalI, SapI, XbaI, XhoI

4.6. Buffers	Source
Restriction enzyme buffers 1.1. 2.1. 3.1. Cutsmart	New England Biolabs, Ipswich (USA)
T4 DNA Ligase buffer	New England Biolabs, Ipswich (USA)
Antarctic Phosphatase buffer	New England Biolabs, Ipswich (USA)
Phusion HF buffer	New England Biolabs, Ipswich (USA)
Phusion GC buffer	New England Biolabs, Ipswich (USA)
Thermo Pol Buffer	New England Biolabs, Ipswich (USA)
Agarose Gel Buffer	
Bromphenol Blue	Roth, Karlsruhe (Germany)
Xylen-Cyanol FF	Merck, Darmstadt, (Germany)
Glycerol	Roth, Karlsruhe (Germany)
H2O	
Electroporation Buffers (non-Neon)	
KCl	Merck, Darmstadt, (Germany)
MgCl	Roth, Karlsruhe (Germany)
Na2HO4/NaH2PO4 pH7.2	Merck, Darmstadt, (Germany)
Mannitol	Merck/Sigma-Aldrich GmbH, Munich (Germany
Neon electroporation Buffer	Invitrogen, provided with kit

4.7. Electrophoresis Ladders	
0.6 kB Ladder	
1 kb ladder	Thermo Fisher Scientific, Waltham (USA)
2 log Ladder	New England Biolabs, Ipswich (USA)

4.8. Disposables	
Cell culture plates	TPP, Trasadingen (Switzerland)
Cell culture flasks	TPP, Trasadingen (Switzerland)
Cell scrapers	TPP, Trasadingen (Switzerland)
Conical centrifuge tubes 15ml/50ml	BD Biosciences, Franklin Lakes, USA
Eppendorf tubes	Eppendorf, Hamburg
PCR tubes 0.2ml	Eppendorf, Hamburg
Tubes, 5ml 75x12mm, PS (for luminometer)	Sarstedt, Nümbrecht

4.9. Software	
GraphPad Prism 9.0.1	Graphpad Software, La Jolla (USA)
Snapgene 5.2.5	GSL Biotech LLC
Clone Manager	Sci-Ed, Denver

4.10. Plasmids and vectors

Entry clones	Purpose	Flanked
pENTR221-Globin2f	Entry clone for Globin Tausch, creation of CCL5 rep.	attL1.R5
pENTR221-Globin3f	Entry clone for Globin Tausch, creation of CCL5 rep.	attL1. L4
pENTR221-Cherry	Entry clone for Cherry	attR4,R3
pENTR221-iRFP2f	Red Fluorescent Protein	attL5, L2
pENTR221-iRFP3f	Red Fluorescent Protein	attR4,R3
pENTR221-IRES Puro	Internal ribosome entry site	attL3, L2
pENTTR221CAGPro2f	CAG Promoter	trad. ligation
pENTTR221CAGPro3f	CAG Promoter	trad. lig.
pENTR221-TGFv2	TGF-β Promoter	attL1.R5
pENTR221-RantesPro	CCL5 promoter	attL1. R5
pENTR221-sGFP		attL1. R5

Dest Vectors	Purpose	Flanked
pCDNA6.2PLITS-SF Dest	for IRESPuro, iRFP and CAG Dest vectors	
pcDNA6.2ITRHygro	Backbone for TGF	
CAG3f SF Dest		
iRFP3f SF Dest		
pcDNA6.2ITR-IRES-Puro-Dest	Ligation of IP,Overhangs, NeoDest	
CAG2F Hygro dest		
iRFP 2f Hygro dest		
pCDNA6.2PLITSRNEO_Dest	For IRES Puro	

Expression Clones	Purpose	Flanked
pCDNA6.2ITRNEO_Waf1Pro_Luc	Gaussia Luc	attB1.B2
pCDNA6.2ITRNEO_Waf1Pro_iRFP	Red Fluorescent Protein	attB1.B2
pCDNA6.2ITR-CAGPRo-iRFP-IRES-PURO	Red Fluorescent protein with a CAG Promoter, Puromycin resistance	attB1.B2
pCDNA6.2ITR-Hygro_CAGPRo-iRFP	Red Fluorescent protein with a CAG Promoter, Hygromycin resistance	attB1.B2
pCDNA6.2PLITR-CAGPro-Cherry-IRES-PURO	Cherry fluorescent protein, CAG promoter, Puromycin resistance	attB1.B2
pCDNA_Gluc3_CMVMin_TGF2	Pre-established, traditional cloning	
pCDNAGluc3_RANTES	CCL5 promoter, Gaussia reporter gene, Blasticidin resistance	

5. Methods

5.1. Experimental design

For experiments two plates were used for each run, each condition represented by three wells on each plate containing the same number of cells for each experiment. The number of cells seeded into each well varied according to cell type, as well as number of cells in the culture from which they originated. For experimental conditions the medium in culture plates was substituted by empty DMEM Medium 24 hours before plating, as the presence of FCS, thrombocytes and Penicillin-Streptomycin might induce expression of the reporter. Stimulation with chosen compounds was done at the moment of plating. Cells were kept in an incubator at 37˚ C, either at 21% O_2 or 1% O_2. Luminescence measurements were performed at 24h and 48h after plating.

Negative controls were represented by unstimulated cells. TNFα and IFN-γ were cells stimulated by TNFα and IFN-γ, based on the data from the literature discussed above.

5.2. Cell culture

5.2.1. Culture conditions, splitting and medium changes

Cells were cultured in a 37°C, 5% CO2 and 21% O2 laminar flow incubator at all times, except for experiments involving hypoxia. The hypoxia incubator (from producer ThermoFisher[224]) used Nitrogen in order to generate an O2 concentration of 1%, while maintaining CO2 concentration.

Experiments and plating were performed under room temperature with reagents thawed out to room temperature before use, except in the case of freezing of cells with DMSO, in which case this was done on an ice box to avoid DMSO toxicity.

Splitting and platting of cells involved removing DMEM medium and washing cells with PBS. The cells were the loosened from the flask or plate using Trypsin-EDTA Solution, under microscope control. After adding 10% FCS DMEM Medium, the cells were centrifuged in Falcon tubes, forming a pellet. After removal of the mix of Trypsin EDTA and DMEM, the pellet was resuspended in medium (different mediums, according to cell type and experiment involved) and either plated or seeded into a culture flask or dish. If the cells were being selected, antibiotics were then added according to defined concentrations. For Hek293, splitting was done every other day, as was done with L87 cells. Medium changes were done every day.

5.2.2. Cell culture for primary mesenchymal stem cells

The mesenchymal human bone marrow stem cells used were obtained from the company Apceth and were donated by a 37 year old healthy male patient (hbMSCAp172_1). These were cultured in MSC Medium containing DMEM with 10% FCS, 1% PS heparin and 5% thrombocyte concentrates (see materials section).

MSC were split depending on the homogeneity of the coating of the culture flask or dish, which was checked under the microscope daily. Within certain experiments, medium changes were done at certain intervals. This is described later.

5.2.3. Antibiotic selection of electroporated cells

In order to determine the lethal concentration of the planned antibiotics on non-resistant cells, an experiment using a dilution row was performed. This was done for cell lines hbMSC, L87 and Hek293 for Blasticydin, Hygromycin, Zeocyn, Puromycin, depending on necessity. For this, vials with different concentrations of antibiotics were kept in culture and observed for cell vitality daily, with medium change every other day. The following necessary concentrations were established:

	Blasticydin	Hygromycin	Puromycin	Zeomicyn
hbMSC	2.0 µg/ml	40 µg/ml	0.6 µg/ml	300µg/ml
Hek293	5.0 µg/ml		5.0 µg/ml	
L87	6µg/ml	200µg/ml		

After electroporation, cells were put under antibiotic selection after 24h. Then, a medium change performed with first 50% of the maximum antibiotic dose, which was kept until the next medium change, 72h after electroporation. At this point, the full antibiotic concentration was added. When cells were seeded for experiments, the antibiotic was not added into the medium at resuspension of the pellet, so an to avoid interactions with the substances used.

5.2.4. Counting cells

When necessary for plating of cells for experiments, cells were counted. This was done using the same process described above to loosen the cells. An aliquot was then mixed with tryptane blue in order to stain the cells and filled into a Neubauer counting chamber. One such chamber holds 0.1 µl of fluid per square. Cells were counted in all four squares and averaged. Depending of desired number of cells, the volume necessary was then calculated, accounting for dilution, using the following calculation:

Concentration of cells (units/ml) = cells per square x 10^4 x factor of dilution

5.2.5. Freezing of cells

Freezing of cells was used to preserve cloned cell lines. This was done in an ice box, so as to keep the cells as cool as possible prior to freezing, avoiding DMSO toxicity. For this, cells were loosened as described above, transferred into a cryo-tube and received a specially formulated medium containing 55% DMEM, 35% FCS and 10% DMSO. The cryo-tubes were then quickly stored in a isopropanol container and into a -80°C freezer. After 24h, if long time storage was wished, the tubes could be transferred into liquid nitrogen containers.

In order to thaw cells up, the contents of the cryo-tube were transferred into large volumes of Medium at room temperature, so as to dilute the DMSO, with a subsequent change of medium within 24h.

5.3. Microbiology

5.3.1. Preparation of competent E. coli MACH-1

In the cloning process, produced plasmids had to be propagated. This was done using E.coli which were chemically competent, which are able to take up plasmids due to increased permeability of their membrane after chemical treatment. While these can be acquired commercially, they were produced in our laboratory.

MACH-1 E. coli are used for their high transformation efficiency. For preparation, they were inoculated into LB Medium and incubated overnight at 37°C until reaching a concentration of 0.375 in terms of OD600nm. They were then divided into 50µl aliquots, which were incubated on ice for 5 minutes and subsequently centrifuged for 10 minutes at a speed of 1600G, in order to separate the supernatant. Bacteria were then resuspended in cold $CaCl_2$ solution. Once again, the aliquots were centrifuged under the same conditions and the supernatant discarded. This step was repeated twice with ice-cold $CaCl_2$. Thereafter, the pellet of bacteria was finally resuspended in 2ml $CaCl_2$. divided into 50µl aliquots and stored in -80°C.

5.3.2. Freezing and thawing of bacteria

If, after transformation, it was wished to storage an E.coli strain, those could be frozen to -80C. To do so, 900µl of liquid overnight culture were mixed with 100µl of freezing solution for bacteria (for exact contents, see materials). When frozen E.coli were needed, a measure of frozen cells could be put directly onto an agar plate and cultured overnight in a 37C incubator. One of the resulting colonies was

selected and inoculated into a liquid culture using LB Medium.

5.3.3. Preparation of agar plates

Agar plates were used in bacterial culture. To prepare those, 1.5% agar was added to a dosis of LB medium and subsequently autoclaved. If necessary, antibiotics were added in the following concentrations: Kanamycin 50µg/ml; Chloramphenicol 25µg/ml. The plates were stored in 4°C refrigerators.

5.3.4. Transformation of E.coli

E.coli were transformed with plasmid sequences in order to amplify DNA quantities. To do so, firstly the LR/BP reactions were stopped (see Gateway cloning) using proteinase K enzyme. 2µl-10µl of the plasmid DNA were added to one vial containing 50-100µl Mach-1 chemically competent E.coli and incubated for 30 minutes. The bacteria were then heat-shocked at 42°C for 45 seconds, and the immediately the tubes were transferred onto ice. 250µl of SOC medium were added to each vial, which was then shaken for 1 hour at a 37C temperature. After this time, the cells were spread onto a pre-warmed agar plate containing antibiotics, ensure well-spaced colonies. If the transformation was for entry clones, the antibiotic selection was done with Kanamycin, as that is resistance contained in the EntryDest plasmid. If the involved vector was an expression vector, we used Ampicillin.

5.4. Cloning

5.4.1. Gateway cloning technology

5.4.1.1. Outline

Gateway cloning is a commercially available technology established since the 1990's, which utilizes the recombination properties of Phage λ and E.coli intergrase, together with a set of defined sequences flanking DNA in order to allow for efficient and easy recombination and generation of expression clones. It is based upon the generation of a so-called entry clone (The nomenclature for the overhangs, reactions and clones is proprietary to Invitrogen),which contains a gene if interest embedded into a standardized donor plasmid provided by the company Thermo Fischer, done through a BP-reaction (see ahead). Different entry clones can then be combined in an LR-reaction and integrated into a destination vector of one's choice[225–227].

Figure 2 outlines the basic principles of Gateway technology. In the first step (A), the sequence of interest is flanked with the known attB1/2/5 sequences using PCR. These are homologous sequences. The resulting product then undergoes the BP reaction, in which it is ligated into compatible donor vectors, which contain the matching attP1/5/5r/2 sequences (B), thus generating entry clones (C). These can then be used in the LR reaction (D), in which the genes of interest contained in each entry clone are then integrated into a single plasmid, the backbone of which is a provided plasmid with compatible attR1/2 sequences. The attL5 and attR5 overhangs of the involved genes are compatible to each other and thus the end product contains the sequences immediately following each other in the resulting expression vector (E). In between steps, plasmids are amplified using E.coli transformation. The donor vectors provided contain suicide genes, (ccdB), which are replaced during the recombination reaction. It that doesn't take place, i.e., the product is faulty, the transformed E.coli dies[228].

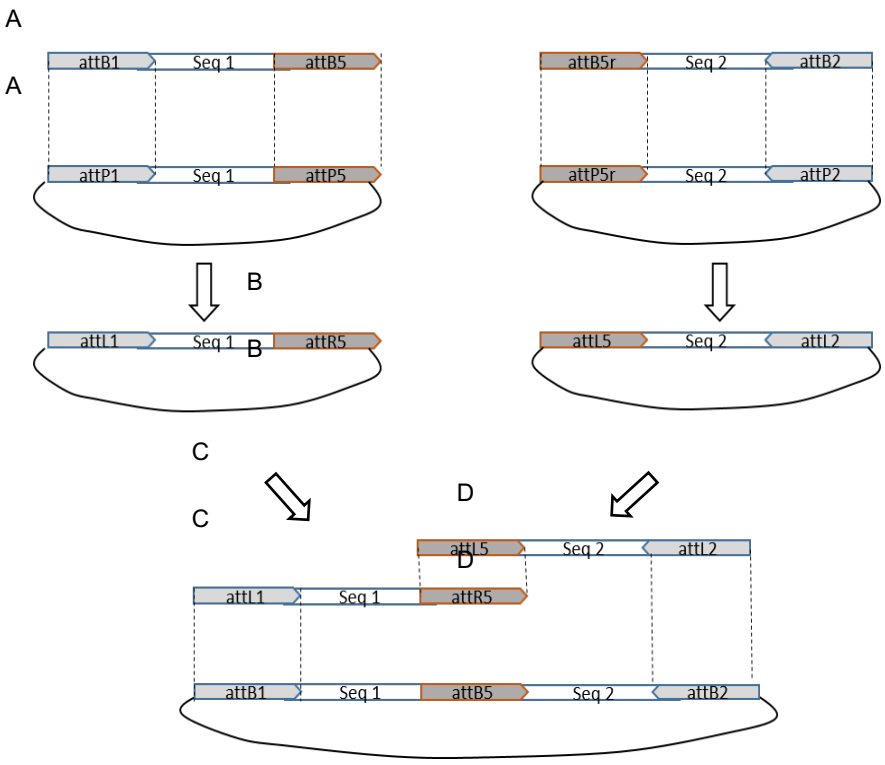

Figure 2: schematic representation of the Gateway recombination steps. *Gateway cloning is a commercially available technology which utilizes a set of defined sequences (termed "attB/ attP, attR and attL) flanking DNA in order to allow for efficient and easy recombination and generation of expression clones, in a mix-and-match principle. In the first step (A), the sequence of interest is flanked with the known attB1/2/5 homologous sequences using PCR, then ligated into compatible donor vectors, which contain the matching attP1/5/5r/2 sequences (BP Reaction) (B), thus generating entry clones (C). The destination vectors used for the entry clones ("pSB Dest" vectors) had been previously generated and made gateway-compatible, and contained antibiotic resistance for later selection of transformants. These can then be used in the LR reaction (D), in which the genes of interest contained in each entry clone are then integrated into a single plasmid, using homologous attR1/2 sequences, resulting in the expression vector (E).*

5.4.2. Specific steps of the generation of entry clones through BP-reactions

Firstly, in order to make sequences of interest compatible Gateway cloning technology, known sequences for the attB-overhangs were added using PCR (see above). The templates for PCR were commercially acquired and contained the homologous sequence to attB, which is attP in the Gateway system.

The MultiSite Gateway Pro Plus Kit was used. The reactions were performed according to the manufacturer's protocol. In broad strokes, the attB-flanked DNA product was mixed in a 1:1 volume with purchased donor vector, TE buffer and BP Clonase. This was incubated at 25°C for an hour and the reaction then terminated using proteinase K.

The resulting plasmid was transformed into Mach1 E.coli, which were selected with Kanamycin. Kanamycin resistance is contained in the standardized donor vector.

To assess correctness of the reaction, test digestions were performed, then the resulting clone was sent to sequencing.

5.4.3. Specific steps of the generation of expression vectors through LR-reactions

Our final expression vectors were generated with two entry clones, one for a promoter sequence and one for a reporter construct, respectively. The destination vectors used ("pSB Dest" vectors, see below) had been previously generated and made gateway-compatible, and contained antibiotic resistance for later selection of transformants. LR reactions were carried out according to manual instructions in the MultiSite Gateway Pro Plus Kit[228].

The LR reactions were performed by adding varying volumes of entry clone (depending on length), 1µl destination vector and TE buffer to amount to a total of 8µl. Analogously to the BP reaction, 2µl LR Clonase were added, the solution was incubated at 25°C for one hour and the reaction stopped using Proteinase K. More details are contained in the user's manual.

5.4.4. PCR

In order to create oligomers with compatible overhangs for Gateway cloning or enzyme digestions, PCRs were performed. The ordered primers were resuspended with a dilution of 1:10. For each well, 1µl each of the forward and reverse primer was added to 4µl dNTPs (at a concentration of 1.25mM), 0.24µl of Polymerase, 5µl buffer and water. PCRs were done using a Polymerase with 3'-to-5' exonuclease function, available commercially. The times and temperature settings of the PCR machine are listed below. After performing the PCR, the samples were added together, mixed with a loading

buffer and subjected to gel electrophoresis.

Table 1

Denaturation	98°C	5'30s
Annealing	According to primer	30s
Extension	72°C	Depending on lenght
Final extension	72°C	10 in

5.4.5. Separation of DNA fragments by electrophoresis

After digestion, DNA fragments were separated by means of electrophoresis. The gels used ranged between 0.6% and 2% agarose content, depending on molecule size. To prepare the gels, 0.5x TBE buffer was mixed with ethidium bromide. After heating, gels were poured into gel chambers and let cool.

After cooling of the gel, the DNA samples were mixed with a loading buffer. A size standard for comparison (either 1log or 2log ladder) was also mixed with loading buffer. Electrophoresis itself was performed under 170V. The resulting gel was then examined under UV light, photographed and, if DNA was to e purified, the corresponding band was cut out of the gel and purified with a kit, as described below.

5.4.6. Gel extraction and purification of DNA

After electrophoresis and cutting out the bands of interest, DNA was extracted from the gel and purified using the QIAquick PCR Purification Kit, according to manufacturer's instructions. If the resulting DNA fragment after digestion had compatible overhands, the plasmid 5' ends were dephosphorylated using Antarctic phosphatase (5 IU per pmol).

5.4.7. Determination of DNA and RNA concentrations

After purification, the concentration of DNA was determined using a Nanodrop ND-1000 spectrophotometer, according to the manufacturer's instructions and blanked with 2µl of the water in which DNA was diluted.

5.4.8. Design of short synthetic DNA elements

In situations where oligomers (<200bp) of single strand DNA were needed for the assembly of reposter vectors, those were designed and ordered commercially (Thermo Fischer Scientifc, USA), with overhangs compatible to the restriction enzymes planned for ligation into a backbone.

5.4.9. Ligation of DNA fragments

The oligomers were resuspended in a concentration of 200µM, and 5µl of top and bottom oligomer were mixed with 2µl annealing buffer and water. This was heated up to 98°C for 4 minutes. After cooling, time in which the oligomers annealed, 1µl of this solution was diluted in 30µl H2O. 5µl of this diluted solution were mixed with 3µl of a DNA solution containing the digested Backbone with compatible overhangs, and 1µl of t4 DNA ligase and its respective buffer were added. Ligations were performed overnight in room temperature. After ligation, the solution was transformed into Mach1 E.coli (see above), the purified using Mini Preps and test-digested.

5.4.10. Restriction digestion of DNA

The restriction digestions of DNA were performed with 1IU of the respective restriction enzyme per µg DNA to be cut per hour, occasionally using more time and reagents if there were especially large quantities of DNA. The solutions were prepared so as to contain at maximum 10% of enzyme, so that after calculating the amount of enzymes used plus the amount of DNA and buffer, the remainder of the volume was filled up with H20 so as to total 10x the amount of enzymes. The buffers used were enzyme-specific and 10x concentrated. The chosen temperatures were according to manufacturer instructions. The restriction enzymes used are listed under materials.

5.4.11. Test digestions

In order to verify that the ligations had been successful, test digestions were performed with enzymes that would cut once in the backbone and once in the ligated fragment, producing fragments with expected size. To identify such sites and the corresponding enzymes CloneManager Software was used. Temperatures and buffers for the chosen enzyme were profided using the double digest finder website from New England Biolabs. Selected clones were then transformed into E.coli, as described above.

5.4.12. Isolation of plasmid DNA from transformed bacteria

After transformation into Mach1 E.coli and incubation of a culture, DNA Plasmids were isolated and purified using Innuprep Plasmid Mini Kit and ZymoPure Plasmid Maxi Kit, which are commercially available, depending on the culture volume. In general, the process relies on lysis of the bacteria and passing the solution through a filter membrane, which binds plasmid DNA, while precipitating bacterial DNA. Subsequently, the bound DNA is washed and eluted, yielding purified plasmids. The specific process was done according to manufacturers' instructions. As described with ligation, a test digestion was then performed to verify that the DNA had the expected length after digestion.

5.5. Stable integration using Sleeping Beauty transposons

In 1997, an inactive transposon/transposase system from salmon species was re-engineered to generate a synthetic active system, which was termed Sleeping Beauty (SB)[229]. While in nature a transposons efficacy is limited by the fact that the 'host' DNA would not survive if mutations and insertions were done to excess, in vitro the efficiency of SB has been increased so as so to obtain the highly active enzyme used today[230]. This system has since then been used to successfully integrate desired sequences into the Genome of multiple vertebrate species, without the limitations of viral systems, and showing prolonged expression of said genes[231,232].

A transposon/transposase system is used for stable integration of desired sequences into cell DNA. It is constituted by two basic elements: a short, repeating flanking sequence of DNA, which consists of short rep inverted terminal repeats (ITR), and a transposase protein, which can be coded between said fragments[8,]. The tranposase protein has the ability to excise the gene sequence contained between ITR (which may include the transposase itself, in which case it is an autonomous system) and insert it into any point of the genome[9].

In our work, we used the SB system widely a as non-autonomous element. A vector containing the SB100 transposase enzyme was gifted to our laboratory by Zsuzsanna Izsvak (Addgene plasmid number 34879). ITR Repeats for use in connection with the enzyme were contained in the Destination vector used.

5.6. Electroporation

Transfection is an umbrella term to describe various methods of introducing foreign DNA/RNA into cells[235]. One of those is electroporation, a technique by which exposing a cell to an electrical current causes an increase of membrane permeability and allows nucleic acid molecules in the environment to enter the cell. This has been shown to be a highly efficient process, albeit highly toxic for the cells[236,237].

5.6.1. Electroporation transfection using the Lonza Amaxa® System

Until Aug. 2015, we conducted electroporations using the Lonza Amaxa® nucleofector IIb. This was done for both Hek293 and hBMSC. To do so, cells were first loosened with trypsin, centrifuged and resuspended, then separated into aliquots containing 1x 106 cells each. This was again centrifuged, the supernanant discarded, and the cells resuspended in a pre-defined buffer, (buffer 1M, containgn 5 mM KCl; 15 mM MgCl2; 120 mM Na2HPO4/NaH2PO4 pH7.2; 50 mM Mannitol). After adding 2µl of each plasmid to be eletroporated and double as much SB100 enzyme, 100µl of the resulting mix were pipetted into a cuvette. The cuvete was the exposed to an electric current in the nucleofactor machine , using the according program for each cell type, provided by manufacturer's instructions (U23 for stem cells, Q001 for Hek293). Afterwards the cells were immediately transferred to a vessel and supplied with DMEM with 10% FCS, 1% PS, 5% TK and 250µl Heparine). After 24h, a medium change was performed and antibiotic selection was started.

5.6.2. Electroporation transfection using the Invitrogen® Neon System

Because of relatively low efficiencies and a cumbersome process of electroporation using the Amaxa system, from Aug 2015 onwards, we performed transfections by electroporation using the NEON ® System from Invitrogen[238]. This required the establishment af new electroporation protocols for MSC.

The electroporations done using the NEON System from Invitrogen[238] required the establishment of new protocols for hBMSC. In our experience, this method of electroporation has proven to be more efficient and less toxic towards cells, as well as more practical, for it only needs one kit of buffers, independently of cell type.

In accordance to the supplier's instructions1. cells were loosened with trypsin, centrifuged and resuspended and counted, the separated into 300.00 to 500.000 cells per aliquot. They were then centrifuged again, the supernatant discarded and resuspended in 10µl of buffer R (contained in the commercially available kit). We then added 1µg (therefore a variable volume, depending on plasmid concentration) of each plasmid of interest and 2µg if SB100 plasmid. As The NEON System works with special pipettes, which allow the transmission of a current, these were used to take up the resulting mix and were immediately placed into the machine. Parameters were chosen according to cell type, as seen on table 2. While the manufacturer provides suggestions for some cell types, at the time we designed our own protocol for hBMSC. After putting the cells trough the current, they were transferred into a pre-prepared vessel containing medium. This was repeated using the same pipette tip (which contains c.a. 10µl), until all the solution was used up.

Tips could be reused after cleaning and regenerating the, using the protocol by Brees and Fransen[239].

Table 2

H9 Human Embryonic Stem Cells					
Voltage (v)	**Pulse nmr**	**Width (ms)**	**Cell density**	**Efficiency**	**Viability**
1100	1	30	10^7/ml = 100.000 in 10µl	29%	99%
1050	2				
BGO1V Human Embryonic Stem Cells					
Voltage (v)	**Pulse nmr**	**Width (ms)**	**Cell density**	**Efficiency**	**Viability**
1200	1	30	10^7/ml = 100.000 in 10µl	33%	92%
	2	20		21%	90%
Mouse Embryonic Stem Cells/ Adipose-derived Stem Cells					
Voltage (v)	**Pulse nmr**	**Width (ms)**	**Cell density**	**Efficiency**	**Viability**
1400	10	3	10^7/ml = 100.000 in 10µl	88%	96%
1200	20	2		79%	99%

For hBMSC, the standard programme chosen was based on H9 Human Embrzonic Stem Cells: Voltage: 1050v; Width: 30ms; Pulses: 2.

5.7. Seeding and stimulating of cells for experiments

In order to perform luminometric experiments, cells that had been selected with antibiotic for at least 4 passages were detached using Trypsin-EDTA, resuspended, counted and then diluted so as to obtain a certain number of cells per 50µl. Usually that was 20000 cells/50µl, but depending on the experiment, cells were seeded in 100µl, or higher concentrations of cells were needed. This is indicated at the description of each experiment. They were then plated into a 96-well plate. When plating cells, no antibiotic was used. After 2 hours, the test substance was added. The dilution of test substances was choses so as to not exceed 4% of total well volume for a defined concentration (for example, 2µl TGF-β in a 50µl well). The assays were performed at timepoints 24h, 48h and sometimes 72h.

Stimulation with hypoxia was done using a hypoxic incubator with 1% O_2 air concentration. While the simulation of hypoxia would also have been possible using cobalt chloride, as outlined above, and the response of the constructs to $CoCl_2$ had been established, we ultimately preferred the hypoxic incubator, as potential interactions of Cocl2 itself with the other substances used for stimulations could not be excluded. Furthermore, O_2 concentrations ranging from 2% to 0.5% were tested (data not shown). While there was no statistically significant difference in response between 1% and lower concentrations, exposure 2% did not yield as high a response se as to 1% O_2. Preliminary tests done by exposing cells to hypoxia for up to 72h resulted in equal or less activation of the promoter, as cells were overgrown and cell death impaired results (not pictured).

5.7.1. Overexpression

For some proof-of-concept experiments, overexpression was needed. To this end, constructs contained a Tet-repressor element and an inducible promoter (CMV/TO). The stimulation of cells with doxycycline (a tetracycline) leads to an unhindered transcription of said promoter, which in the unstimulated state is blocked by the Tet-protein. Cells containing such constructs were stimulated with 1µg/ml Doxycycline and activity was measured 24 and 48 hours later.

5.8. Luciferase Assays

For the main experiments, we used Gaussia luciferase as a reporter. Luciferases are enzymes which, upon reaction with their substrate, produce light, which can be measured photometrically. In this instance, the Gaussia protein has a signal sequence added to the 5' end of the construct allowing the protein to be secreted into the extracellular space. The substrate of the luciferase enzyme of the bacterial species Gaussia is coelenteramide[240,241]. The constructs were planned so as to have different inducible promoters coupled with Gaussia luciferase gene, which, upon activation of the promoter gets transcribed and leads to the production of a luciferase enzyme.

After stimulation of cells, 20µl aliquotsof supernatant were taken from each well at 24h and 48h. using a luciferase assay kit from BioLux (see materials), a mix of coelenteramide and buffer solution was prepared. 50µl of this solution were the added to the 20µl supernatant and, after 45 seconds incubation, photometric activity was measured using a Berthold Lumat LB 9507 Luminometer[240].[2]

5.9. Confocal fluorescence microscopy

Many of our construct used fluorescent proteins in the proof-of-concept experiments to assess stable integration. In particular, we utilized superfolding green fluorescent protein (sGFP), which can be seen in Figure 5, section 6.1.1.1. Fluorescent proteins have the characteristic of emitting light of a known wavelength upon exposure to a stimulant light, which also has a know wavelength. The emitted light can be detected by confocal microscopy[242]. For that, cell plates were examined under the Leica DM IL LED Inverted Microscope[243], and representative spots were chosen. Pictures were taken with bright field and then fluorescent lighting, overlays were generated with the ImageJ software[244].

5.10. Statistical analysis

To assess the statistical significance of results, unpaired, parametric student´s t-test were used. Significant results were considered as a p value under 0,05. Calculations were performed using the Prism 9 Software, Version 9.01 from the Company GraphPad.

6. Results

6.1. Establishment of a platform for efficient engineering of primary MSCs trough generation of a Gateway compatible reporter vector based on commercial Destination vectors

The experiments performed relied on stable integration of the various expression constructs using Sleeping Beauty-based approaches including the integration of ITR elements into the two vector backbones. These were shown to work by transfecting Hek293 and hBMSC with constitutively expressed sGFP, and through photometric assessment of expression of Gaussia luciferase reporter driven by an inducible synthetic HIF promoter over multiple passages. Both experiments showed no significant decrease in expression, allowing reliable stable transfection of the CCL5, 3AB and MegaRantes reporter constructs later on (sections 6.2. 6.3, 6.4).

To allow for efficient stable integration, ITR sequences were introduced into the pCDNA6/TR TET-Repressor expression vector backbone (based on the commercially available Invitrogen pCDNA6 dest vector, gateway compatible with the addition of Tet-Repressor controlled elements) by adding PciI and SgrDI cleavage sites to the ITR sequences by PCR, and subsequently performing a digestion with said enzymes followed by isolation and ligation. The new vector was named pCDNA6/TR-ITR (Figure 3, table 4.10). Afterwards, gene sequences cloned into that vector through the Gateway system could be efficiently stably integrated into host cell genomes using SB transposase technology.

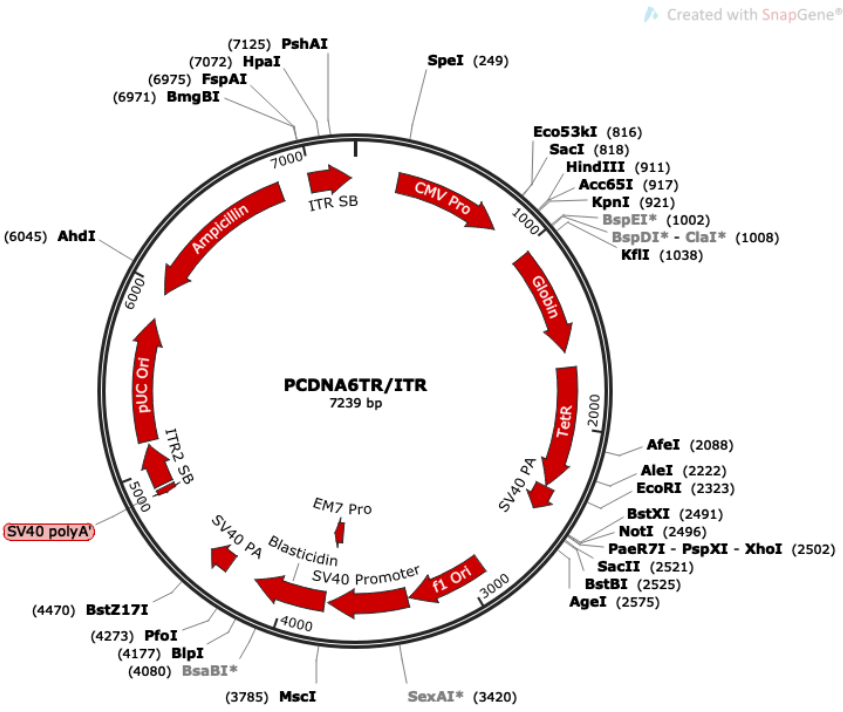

Figure 3: Destination vector used for the entry clones *It was made gateway compatible and contained a blasticidin resistance for selection, as well as ITR sequences for Sleeping Beauty transposase stable integration ITR: internal tandem repeats, as recognition sequence for the Sleeping Beauty transposase; Blasticidin: Blasticidin resistance gene*

As a further step, a *Gaussia* luciferase reporter gene was introduced to the destination vector where the promoter of interest (i.e. CCL5, 3AB, MegaRantes, as described below) was used to drive the reporter gene that was actively secreted into the growth media.

6.1.1.1. Stable integration: CAG sGFP IPDest

The CAG sGFP IPDest vector was engineered with the purpose of establishing the general efficacy of stable integration using the transposase system. The vector was generated using Gateway cloning as described in section Materials and Methods using a constitutively active chicken beta actin promoter driving a GFP (green fluorescent protein) sequence. In order to select the cells expressing the construct an IRES sequence was added followed by a puromycin resistance gene. The resulting vector was transfected into both human bone marrow stem cells and Hek293 cells using Neon nucleofection and selected with 0.6µg/ml Puromycin for 2 passages.

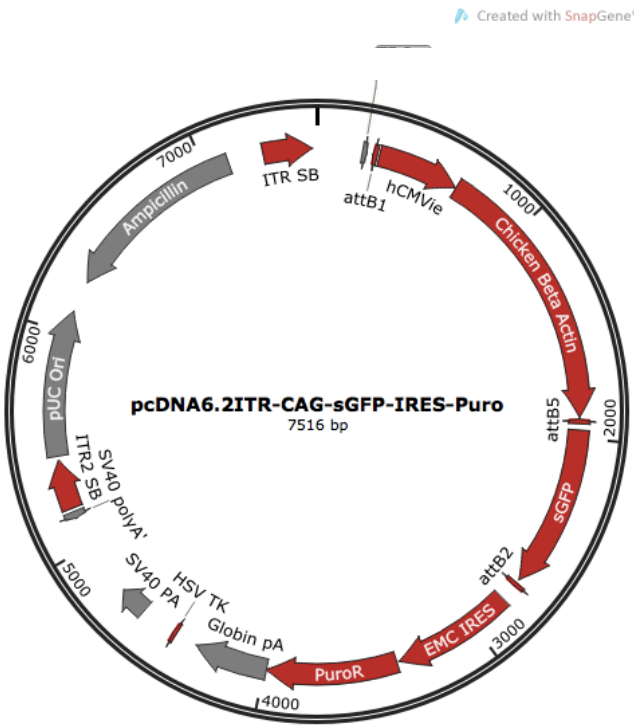

Figure 3 :Structure of the sGFP reporter construct used to establish stable integration. *The CAG sGFP IPDest vector was engineered with the purpose of establishing the general efficacy of stable integration using the transposase system, using a constitutively active chicken beta actin promoter driving a GFP (green fluorescent protein) sequence. In order to select the cells expressing the construct an IRES sequence was added followed by a puromycin resistance gene. ITR: internal tandem repeats, as recognition sequence for the Sleeping Beauty transposase; sGFP: green fluorescent protein; EMC IRES: Internal ribosomal entry site; PuroR: Puromycin resistance gene*

Fluorescence Microscopy was then performed at regular intervals to assess whether the cells would stably express the green fluorescent protein throughout continuous passaging. At the same time, through comparison of the pictures with and without fluorescence, we could assess the proportion of living cells expressing the gene.

As illustrated below, both in hBMSC and Hek293 cells a strong and consistent expression of sGFP was observed that did not fade through passages and was present homogenously.

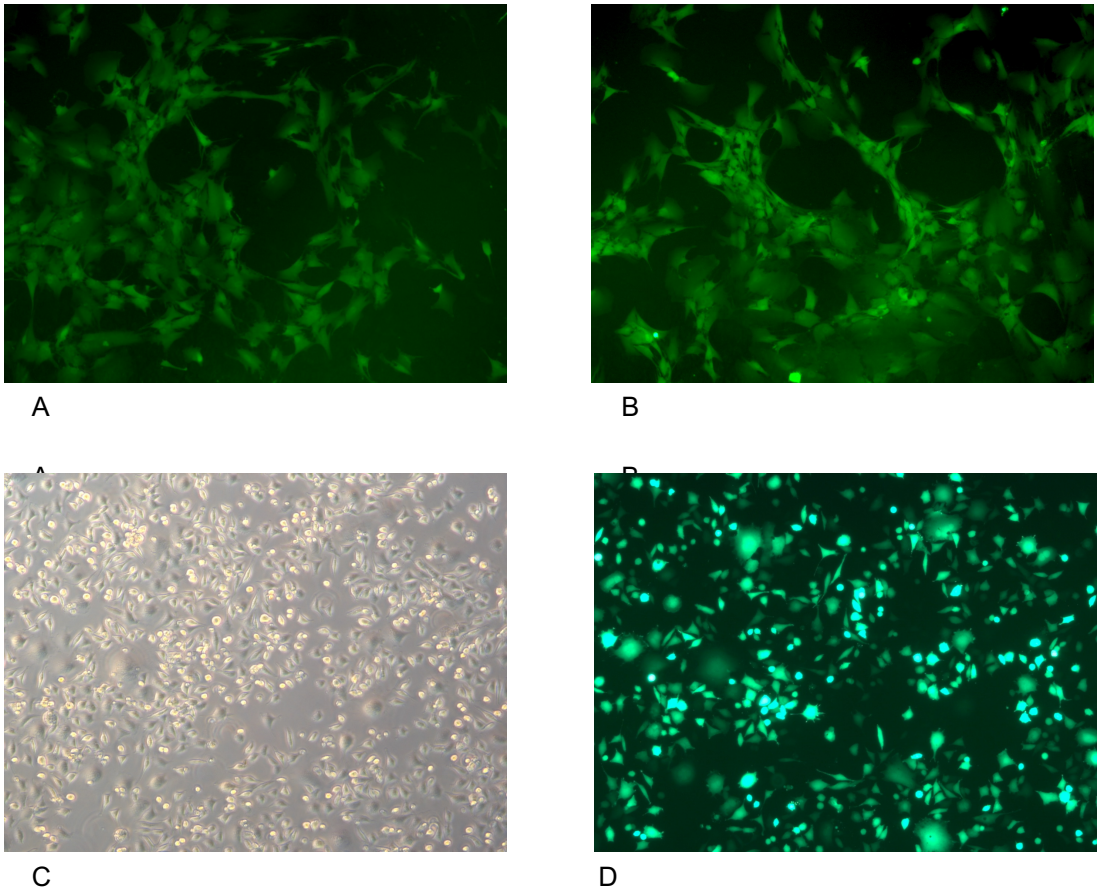

Figure 4: Fluorescence microscopy of cells containing a stably integrated sGFP construct over time. *The effectiveness of stable integration was assessed by transfecting the constitutively active sGFP vector Hek293 cells using Neon nucleofection and selected with 0.6µg/ml Puromycin for 2 passages. Fluorescence Microscopy was then performed at regular intervals to assess whether the cells would stably express the green fluorescent protein throughout multiple passages. A strong and consistent expression of sGFP was observed that did not fade through passages, and was present homogenously A-B: different dishes containing CAG sGFP Hek293 at time of transfection; C: ten passages later, cells are still alive; D: ten passages after transfection, Hek293 cells continue to consistently express sGFP*

6.2. Investigation of the effects of different stimuli on the CCL5 promoter in Hek293 cells and hBMSC

6.2.1. CCL5 reporter construct

To investigate the influence of different pro-tumorigenic stimuli on the CCL5 promoter, a construct containing a *Gaussia* luciferase reporter coupled to the native human CCL5 promoter was established. The plasmid for the backbone was pcDNA-Gluc3-Rantes and had been previously cloned in our lab by C Jaeckel and N. Ehni. The construct also contained SB ITR sequences allowing for stable integration into the cell genome when using the Sleeping Beauty transposase and a selection gene, a platform established by our group.

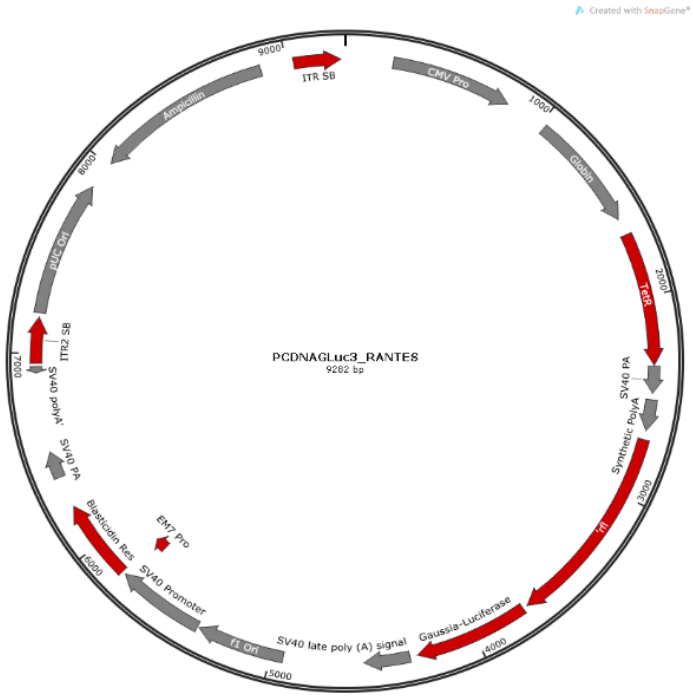

Figure 5: Structure of the CCL5 Gaussia luciferase reporter construct. *To investigate the influence of different pro-tumorigenic stimuli on the CCL5 promoter, a construct containing a Gaussia luciferase reporter coupled to the native human CCL5 promoter was established (pcDNA-Gluc3-Rantes). The construct also contained SB ITR sequences allowing for stable integration into the cell genome when using the Sleeping Beauty transposase and a selection gene.*

Red items in the chart: ITR SB: internal tandem repeats for Sleeping Beauty transposase; ´rfl: CCL5/Rantes promoter, followed by Gaussia luciferase gene; EM7 pro: EM7 procaryotic promoter, which is constitutively active coupled to Blasticidin Res (resistance), so that the resistance can be expressed in E.coli bacteria; SV40: In the hBMSC, the promoter used to constitutively express Blasticidin resistance, so that expression can be independent of CCL5 activation.

6.2.2. Experiments on Hek293 cells as proof of concept

To establish the functionality of our constructs they were first tested on human embryonic kidney (Hek293) cells (see Materials). These cells are easily kept in culture and transfection protocols are well established for them. Furthermore, they can be kept for many passages without exhibiting senescence signs, therefore being ideal for the establishment of the construct and experiment conditions.

6.2.3. Effect of TNFα and IFN-γ on CCL5 promoter in Hek293 cells

To assess the responsiveness and functionality of the CCL5 reporter construct, we transfected Hek293 cells with the Rantes *Gaussia* Blasticidin construct and upon plating in a 96 well plate, stimulated the cells with 15ng/ml TNFα, 45ng/ml IFN-γ individually and in combination. For each stimulation three wells of a 96 well plate were used with 20,000 cells per well, conditioned media was removed after 24 and 48 hours and tested for luciferase activity.
The CCL5 reporter construct was found to be functional and responded to the inflammatory stimuli. TNFα and IFN-γ stimulated promoter activity was seen to increase over time. Numerically, stimulation with these substances increased activity by 12-fold, when comparing the stimulated samples at 48h with the unstimulated ones at 24h (p value <0.0001). The 48h time point was used for subsequent experiments.

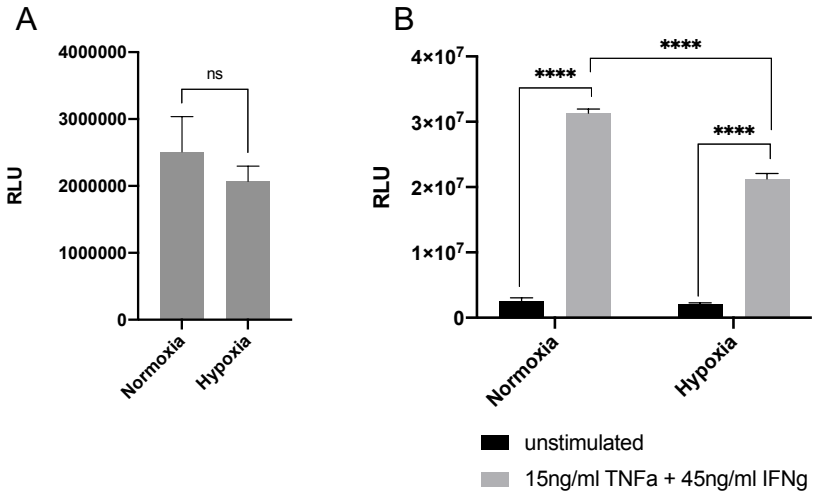

Figure 6: Rantes Gaussia Blasticidin reporter Hek293 cells in normoxia and in hypoxia, then effect of addition of inflammatory stimuli and hypoxia. *(A): Rantes Gaussia Hek293 reporter cells stimulated solely with hypoxia: (48h) For the evaluation of potential hypoxic effects on CCL5 transcription in Hek293 cells, we compared the induced transcription of luciferase of cells containing the construct in normoxia and hypoxia (1% O2). Cells were plated in a 96 well plate, in triplets at 20,000 cells per well, and kept in a 1% O2 incubator for 48h. The control group was kept under normoxia at 37°C. Both groups showed a base luciferase transcription on the order of 1 x 10^6 RLU after 24h. Exposure of the cells to hypoxia in the absence of inflammatory stimulation did not result in an increase in measured luciferase activity, in fact, it decreased it (17% decrease, albeit statistically non-significantly, p value 0,253)*
(B) : Rantes Gaussia Blasticidin Hek293: cells exposed to TNFα and IFN-γ in normoxia, as compared to hypoxia. Incubation conditions were identical to those previously described. The exposure to hypoxia led to a decreased absolute measurement of RLU (measurements after 48h) – 32% less activity in the TNFα, IFN-γ and hypoxia group, as compared to the TNFα, IFN-γ and normoxia group (p < 0.0001)

6.2.3.1. Effects of hypoxia on the CCL5 promoter in Hek293 cells

Hypoxia was a factor investigated for its potential influence on the CCL5 promoter. To establish appropriate conditions to assess the effects of hypoxic stress, a hypoxic incubator was used in concert with a HIF-based synthetic promoter driving the *Gaussia* reporter. We compared the induced transcription of luciferase of cells containing the construct in normoxia and hypoxia, as well as simulation of a hypoxic state using cobalt chloride. The construct responded to both $CoCl_2$ and incubation in 1% O_2 in a dose or time dependent manner (not shown).

For evaluation of potential hypoxic effects on CCL5 transcription in Hek293 cells. In the first experiment, cells were plated in a 96 well plate, in triplets at 20,000 cells per well, and kept in a 1% O_2 incubator for 48h. The control group was kept under normoxia at 37°C.

Both groups showed a base luciferase transcription after 24h. Exposure of the cells to hypoxia in the absence of inflammatory stimulation did not result in an increase in measured luciferase activity (data not shown).

As a next step, we tested whether hypoxia could have an additive effect when included with inflammatory stimuli. Hek293 containing the Rantes *Gaussia* construct were exposed to hypoxia and normoxia and additionally stimulated with the inflammatory stimuli. Incubation were identical to those previously described.

In wells containing the TNFα and IFN-γ, under hypoxic conditions the Gaussia luciferase activity

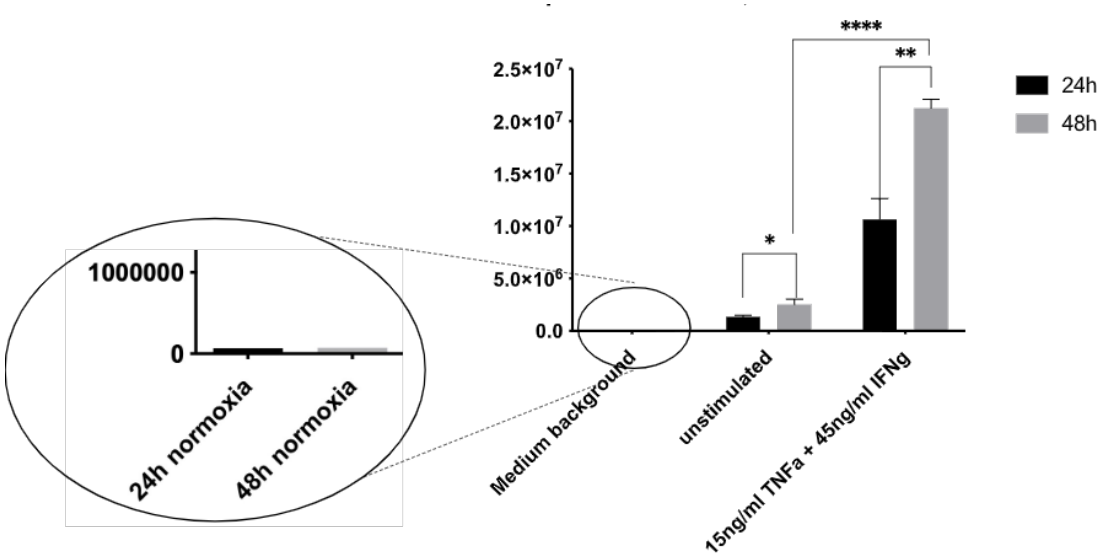

Figure 7: CCL5 Gaussia reporter Hek293 cells exposed to the pro-inflammatory stimuli TNFα and IFN-γ. *To assess the responsiveness and functionality of the CCL5 reporter construct, we transfected Hek293 cells with the Rantes Gaussia Blasticidin construct and, upon plating in a 96 well plate, stimulated the cells with 15ng/ml TNFα, 45ng/ml IFN-γ or both, as TNFα and IFN-γ . For each stimulation three wells of a 96 well plate were used with 20,000 cells per well, conditioned media was removed after 24 and 48 hours and tested for luciferase activity.*
The CCL5 reporter construct was found to be functional and responded to the inflammatory stimuli. TNFα and IFN-γ stimulated promoter activity was seen to increase over time. Numerically, stimulation with these substances increased activity by 12-fold, when comparing the stimulated samples at 48h with the unstimulated ones at 24h (p value <0.0001). The 48h was used for subsequent experiments. Statistics: unpaired stundents t-test

increased less than it did under normoxia – i.e., hypoxia may have had an inhibitory effect on *Gaussia* luciferase expression. In terms of fold change: in the normoxia group, stimulation with TNFα and IFN-γ led to a 12-fold increase in luciferase activity after 48 hours (unstimulated: 2.5 x 10^6 RLU, after addition of positive control 3 x 10^7 RLU). While in the group of cells exposed to hypoxia there was also an increase through stimulation with TNFα and IFN-γ (average unstimulated: 2 x 10^6 RLU, positive control: 2 x 10^7 RLU, $p < 0.0001$). In direct comparison, that translates to 32% less activity in the hypoxia samples as compared to the TNFα and IFN-γ in normoxia ($p < 0.0001$). The wells were observed under the microscope after the experiments to verify that the cells remained viable in response to the treatments.

6.2.3.2. Effects of TGF-β stimulation of the CCL5 promoter in Hek293 cells

Hek193 cells transfected with Rantes *Gaussia* were stimulated with increasing concentrations of TGF-β: 10ng/ml, 100ng/ml and 400ng/ml. Stimulation did not show induced *Gaussia* activity independently of concentration.

To evaluate the role of combined factors linked to the environment of the TME, the experiment was repeated under hypoxic conditions (Fig. 8). The differences observed between the different TGF-β groups in normoxia and hypoxia were not statistically significant, nor was there any significant increase when comparing these groups to the unstimulated cells in normoxia. Therefore, the addition of TGF-β to hypoxia did not show any additive effect. Independently of the addition of TGFβ, once again we saw a marked decrease in activity of the TNFα and IFN-γ in hypoxia (32% decrease, p <0.0001), underscoring the results obtained before.

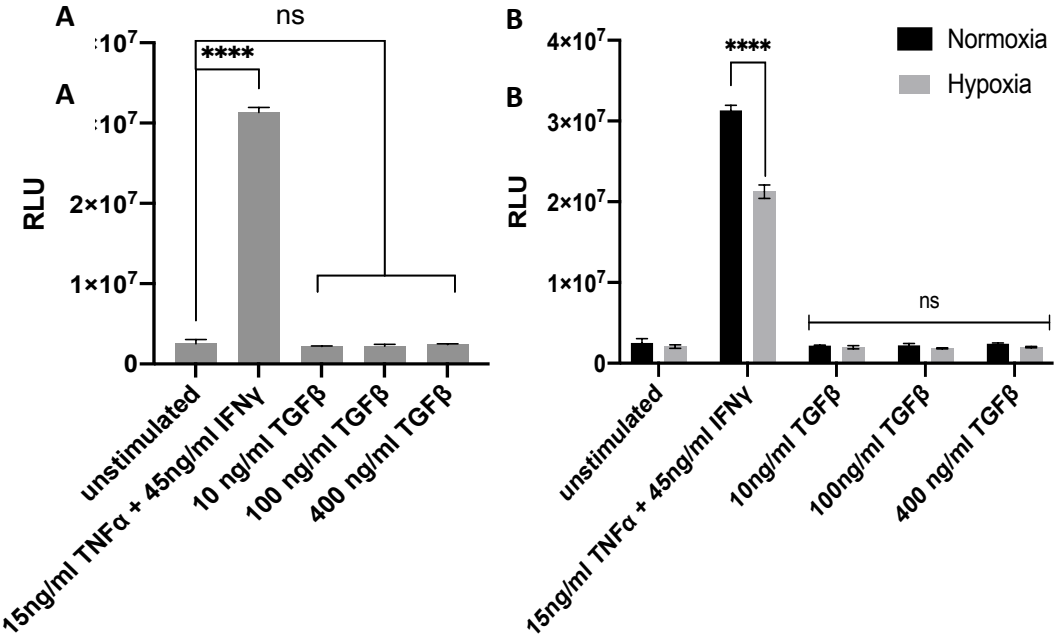

Figure 8: Rantes Gaussia Hek293 reporter cells' response to TGF-β, in normoxia and hypoxia. *(A) Hek293 cells transfected with Rantes Gaussia reporter construct were stimulated with increasing concentrations of TGF-β: 10ng/ml, 100ng/ml and 400ng/ml. Stimulation did not show induced Gaussia activity independently of concentration. (B) To evaluate whether a synergy between TGF-β and hypoxia might exist, the experiment was done in parallel under hypoxic conditons. The concomitant stimulation with TGF-β and hypoxia did not show any statistically significant effect, independently of TGF-β concentration use. There was also no significant increase when comparing these groups to the unstimulated cells in normoxia. Once again we saw a marked decrease in activity of the TNFα and IFN-γ in hypoxia (32% decrease, p <0.0001), underscoring the results obtained earlier. Statistics: unpaired students t-test*

6.2.3.3. Assessing Hek293 cell responsiveness to TGF-β: proof of concept with a SMAD Reporter

To validate the ability of Hek293 cells to respond to TGF-β stimulation, the cells were transfected with a SMAD *Gaussia* Luciferase reporter construct. The plasmid containing the construct also contained SB ITR sequences allowing it to be stably integrated into the cellular genome. To assess Hek293 responsiveness to TGF-β, the cells were transfected with the SMAD reporter construct using AMAXA nucelofection and selected with blasticidin for 2 passages. They were plated in triplets into a 96-well plate at 20,000 cells per well and stimulated with different concentrations of TGF-β: 10ng/ml, 100ng/ml and 400ng/ml in the 50µl medium present in each well. Figure 9 shows the results. Hek293 are capable of responding to TGF-β stimulation, although no significant difference in luciferase activity was observed between different dosages of stimulation.

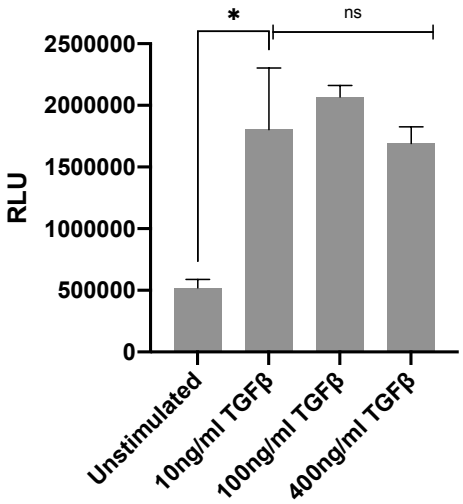

Figure 9: Hek293 cells containing a SMAD Gaussia reporter construct. *Cells were plated in triplets into a 96-well plate at 20,000 cells per well and stimulated with different concentrations of TGF-β: 10ng/ml, 100ng/ml and 400ng/ml. Hek293 are capable of responding to TGF-β stimulation (maximum of X-Fold increase after 48h in the 1000nh/ml group, compared to unstimulated cells, p<0,005), although no significant difference in luciferase activity was observed between different dosages of TGF-β. Therefore, Hek293 cells possess the cellular apparatus to react to TGF-ß. Statistics: unpaired students t-test*

6.2.3.4. Conclusions drawn from the experiments on Hek293

The first objective of the production of Rantes *Gaussia* Blasticidin Hek293 cells was to assess the integrity and functionality of the CCL5 reporter construct. In that aspect, it is possible to characterize the construct. We also showed that the construct responds to inflammatory cytokines TNFα and IFNγ with a 3- to 4-fold increase in activity in normoxia. While the SMAD reporter assays showed that Hek293 cells could respond to TGF-β stimulation, but there was no clear effect on the CCL5 reporter construct. Thus, in Hek293, CCL5 transcription does not appear to be influenced by TGF-β.

In all experiments involving hypoxia, Hek293 Rantes-*Gaussia* showed diminished luciferase activity when compared to the same cells in normoxia. Therefore, these important TME factors do not add to the activation of the CCL5 promoter in Hek293 cells[115].

6.2.3.5. Response of CCL5 Gaussia transfected hBMSC to inflammatory stimuli

Having validated our reporter constructs in Hek293 cells, we then sought to establish response of CCL5 in hBMSC under different relevant conditions. Importantly, it is well established that the CCL5 promoter responds differently in the various cells that express the gene.

Donor human bone marrow cells from the company Apceth (cell line Ap172_1) in passage 1 were transfected with the Rantes Gaussia Blasticidin construct and selected for two passages. They were

plated into 4 wells for each condition, at 16,000 cells per well. The same concentrations of TNFα and IFN-γ were used as in the Hek293 experiments.

Stimulation with TNFα resulted in a 2-fold increase within 48 hours (p 0.006). Stimulation with IFN-γ alone did not yield a significant increase in RLU (1.32- fold, p 0.18), while the addition of both cytokines led to a 2.8-fold increase in *Gaussia* luciferase activity (p 0.0026).

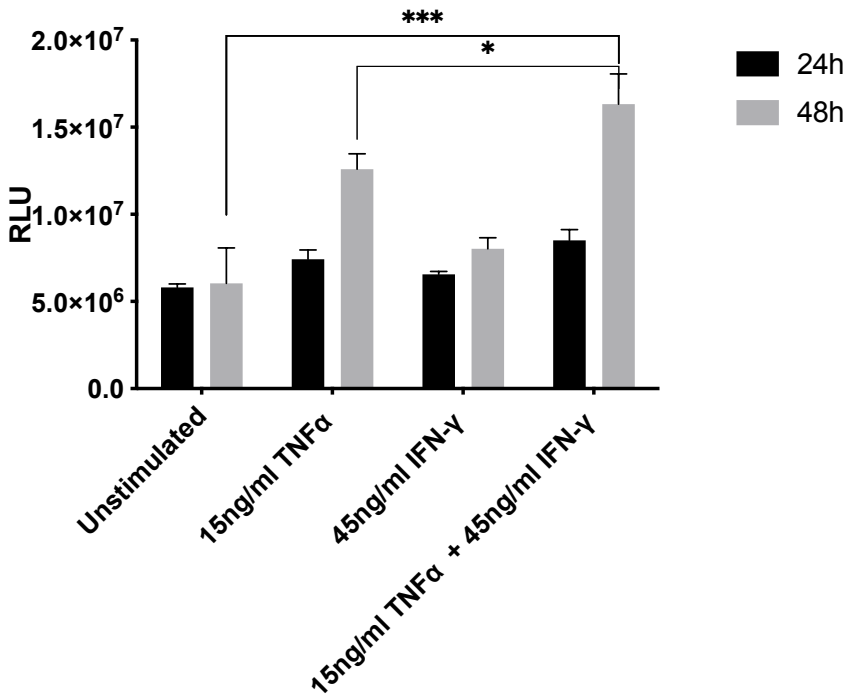

Figure 10: hBMSC containing the CCL5 Gaussia reporter construct: stimulation with of TNFα and IFN-γ in normoxia*. To establish response of CCL5 in hBMSC under different tumor conditions, we stimulated cells containing the CCL5 reporter construct with the pro-inflammatory cytokine TNFα. IFN- γ, and both together. These have been described in the literature to have synergistic action on the CCL5 promoter in other cell types, e.g. endothelial cells. After antibiotic selection, hBMSC were plated into 4 wells for each condition, at 16,000 cells per well. The same concentrations of TNFα and IFN-γ were used as in the Hek293 experiments. Stimulation with TNFα resulted in a 2-fold increase within 48 hours (p 0.006). Stimulation with IFN-γ alone did not yield a significant increase in RLU (1.32- fold, p 0.18), while the addition of both cytokines led to a 2.8-fold increase in Gaussia luciferase activity (p 0.0026). The TNFα + IFN-γ wells had a 2.8-fold higher Gaussia activity, on average: p value 0.0026)*

6.2.3.6. Effects of hypoxia on the CCL5 promoter in hBMSC, alone and in addition to inflammatory stimuli

As was seen with the CCL5-Gaussia Hek293 cells, there was no significant increase in CCL5 reporter activity resulting from hypoxia exposure alone. When examining the effects of hypoxia and inflammatory stimuli we observed no changes in promoter activity relative to the response seen with TNFα and IFN-γ. Unlike in Hek293 cells, exposure to hypoxia did not result in a diminished *Gaussia* luciferase activity.

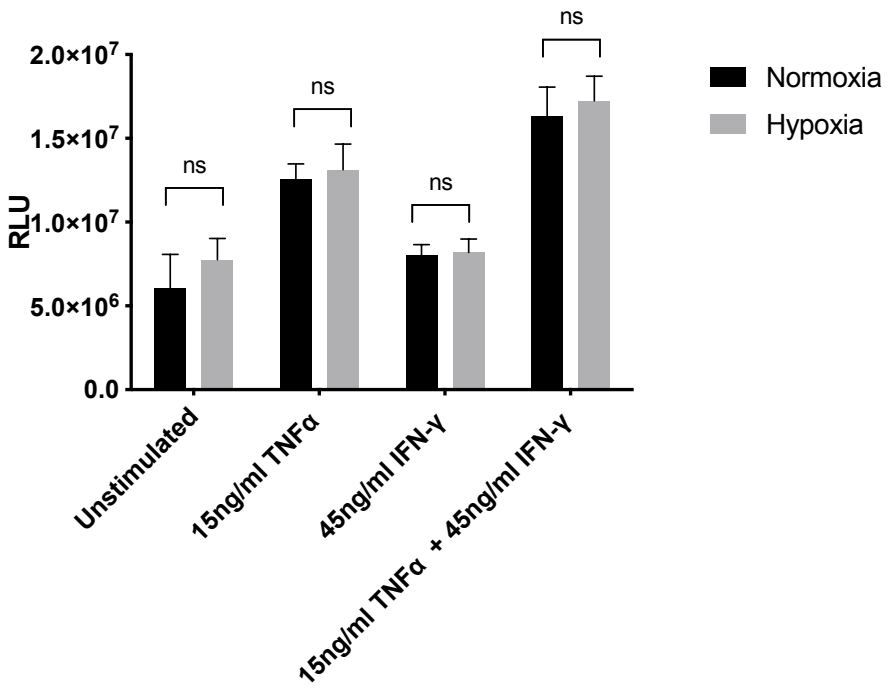

Figure 11: hBMSC containing the CCL5 Gaussia reporter construct: stimulation with of TNFα and IFN-γ in hypoxia*. hBMSC cells containing the CCL5 reporter construct with the pro-inflammatory cytokine TNFα. IFN- γ, and both together, now under hypoxic condition (1% O_2) for 48h (4 wells for each condition, at 16.000 cells per well)(results for the 24 hour measurements not shown). As was seen with the CCL5-Gaussia Hek293 cells, there was no significant increase in CCL5 reporter activity resulting from hypoxia exposure alone (stats). When examining the effects of hypoxia and inflammatory stimuli we observed no changes in promoter activity relative to the response seen with TNFα and IFN-γ. Unlike in Hek293 cells, exposure to hypoxia did not result in a diminished Gaussia luciferase activity. Statistics: unpaired student's t-test.*

6.2.3.7. Evaluation of TGF-β stimulation on the CCL5 promoter in hBMSC in normoxia and hypoxia

To evaluate the CCL5-reporter engineered MSC response to TGF-β alone, cells in p2 were transfected with the CCL5 promoter-reporter using NEON nucleofection and selected. Cells were plated at 18,000 cells per well, and treated with 100ng/ml of TGF-β, and tested in 21% and 1% O_2. Change in RLU was not statistically significant.

Hypoxia had no effect on CCL5 promoter activity in cells stimulated with TNFα and IFN-γ, nor did it cause any increase in luciferase activity on the cells additionally stimulated with TGF-β (p value for the comparison between hypoxia and normoxia for positive control only: 0.85; p value for the same comparison in the TNFα + IFN-γ + TGF-β group: 0.79). If we compare unstimulated cells in normoxia to the TNFα and IFN-γ in hypoxia, the activity increase was 3.8-fold (p 0.002). The increase in activity in the cells stimulated with TNFα and IFN-γ, hypoxia and TGF-β, when compared to unstimulated cells in normoxia was 4.0-fold (p 0.002). Therefore, the addition of TGF-β did not influence activity in any way in the observed time period.

6.2.3.8. Proof-of-concept: evaluation of TGF-β responsiveness in MSC using the SMAD reporter construct

To demonstrate that hBMSC have the cellular apparatus needed to respond to TGF-β, we utilized a SMAD *Gaussia* reporter to assay luciferase activity upon addition of TGF-β1 to medium, both in Hek293 and hBMSC. The statistically relevant different in RLU measured permits us to draw conclusions on the effects of TGF-β on the investigated promoters themselves.

AP172_1 hBMSC cells were transfected in passage 4, selected with blasticidin for 2 passages and then plated at 11,000 cells per well in triplets. After plating, the medium contained no more antibiotic. Stimulation was done concomitantly with plating. TGF-β response in hBMSC was low with an increase of 1.24 and 1.2-fold for the two respective TGF-β concentrations, but it was statistically significant with p values of 0.02 and 0.017, respectively. (Figure 12).

This experiment was also performed under hypoxia, to verify whether a synergistic response might take place, but no statistically significant change could be demonstrated (p value for the comparison of hypoxia vs. normoxia in the 10ng/ml group: 0.47; in the 100ng/ml group, that value was 0.24).

6.2.4. Conclusions from results in hBMSC CCL5 reporter cells

The experiments conducted in hBMSC added significantly to the objective of characterizing CCL5 activation in the context of tumor environment-relevant signals. We were able to establish that the construct is inducible with TNFα and IFN-γ. Increased activity increased upon stimulation with TNFα and a lower increase was seen when stimulated with IFN-γ alone. An additive increase was seen upon stimulation with both agents. *Gaussia* luciferase increased over time and peaked around 48h. The CCL5 promoter did not show a significant response to hypoxia in hBMSC, on its own, or in addition to other stimuli. The hBMSC CCL5-Gaussia cells did not show any statistically significant increase in luciferase activity in response to TGF-β. That was true both to TGF-β stimulation alone, as it was in conjunction with TNFα and IFN-γ, in normoxia and in hypoxia.

Through parallel experiments using SMAD and HIF-based synthetic reporters, we ascertained that hBMSC are able to respond to TGF-β and hypoxia.

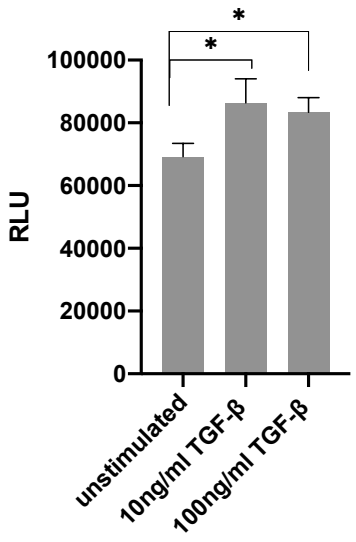

Figure 12: SMAD Gaussia Blasticidin reporter hBMSC exposed to different concentrations of TGF-β, in normoxia (48h)**:** *To assert that hBMSC had the cellular apparatus to respond to TGF-β, we utilized a SMAD Gaussia reporter construct. HBMSC cells were transfected in passage 4, selected with blasticidin for 2 passages and then plated à 11.000 cells per well, in triplets. TGF-β response in hBMSC showed an increase of 1.24 and 1.2-fold for the two respective TGF-β concentrations, as compared to unstimulated cells but it was statistically significant, with (p values of 0.02 and 0.017, respectively, students t-test). Therefore, we conclude that hBMSC themselves are capable of responding to TGF-β, and the lack of response seen with the CCL5 reporter construct was connected to the CCL5 promoter itself, not the cellular apparatus. This experiment was also performed under hypoxia, to verify whether a synergistic response might take place, but no statistically significant change could be demonstrated (p value for the comparison of hypoxia vs. normoxia in the 10ng/ml group: 0.47; in the 100ng/ml group, that value was 0.24. Statistics used: students t-test)*

6.3. Investigation of the effects of different stimuli on a synthetic promoter based on previously identified CCL5 promoter elements (3AB) in hBMSC

The AB region of the CCL5 promoter contains tandem binding sites for heterodimeric and homodimeric NFκB that were previously shown to help drive the responsiveness to TNFα and IFN-γ stimulus in other tissue settings. A synthetic promoter was then designed containing three tandem sequences of the (R)AB region (3AB) linked to a minimal CMV promoter driving the Gaussia reporter gene. With this approach, we hoped to better evaluate the specific response of the promoter, as well as to determine if the strength of the response could be enhanced. If this were the case, the synthetic promoter might be useful for the delivery of therapeutic transgenes within the tumor micromilieu.

This design was conceived in consideration of the fact that the presence of different promoter modules in the natural CCL5 promoter may lead to contradictory reactions in different cells and contexts, which, we hypothesized, may have led to unexpected results in the previous experiments.

The 3AB construct (pcDNAGluc3CMVmin3AB) was cloned using restriction digestion and ligation. Oligonucleotide sequences of the multimer were ordered from Invitrogen, annealed using PCR, cut with enzymes BsrGI and NotI and ligated into a backbone containing Gaussia Luciferase (pcDNAGluc36xAP1), which had previously been cut using PstI and Xho for matching overhangs. The backbone contained ITR SB100 sequences for stable integration and a gene for Blasticidin resistance for selection. The construct also contained SB ITR for stable integration.

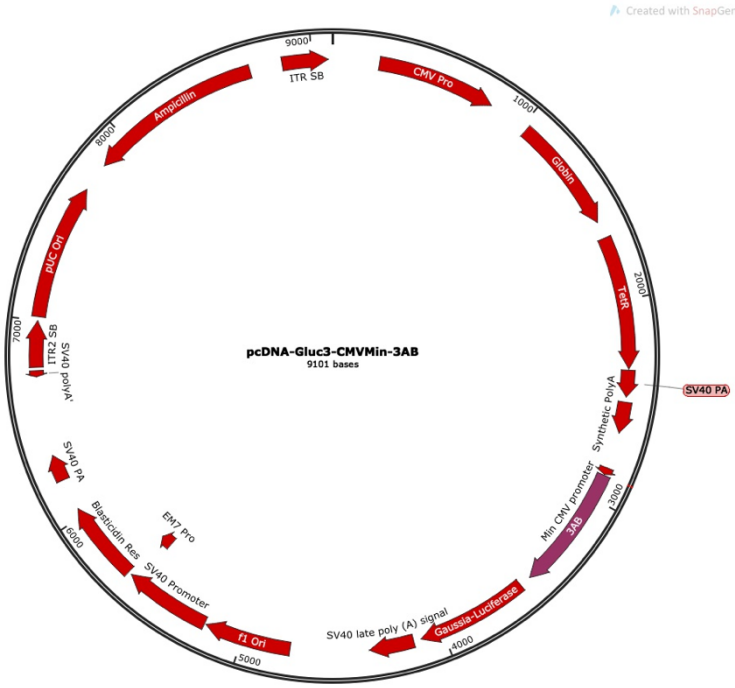

Figure 13: Structure of the reporter construct for the synthetic promoter 3AB, using Gaussia as a reporter gene. *The construct was termed pcDNAGluc3CMVmin3AB. The R(A)(B) regions of the CCL5 are involved in the activation of the promoter through the NFκB pathway, which drive the responsiveness to TNFα and IFN-γ stimulus in other tissue settings. Thus, we designed containing three tandem sequences the (R)AB region (3AB) linked to a minimal CMV promoter driving the Gaussia reporter gene, hoping to enhance response to pro-inflammatory stimuli. The vector contained ITR SB100 sequences for stable integration and a gene for Blasticidin resistance for selection.*
ITR SB: internal tandem repeats for Sleeping Beauty transposase; 3AB promoter, followed by Gaussia luciferase gene; EM7 pro: EM7 procaryotic promoter, which is constitutively active coupled to Blasticidin Res (resistance), so that the resistance can be expressed in E.coli bacteria; SV40: In the hBMSC, the promoter used to constitutively express Blasticidin resistance, so that expression can be independent of CCL5 activation.

6.3.1. Effect TNFα and IFN-γ on 3AB Gaussia Blasticidin hBMSC

Having determined that the CCL5 promoter can be activated by a combination of TNFα and IFN-γ in human MSC, these factors were then used to assess whether 3AB is a functional promoter in Apceth 172_1 hBMSC. Cells were transfected in P1, selected, and were in passage number 4 when experiments took place. Cells were then plated into a 96-well plate as described in methods, 20,000 cells per well. As was seen with the native CCL5 promoter, there was a response to TNFα (5.7- fold, p value 0.0032), a mild one to IFN-γ (1.2 fold, p 0.018), and again a more than additive effect in combination (12-fold increase in 48h, p value <0.0001).

If we compare these results to the CCL5 reporter, the pattern is the same, but there is a difference in the base activity for 3AB, when not stimulated was much lower, around 1.8 x 10^5, compared to 3.2 x 10^6 in the CCL5 promoter. Furthermore, the CCL5 promoter reached maximum TNFα and IFN-γ stimulation reached at 2.7-fold that of the unstimulated cells, but over 12-fold stimulation was seen with the synthetic 3AB promoter.

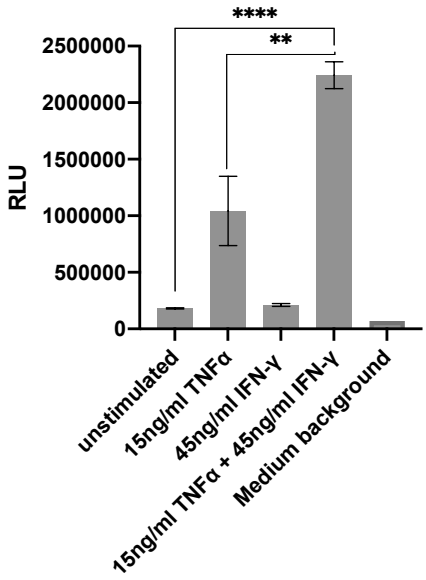

Figure 14: 3AB Gaussia reporter hBMSC stimulated with TNFα and IFN-γ, in normoxia, after 48h. *Cells were transfected in P1, selected for 3 passages, then plated into triplets of 20,000 cells per well. Gaussia luciferase activity measure after stimulation with TNFα alone was 5.7- fold, compared to unstimulated cells (p value 0.0032); stimulation with IFN-γ alone yielded a 1.2 fold in the same comparison (p 0.018): the combination of both substances showed a synergistic activation of the promoter, with Gaussia luciferase activity 12-fold, compared to unstimulated cells, (p value <0.0001), an increase which is more than the sum of the individual increases under the stimulants alone. Base activity for 3AB, when not stimulated was much lower, around 1.8 x 105, compared to 3.2 x 106 in the CCL5 promoter, and the fold increase with double stimulation with TNFα and IFN-γ was higher for 3AB. While the 3AB experiments used the same cell line as the CCL5 ones, the experiments were not done in parallel, but sequentially, so this can only be an indirect comparison.*

6.3.2. Effects of hypoxia on the 3AB promoter

Hypoxia is thought to influence activation of NFκB. For this reason it was unexpected that no effect was seen when the complete CCL5 promoter was tested. The potential effects of hypoxia on the 3AB synthetic promoter were then assessed as detailed above.

Interestingly, in 3AB, hypoxia clearly amplified the response both to TNFα and IFN-γ alone, and in combination of TNFα and IFN-γ. In the unstimulated samples, there was a 1.8-fold increase in response, with a p value of 0.02. A similar increment was observed in the samples stimulated with IFN-γ alone: the cells kept in hypoxia produced 1.5-fold more Gaussia luciferase, with a p value of 0.02. In keeping with the pattern observed before, the difference was most pronounced in the groups stimulated with

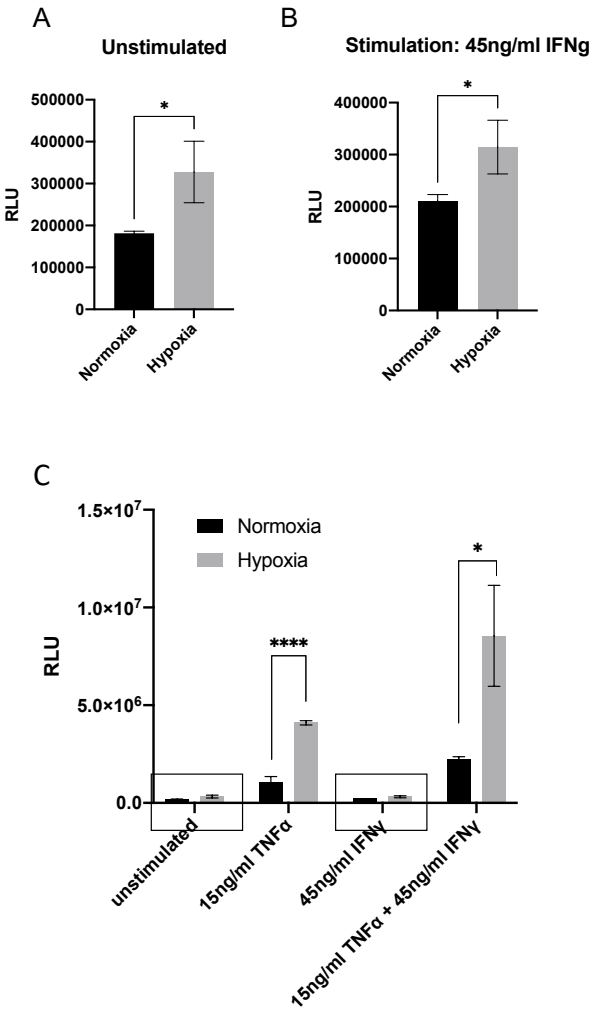

Figure 15: 3AB Gaussia reporter hBMSC stimulated with TNFα and IFN-γ, in hypoxia (1% O_2), after 48h. *Hypoxia is thought to influence activation of NFκB. The potential effects of hypoxia on the 3AB synthetic promoter were assessed in parallel to the experiments in normoxia: cells were transfected in P1, selected for 3 passages, then plated into a 96-well plate as described in methods, 20,000 cells per well. (A): Differently than CCL5, 3AB showed a response to hypoxia on its own: 1.8-fold increase in luciferase activity, measured in RLU (p value of 0.02, students t-test). Additionally, we observed that, in In 3AB, hypoxia clearly amplified the response both to TNFα and IFN-γ individually: TNFα and hypoxia combined resulted in an increase of 3.9-fold, compared to TNFα in normoxia. For IFN-γ (15 B): 1.5-fold more Gaussia luciferase in hypoxia, than in normoxia (p value of 0.02), something not seen with the CCL5 promoter. (C): The combination of TNFα and IFN-γ yielded the maximum response in normoxia, and this was significantly bolstered by exposure to hypoxia, generating a 3,8-fold increase trough oxygen deprivation. In terms of fold response, if compared to unstimulated cells, the group exposed to TNFα, IFN-γ and hypoxia had a 47-fold increase (p value 0.005, student's t-test).*

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TNFα: for the TNFα alone group, exposure to hypoxia increased luciferase expression by 3.9-fold (p value <0.001), while that increase was 3.81 times higher than normoxia in the cells stimulated with TNFα and INFg (p 0.013, owing to a greater standard deviation within these samples). Compared to the basal level of the unstimulated cells in normoxia, that translates into a remarkable 47-fold increase (p value 0.005). In absolute numbers, the activities measured were compatible with those in the previous experiment for the normoxia group, that is, in the order of 10^6. With the addition of hypoxia, the TNFα and IFN samples produced luciferase activity levels comparable with those of the CCL5 reporter cells, reaching almost 10^7 RLU (8.5×10^6 for TNFα and IFN-γ).

6.3.3. Effects of TGF-β stimulation on the 3AB promoter

Seeing as the 3AB promoter had shown a very promising response regarding hypoxia, the next question was whether it would react to TGF-β. These tests were done in parallel with the previous experiment, so the same cells were used, in passage 3 (transfected in passage 1. selected for two passages thereafter), plated in triplets containing 20.000 cells per well. No statistically significant response was observed in response TGF-β, neither at 10ng/ml or 100ng/ml.

6.3.4. Cumulative effect of TGF-β and hypoxia

To further compare the 3AB synthetic promoter to the CCL5 promoter, we examined the impact of the addition of TGF-β might have in a hypoxic setting. This was of special interest, considering the surprising response observed before, where the exposure to 1% O_2 led to a synergistic effect with the TNFα and IFN-γ.

Similar to the experiment performed in normoxia, once again we did not observe a dose-dependency of the luciferase activity measured. If one juxtaposes the data with the results obtained in normoxia, the groups stimulated with TGF-β and hypoxia show a clear increase. For the 10ng/ml TGF-β group, there was a 2.4-fold increase through the addition of hypoxia (p value 0.01), and in the 100ng/ml TGF-β samples, that increase was in the magnitude of 2.2-fold (p value <0.0001). If one compares the effect of the addition of 10ng/ml TGF-β and exposure to hypoxia to the cells that were unstimulated and kept in normoxia, that change reaches a maximum of 2.7-fold (p value 0.009). It is not possible to conclude that this difference was due to TGF-β. We had already observed a dramatic effect of hypoxia alone on the 3AB promoter in the experiments above – in fact, with a much higher fold increase. The results do not allow us to state whether the increase was due to TGF-β of hypoxia.

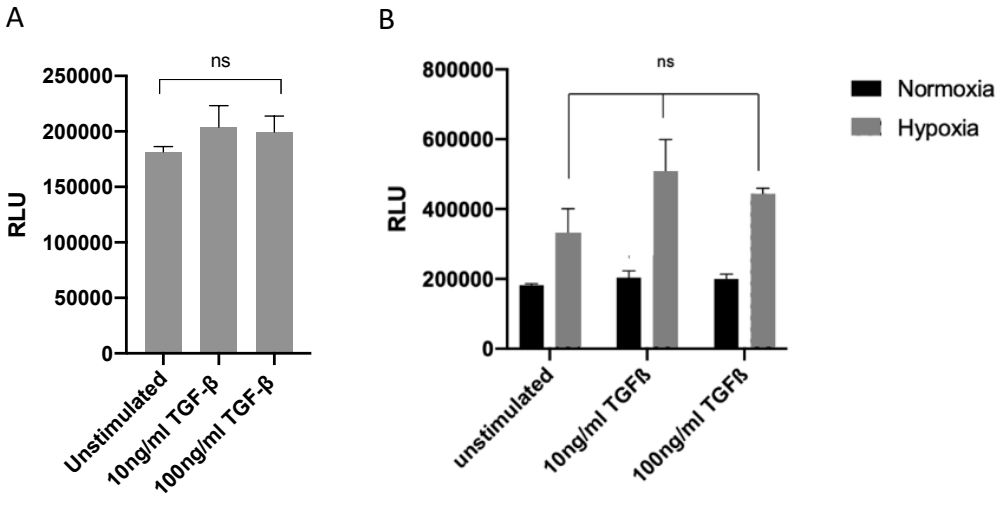

Figure 16: 3AB Gaussia reporter hBMSC stimulated with TGF-β, in normoxia (A) and hypoxia (B). These *tests were done in parallel with the previous experiment, so the same cells were used, transfected in passage 1, selected for two passages thereafter), plated in triplets containing 20.000 cells per well.*
(A): Test done in normoxia showed stimulation of the cells with two different concentrations of TGF-β (10ng/ml nor 100ng/ml) resulted in no statistically significant response (numbers). (B) Furthermore, we examined the impact the addition of TGF-β might have in a hypoxic setting. For the 10ng/ml TGF-β group, there was a 2.4-fold increase through the addition of hypoxia, as compared to the unstimulated cells (p value 0.01), and in the 100ng/ml TGF-β samples, that increase was in the magnitude of 2.2-fold (p value <0.0001). However, when compared to cells stimulated with hypoxia only (columns furthest left in figure B), we conclude that the effects are due to hypoxia exposure, not TGF-β, as hypoxia alone already lead to a Gaussia luciferase activity increase of 2.7-fold (p value 0.009)(statistics: student's t-test).

6.3.5. Addition of TGF-β to TNFα and IFN-γ in normoxia and hypoxia

Cells were stimulated with 10ng/ml TGF-β only, to avoid potentially toxic effects from TGF-β. Once more, we observed a drastic increment in RLU in the positive control group (9.39-fold, p value <0.0001), in the order of magnitude we had come to expect from previous experiments. The group stimulated with TNFα, IFN-γ and TGF-β, on the other hand exhibited a smaller increase in activity compared to the negative controls that being of 6.4-fold ($p < 0.0001$). Thus, the addition of TGF-β to the TNFα and IFN-γ led to a fall of 32% in average RLU (p value 0.0003).

A second plate containing triplets of the same cells and same stimulations was exposed to hypoxia. A similar motive can be recognized here, although it is even more extreme: comparably to the previous tests, the TNFα and IFN-γ led to a 12-fold increase in luciferase measurements (p <0.0001), showing, once more, the added value of hypoxia, while the addition of TGF-β led only to a 6.7-fold increment ($p < 0.0001$). This meant that the samples stimulated with TNFα, IFN-γ and TGF-β had only 53% of the activity measured in the ones without TGF (p <0.0001).

Two conclusions can be drawn: first, independently of whether the cells were unstimulated, received TNFα and IFN-γ or TGF-β, there was always an increment trough the addition of hypoxia. For unstimulated cells, that increase was of 1.2-fold (p 0.02); in the positive control group, it amounted to

1.6-fold (p <0.0001); for TNFα + IFN-γ + TGF-β samples, it resulted in 1.2-fold increase (p 0.028). Maximum fold-increase was achieved when comparing completely unstimulated cells with those receiving TNFα, IFN-γ and hypoxia: a multiplication of 15 times the basal value (p <0.0001).

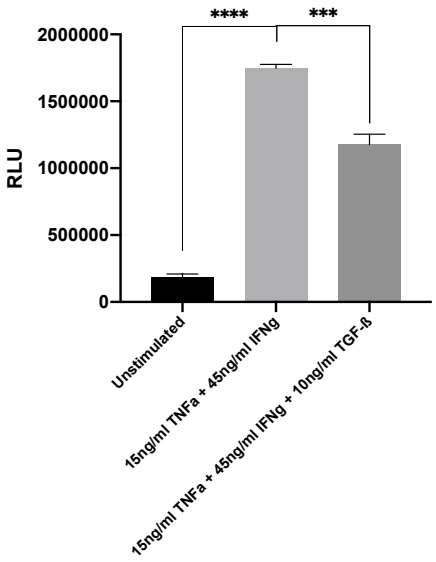

Figure 17: 3AB Gaussia reporter hBMSC response to addition of TGF-β, TNFα and IFN-γ in normoxia. *We examined the effect of the addition of TGF-β to that of the pro-inflammatory stimuli in normoxia. We observed a drastic 9.39-fold increment in RLU in the TNFα, + IFN-γ group (, p value <0.0001). The group stimulated with TNFα, IFN-γ and TGF-β, on the other hand exhibited a smaller increase in activity compared to the negative controls that being of 6.4-fold (p < 0.0001). Thus, the addition of TGF-β to the TNFα and IFN-γ led to a fall of 32% in average RLU (p value 0.0003) (statistics: student's t-test)*

6.3.6. Conclusions of the above experiments: responsiveness of the 3AB promoter to different stimuli

The objective of the creation of this promoter was to allow a more targeted activation for the delivery of therapeutic transgenes within the micromilieu of solid tumors. Therefore, the first objective was to establish its functionality. 3AB showed the same activation pattern of CCL5 upon exposure to TNFα, IFN-γ or both. 3AB proved to have a much lower basal activity in unstimulated cells, which were the same line, same passage as used for CCL5 experiments. Yet, it had a significantly higher fold increase (in the range of 9 to 12 fold for 3AB, compared to 2-3 fold for the CCL5 reporter construct). Also worth mentioning, 3AB did not show any additional activation trough stimulation with IFN-γ alone, other than CCL5.

The exposure to hypoxia had radically different results in 3AB than it did for CCL5, as it responded to it alone and especially in addition to TNFα and IFN-γ.

The tests performed with TGF-β yielded interesting results: alone, TGF-β had no effect on hBMSC transfected with the 3AB reporter construct, but contrary to expectations, the addition of it to hypoxia or

TNFα and IFN-γ led to a detrimental effect on the measured luciferase activity. That was even more pronounced when cells were exposed to hypoxia and TNFα and IFN-γ simultaneously – albeit in these experiments, cells were a passage further than anteriorly, and did not show as much activation as in the previous tests (47-fold increase at maximum, versus 14-fold). Nevertheless, the obvious decrease and the high statistical significance seen in TGF-β groups makes it unlikely that this is due only to a difference in passage.

The in vivo observations of enhanced TGF-β production within tumors and its correlation with tumor progression are deeply dependent on context and presence of a multitude of cell-cell and cell-stroma interactions, which simply are not replicated in virtro. Whatever effect TGF-β has toward tumor progression, it does not seem to be mediated directly or indirectly (e.g., crosstalk with NFκB pathways) by CCL5 or its AB element.

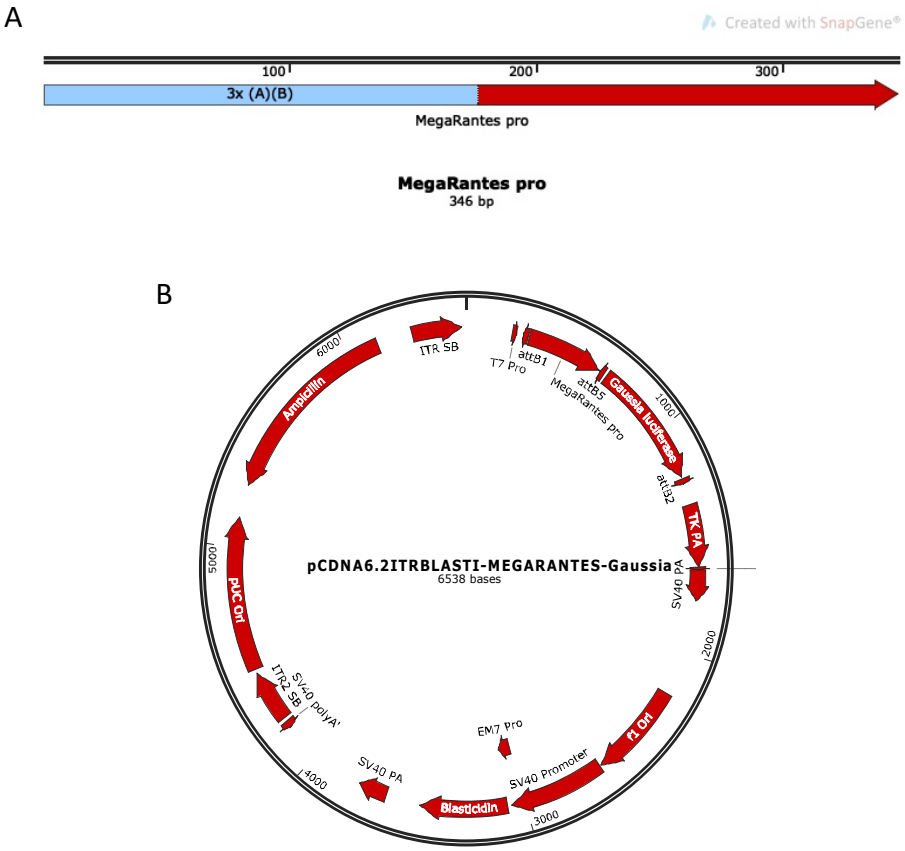

Figure 18: Structure of the synthetic promoter MegaRantes (A), and reporter construct (B). *Structure of the synthetic promoter MegaRantes (A), and reporter construct (B). (A) MegaRantes contained a 3AB element instead of the native R(A) element in the complete CCL5 promoter backbone, hoping to enhance response to pro-inflammatory stimuli, as seen with the 3AB promoter. (B). The reporter construct for MR contained a Gaussia luciferase reporter gene. The vector contained ITR SB100 sequences for stable integration and a gene for Blasticidin resistance for selection.*
ITR SB: internal tandem repeats for Sleeping Beauty transposase; 3x(A)(B): multimer of the (A) and (B) subunits, different from the 3AB promoter, which also contained a CMV Min Pro; *EM7 pro: EM7 procaryotic promoter, which is constitutively active coupled to Blasticidin Res (resistance), so that the resistance can be expressed in E.coli bacteria; SV40: promoter used to constitutively express Blasticidin resistance.*

6.4. Investigation of the effects of different stimuli on the synthetic promoter MegaRantes in hBMSC

The observations made in the experiments with the 3AB promoter added information concerning the activation of CCL5. 3AB offered the possibility of an enhanced synthetic promoter with a lower basal activation and robust activation. As a next step, we attempted to enhance the native CCL5 promoter by replacing a 3AB element in the place of the native R(AB) element in the complete human CCL5 promoter backbone. The promoter was named MegaRantes (MR).

The modified CCL5 reporter construct again drove *Gaussia* luciferase (figure 18 above) and was otherwise identical to the native CCL5 reporter construct. The hypothesis driving the subsequent studies was that this modification would enhance the responsiveness of the promoter to tumor-related stimuli.

6.4.1. TNFα and IFN-γ in hBMSC 172_1 MegaRantes reporter cells

The same human bone marrow cells from Apceth (primary MSCs Ap172_1) were applied here. For evaluation of response to TNFα and IFN-γ the cells were plated into 3 wells for each condition, 20,000 cells per well. The same concentrations of TNFα and IFN-γ were used as previously. Because of a limited number of hBMSC of the same line, stimulation was limited to the combination of TNFα and IFN-γ for maximum response, rather than those individually, since previous tests on Hek293 cells showed an analogous pattern of response to the individual stimuli and synergistic activation trough their combination, as seen with the other promoters.

Concerning maximum activation obtained, the RLU ratios were the lowest of all promoters tested. 3AB had a tenfold higher activation. The response to TNFα and IFN-γ as a positive control yielded a fold-induction of 2.1 times (p 0.0089).

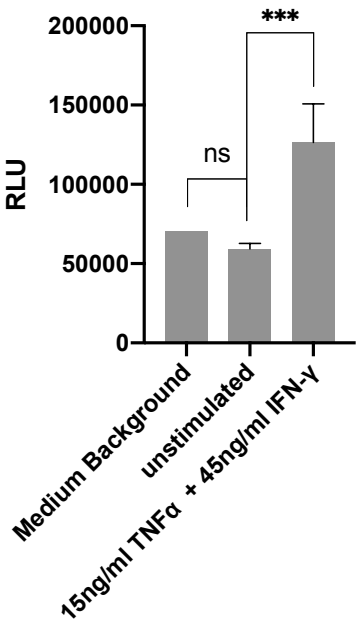

Figure 19: MegaRantes Gaussia reporter hBMSC under stimulation with TNFα and IFN-γ in normoxia. *MegaRantes Gaussia reporter hBMSC under stimulation with TNFα and IFN-γ . Cells were plated into 3 wells for each condition, 20.000 cells per well, same concentration of TNFα and IFN-γ were as used previously. The response to TNFα and IFN-γ combined yielded a fold- induction of 2.1 times (p 0.0089). Because of a limited number of hBMSC of the same line, stimulation was limited to the combination of TNFα and IFN-γ for maximum response, rather than those individually, since previous tests on Hek293 cells showed an analogous pattern of response to the individual stimuli and synergistic activation trough their combination (not shown).*

6.4.1.1. Evaluation of the effects of hypoxia on the MR promoter

Considering the dramatic effect hypoxia had on the 3AB promoter, it was particularly interesting to assess how it would affect the response of MR Gaussia reporter cells.

The transfected hBMSC in passage 4 were plated into triplets for each stimulation in two plates, one in hypoxia and one in normoxia, to permit direct comparison. As before, there was a medium change for empty DMEM 24h prior, and stimulation occurred simultaneously. The graphic comparison of the effect of hypoxia on unstimulated cells (figure 21), as well as in addition to TNFα, IFN-γ shows no difference in activation in neither group.

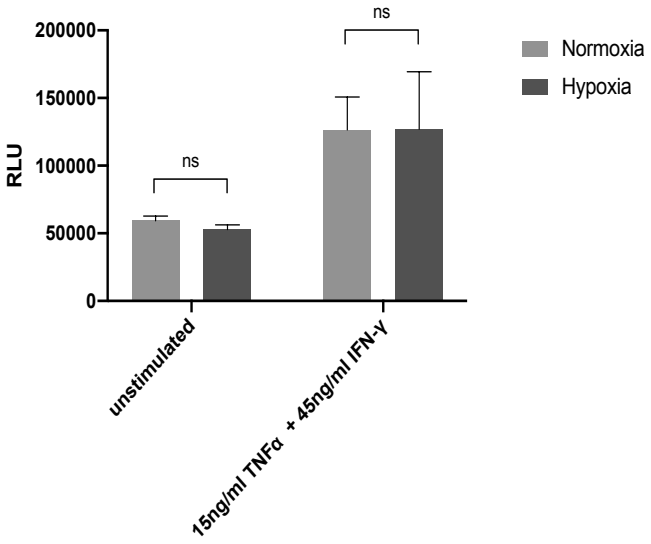

Figure 20: MegaRantes Gaussia reporter hBMSC stimulated with TNFα and IFN-γ under hypoxia, compared to same stimulations under normoxia*. hBMSC in passage 4 were plated into triplets for each stimulation in two plates, one in hypoxia and one in normoxia, triplets of 14 000 cells per well. Hypoxia alone (left) yielded no significant stimulation of Gaussia transcription (p value 0,09). Cells stimulated with TNFα and IFN-γ under hypoxic conditions (right) exhibited no increase in luciferase activity compared to the same stimulation under normoxia (p value 0,9)(student's t-test)*

6.4.1.2. Effects of TGF-β stimulation on the MR promoter under normoxia and hypoxia

As with the other promoters, we stimulated MR with TGF-β to examine its response to it. We observed a decrease in luciferase activity after stimulation with TGF-β that amounted to 28% (p 0.0016). In parallel, the same cells were stimulated with TGF-β and set into the hypoxia incubator for 48h. A tendency towards a decrease in luciferase transcription was observed but was not statistically significant (p 0.09).

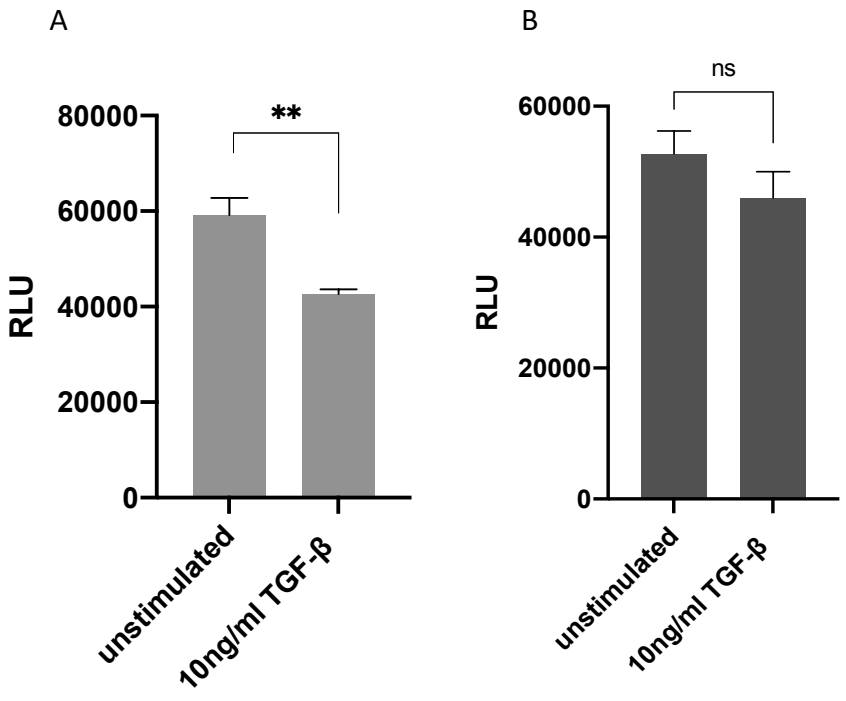

Figure 21: MegaRantes Gaussia reporter hBMSC stimulated with TGF- β under under normoxia (A) and under hypoxia (B). *hBMSC in passage 4 were plated into triplets for each stimulation in two plates, one in hypoxia and one in normoxia, 14 000 cells per well. (A) In normoxia, we observed a decrease of 28% in luciferase activity after stimulation with TGF-β (p value 0.0016). (B) In hypoxia, the trend towards a decrease in luciferase transcription was observed, with a 13% decrease, but this was not statistically significant (p value 0.09)(student's t-test)*

6.4.1.3. Addition of TGF-β to TNFα and IFN-γ in normoxia and hypoxia

To assess whether TGF-β could have a synergistical action when added to inflammatory activatin, we performed a new set of experiments using that combination, both in normoxia and in hypoxia. Cells were then plated and stimulated with TGF-β, TNFα/IFN-γ or both, with a set of unstimulated cells as control. The plates were incubated in ambient air oxygen concentration or at 1% O2. In normoxia, we once more observed that the TGF-β alone group had no significantly increased activation in comparison to the unstimulated cells (in fact, it had a 10% decrease). When TGF-β was added to the TNFα and IFN-γ , an 8.5-fold increase in comparison to the unstimulated cells was observed (corresponding to a 9.4-fold increase to the TGF-β only group), which was statistically significant, with a p value less than 0.0001.

The parallel experiment in hypoxia revealed a similar pattern: while the TGF-β + TNFα + IFN-γ wells had a 12.2-fold increase compared to the unstimulated group ($p < 0.0001$), the cells that had TNFα and IFN-γ only produced 1.37-fold more luciferase (p 0.014), resulting in a 16.7-fold increase compared to unstimulated cells.

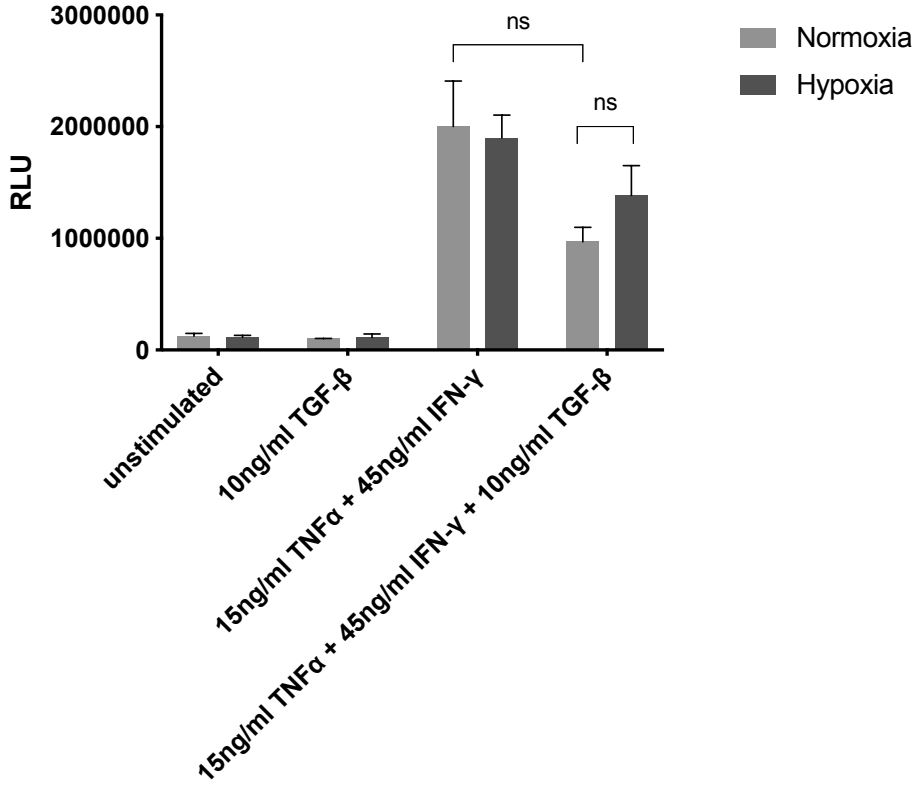

Figure 22: MegaRantes Gaussia reporter hBMSC stimulated with TGF- β, TNFα and IFN-γ, or the combination of all three, under normoxia and hypoxia. *Cells plated in triplets with 14 000 cells per well. In normoxia, we once more observed that the TGF-β alone group had no significantly increased activation in comparison to the unstimulated cells (in fact, it had a 10% decrease, but which was not statistically significant). The TNFα and IFN-γ group in 21% O2 showed the highest activation, with a 16.2-fold increase, compared to unstimulated cells in normoxia. When TGF-β was added to the TNFα and IFN-γ, that increase was only an 8.5-fold. While this difference was not statistically significant, the p value using a student's t-test was of 0.0056. The parallel experiment in hypoxia revealed a similar pattern: while the TGF-β + TNFα + IFN-γ wells had a 12.2-fold increase compared to the unstimulated group (p <0.0001), the cells that had TNFα and IFN-γ only produced 1.37-fold more luciferase (p 0.014)(statistics: student's t-test).*

7. Discussion

In recent years many studies have focused on the role of CCL5 in different malignant diseases. In some tumors, such as gastric and ovarian cancer, CCL5 is associated with tumor progression and a higher rate of metastasis[104]. In prostate cancer CCR5 is upregulated and CCL5 is expressed by prostate cancer cells themselves[127]: In this setting, stimulation with CCL5 was found to induce tumor growth, while treatment with a CCR5 antagonist inhibited tumor growth. In line with these findings, in breast cancer CCL5 expression was found to be elevated in the primary tumor, positive lymph nodes and metastases. Its expression was correlated with an aggressive breast cancer subtype and poor clinical course[130]. Even in hematologic malignancies, especially in multiple myeloma CCL5 seems to play a role in progression[245]. These findings led to the idea that CCL5 may represent a useful vehicle in tumor treatment[104]

An additional important aspect of CCL5 in cancer progression is the observation that the recruitment of hBMSC to the tumor microenvironment and their differentiation induces their expression of CCL5[246]. This biology forms the basis for a novel tumor therapy where the human CCL5 gene promoter has been used to drive therapy gene expression in engineered versions of hBMSC in preclinical and now phase I/II clinical studies, for pancreatic, gastrointestinal and hepatocellular carcinoma[212–214,247]. In the studies, the human CCL5 gene promoter was used to drive expression of the suicide gene Thymidine kinase within the tumor microenvironment, rendering cells in the tumor stroma sensitive to the prodrug Ganciclovir through bystander killing. In one of the preclinical trials, mice with metastatic pancreatic cancer treated with the modified hBMSC exhibited 50% reduction of tumor mass and 60% of the sowed reduction of metastatic burden to some degree[212]. In another study performed with experimental hepatocellular carcinoma, tumor growth was reduced by 56%, as compared to the control group following treatment with the prodrug[131].

The therapeutic strategy for using genetically modified hBMSC as a cancer therapy is centered around the use of the natural tropism of MSCs towards tumors for the delivery of therapy genes. One of the open issues when using this approach is the potential off-site toxicity trough activation of the transgene in non-tumor environments where adoptively applied MSC may accumulate [248]. The specificity of therapy gene delivery in the context of tumor biology can be enhanced by better understanding the activation of the promoter used for transgene delivery by hBMSC within tumors. In this instance, the human CCL5 gene promoter. In human Phase I/II trials with patients with metastatic gastrointestinal adenocarcinoma, half of patients could achieve stable disease, but change in tumor volume varied greatly. On the other hand, in one trial, 36 adverse events and 6 serious adverse events requiring hospitalization were reporter, highlighting the toxicity of such therapies at present[213].

The activation of the CCL5 promotor is complex and varies with cell type. Different subregions of the promoter are involved in the activation of the promoter in the various cell types that have been studied (REFs). The same external stimuli may affect the promoter differently, depending on the cell type. For example TNFα and IFN-γ induce CCL5 expression in mesangial cells, while only TNFα induces CCL5 in fibroblasts. And in case of monocytes, lipopolysaccharides (LPS) induce CCL5 expression while TNFα or IFN-γ have no effect[115]. At the initiation of the present study, the mechanisms underlying the activation of CCL5 in human hBMSCs had not been studied in detail. To this end, the major aim of this thesis was to better understand the potential role of the diverse stimuli present in the microenvironments of solid tumors in the activation of this promoter in hBMSC. A second objective was to potentially

optimize the promoter for a more enhanced or selective delivery of therapeutic transgenes to limit potential side effects to normal tissues.

We postulated that stimulation with TNFα, IFN-γ, hypoxia and TGF-β could play a role in the activation of the CCL5 promoter in hBMSC. The literature suggests that cross-talk between hypoxia and NFκB pathways. Through its action with IKKβ, hypoxia has been shown to intensify cellular sensitivity to stimulation with TNFα [249], [250–252]. Hypoxia and TGF-βhave been previously shown to induce the production of collagen in fibroblasts[200]. Furthermore, the literature suggests cross-talk between the TGF-β pathway, TNFα and IFN-γ activation of the NFκB family proteins, occurring both directly and indirectly through MAPK, and PI3K/Akt/mTor1.2.[78254–257258,]. TNFα and IFNγ have been described to induce SMAD 6 and 7, which are inhibitory SMADs that suppress the anti-inflammatory actions of SMAD 1.5 and 8[165,259]. This interaction between TNFα, IFN-γ and Smad7, and its effects on NFκB suggested that, in the right constellation of stimuli, the summation of TNFα, IFN-γ, hypoxia and TGF-β may influence CCL5 transcription.

Hypoxia is a well described feature of solid tumors and plays an important role in tumor progression[260]. In the literature, the effector protein hypoxia inducible factors (HIFs), are described to interact with the proteins of the NFκB family in a synergistic manner[261–263]. HIF1a has been proposed to activate NFκB [264,265] and to promote degradation of IkB - NFκB inhibitors[266]. NFκB has been shown to bind to the HIF1a promoter and to enhance its action in hypoxic conditions[74]. The CCL5 gene promoter has tandem NFκB binding elements that are present in the immediate upstream of the promoter. These elements have been previously shown to underlay activation of the promoter in most cell types – thus, it was speculated that the gene promoter would be responsive to hypoxia.

TGF-β is an important regulatory factor present in most solid tumor environments, we sought to determine if stimulation with TGF-β would modulate CCL5 expression. On a TME level, the regulatory action of TGF-β upon the immune system is corrupted in favor of the tumor, facilitating immune evasion[267]. Particularly the established link of TGF-β exposure and induction EMT that supports the idea that it may activate CCL5 in the context of the cell differentiation seen in MSCs within tumor environments[268,269]. Both in vitro and in vivo data shows a positive correlation between the levels of both cytokines, and suggests a direct effect of CCL5 on TGF-β-mediated immune escape[171,172].

Interestingly, the results of the study performed here do not support CCL5 promoter activation through hypoxia in hBMSCs. Depending on the type of cell studied, hypoxia proved to diminish the CCL5-driven luciferase reporter activity. The results proved to be different when studying the response of the synthetic CCL5-based promoters to hypoxia. One of these promoters, 3AB was based on the dual NFkB elements (A and B) found in the human CCL5 promoter presented in triplicate with a minimal promoter based on the CMV promoter. hBMSC engineered with the 3AB Gaussia reporter construct showed a marked increase in luciferase transcription under hypoxic conditions, especially when added to TNFα and IFN-γ resulting in a maximum fold increase of 47 times the basal activity. When this triplicate control element was introduced into the native CCL5 promoter to replace the AB elements (MegaRantes) and used as a promoter for the reporter construct, however, the data obtained showed the same pattern as CCL5, with no response to hypoxia seen.

The discrepant results of CCL5, 3AB and MR in regards to hypoxia raise questions as to why that may have come about. AB being binding sites for NFκB family proteins, which are present in all three

promoters investigated, the expectation would be that all would react to it. It is possible that upstream regulatory elements in the CCL5 promoter other than ABpresent in the native CCL5 and MR modified promoter, may restrict the response to hypoxia.

With regards to potential responsiveness to TGF- β stimulation of Hek293 and hBMSC engineered with CCL5, 3AB and MR reporter constructs showed no statistically relevant increase in luciferase activity compared to unstimulated cells under the same conditions. That was true for TGF-β stimulation alone, or in conjunction with TNFα and IFN-γ, in normoxia or hypoxia. In 3AB and MR: the addition of TGF- β to TNFα and IFN-γ and hypoxia diminished Gaussia reporter gene expression.

TGF-β is a regulator of the immune response affecting the inflammatory cascade[270] and inhibiting growth and proliferation[271,272]**.** TGF-β effects are context-dependent[273,274].The conditions under which the experiments were performed in this thesis may not efficiently reflect the complexity of the TME and interactions.

7.1. Comparison of CCL5, 3AB and MR in terms of use as a promoter for delivery of transgenes

The evaluation of the potential of a promoter as a delivery vehicle for therapeutical transgenes must consider its response to different stimuli, its maximum achieved activation in absolute terms, which ideally, would be maximum when exposed to concomitant stimuli.
CCL5 showed a maximum fold induction of 3,81 times the basal transcription of the Gaussia luciferase reporter gene after stimulation with TNFα and IFN-γ , but it did not show increased luciferase activity when stimulated with hypoxia or TGF-β, whether individually or in addition to other stimuli. The response of the 3AB promoter was much different: the maximum fold induction was much higher than that of CCL5, at 47-fold, and it was reached with the addition of TNFα, IFN-γ and hypoxia. It proved reactive to hypoxia, and in a additive level of activation when stimulated with TNFα and IFN-γ . Stimulation with TGF-β, however, either had no effect of even abrogated the activation trough pro-inflammatory stimuli and hypoxia (Figure 20, center). Finally, MegaRantes showed a pattern more consitent with CCL5, reacting to TNFα and IFN-γ , but not to hypoxia or TGF-β, alone or in addition to other stimuli.
In the sense of avoiding expression in off-target sites, 3AB presents itself as the most adequate candidate, for, differently from CCL5, it expresses only a low level of the TT when not stimulated but reaches a potent increase upon simultaneous stimulation of factors present in the tumor micromilieu.

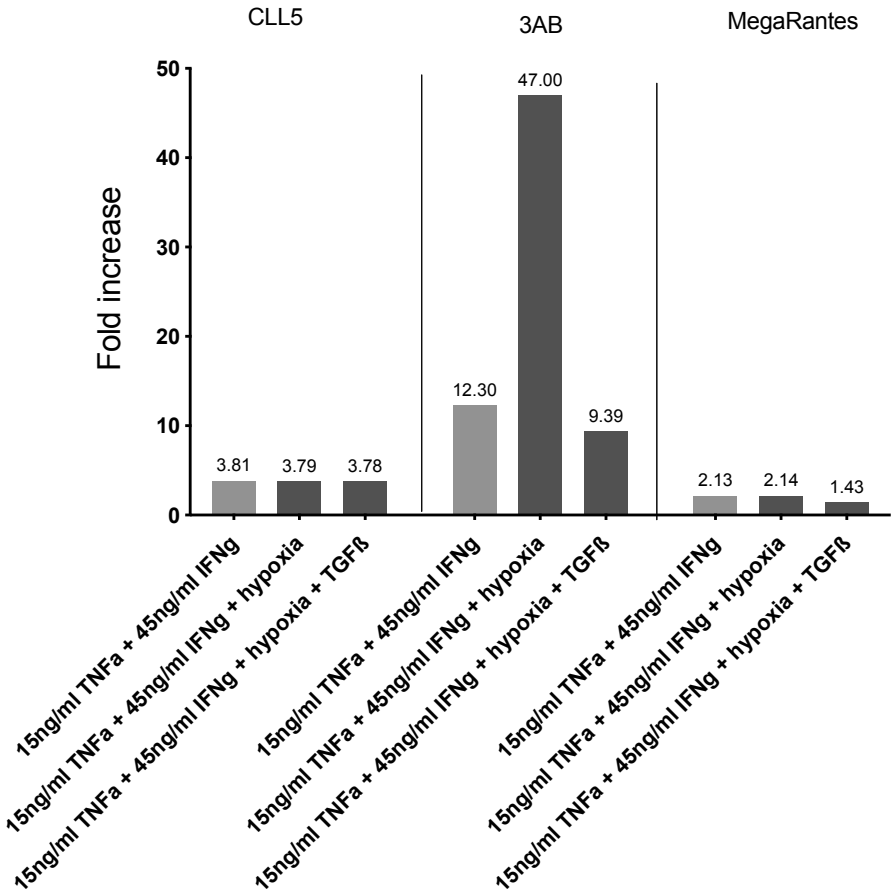

Figure 23: comparison of fold induction obtained through addition of stimuli for the promoters CCL5, 3AB and MegaRantes*, as compared to the Gaussia luciferase activity measured in unstimulated reporter hBMSC for those promoters. Left: CCL5 showed a maximum fold induction of 3,81 times the basal transcription of Gaussia luciferase under stimulation with TNFα and IFN-γ , but no relevant additive effect was observed by adding hypoxia or TGF-ß. Center: The response of the 3AB promoter was much different: the maximum fold induction was much higher than that of CCL5, at 47-fold, and it was reached with the addition of TNFα, IFN-γ and hypoxia. In contrast, adding TGF-ß dramatically reduced that response. Right: MegaRantes reacted similarly to CCL5, having had no relevant response to hypoxia or TGF-ß, alone or in addition to other stimuli.*

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7.2. On experimental design and methods employed

It is important to consider the potential limitations of the experiments performed here. hBMSC in culture are subjected to senescence and accompanying phenotype changes, an artefact of *in vitro* culture conditions[275]. This also affects their response to stimuli: in different sub-populations of hBMSC within the bone marrow niche, depending on their differentiation stage and immune phenotypical marker expression, radically different responses to hypoxia have been observed[276]. So even within the same passage, differences in experiment results may arise due to culture conditions. In addition, some data suggests hBMSC may lose some of their stem cell defining characteristics and tropism through transfection[221]. That issue underlies not just the experiments described here, but the general approach of using MSC as delivery vehicles.

Consideration must also be given to the viability of cells that underwent hypoxic conditions. The current experimental design could not account for dead cells or a possible metabolic reduction through hypoxia. While we did observe living cells in the wells through microscopy, this was not a quantitative

measurement. Alternative approaches would be to include staining (e.g. DAPI staining[277]) or FACS analysis (e.g. 7-AAD analysis[278]), which would show living cells..

Another possible factor that could influence the CCL5 promoter response to hypoxia may be the time of exposure: HIF1 is expressed within a few hours of exposure to hypoxia (0.5 to 6 hours)[279]. Yet in tumors, cells are exposed to chronic hypoxia, which is not reflected in the timepoints chosen. HIF2, for example is expressed only after at least 42 hours of exposure[280].

7.3. Open questions and proposal of further experiments

The data reported here provide information on the activation of the human CCL5 promoter through signals present in the TME and proposes a new option for a promoter for the delivery of therapeutic transgenes thought hBMSC in the form of the 3AB promoter. The results obtained with the 3AB construct are promising and suggest that in vivo models may provide more specific information as seen in our previous work.

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Affidavit

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