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Engineered mesenchymal stem cells as therapeutic vehicles for tumor therapy: Angiogenesis-based targeting and the influence of thyroid hormones

Dissertation

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Dedicated to

My mom, my dad, my grandma, my sister, my boyfriends and very good friends, who raised me, taught me and stood by me in all different life situations and especially in all different episodes of this thesis writing process and my dad and great granddad as role models who I follow with writing a doctoral thesis.

Table of content

Publications

A vector platform for the rapid and efficient engineering of stable complex transgenes, Jackel, C., Schmitt-Nogueira M, Ehni N, Kraus C, Ranke J, **Dohmann M**, Elfriede Noessner and Peter J. Nelson, Sci Rep, 2016. 6: p. 34365.

Integrin alphavbeta3-Mediated Effects of Thyroid Hormones on Mesenchymal Stem Cells in Tumor Angiogenesis, Schmohl, K. A., Mueller, A. M., **Dohmann, M.**, Spellerberg, R., Urnauer, S., Schwenk, N., Nelson, P., Spitzweg, C. (2019). Thyroid, 29(12), 1843-1857. doi:10.1089/thy.2019.0413.

Zusammenfassung

Neue Ansätze in der Krebstherapie verbessern langsam die Behandlungsergebnisse von Krebspatienten. Der Einsatz von gentechnisch veränderten mesenchymalen Stammzellen (MSCs) wird derzeit als Möglichkeit zur gezielten Therapieentwicklung von Tumoren untersucht. Dieser Ansatz macht sich den starken Tumortropismus von mesenchymalen Stammzellen zunutze, um therapeutische Gene oder Zytokine, die toxisch sein können, tief in das Tumormilieu zu bringen.

In dieser Arbeit wurde der Effekt von Schilddrüsenhormonen auf diese mesenchymalen Stammzellen sowie deren Einfluss auf die Tumorangiogenese untersucht.
Zu diesem Zweck wurde ein *in-vitro* Angiogeneseassay etabliert. (Siehe Resultate 4.1.)
Parallel dazu entwickelten wir, unter Verwendung einer neuen Klonierungsplattform Reportertransgenen, die mit Tumorhypoxie und Angiogenese-Biologie verknüpft sind. (Siehe Resultate 4.2.)
Die dadurch entwickelten stabilen Transgene transfizierten wir im Folgeschritt in primäre humane mesenchymale Stammzellen. Die Transgene, die entwickelt wurden, waren zum einen ein synthetisches Hif-1α- Promoterelement, das sensibel auf Hypoxie reagieren kann, ein menschlicher VEGF-Promoter, der für seine Aktivierung in früher Angiogenese bekannt ist, und der Tie2 -Promoter, der die Expression des TEK-Rezeptor-Tyrosinkinase-Gens steuert und eine wichtige Rolle in den späten Angiogenese spielt. Hinter diese Promotoren wurde Gaussia-Luciferase als Reporter eingebaut, um die Aktivierung als Lichtreaktion messen zu können.
Während das synthetische Hif-1α-responsive Promoterelement in MSCs eine signifikante Induktion unter Behandlung von Cobalt Chlorid ($CoCl_2$), einer Substanz, die hypoxische Bedingungen imitiert, zeigte, konnte dies zwar auch für MSCs, die mit dem VEGF- Promotor transfiziert waren, nachgewiesen werden, aber nicht in einer dosisabhängigen Weise wie für Hif-1α. Die Stimulation mit $CoCl_2$ der MSCs, transfiziert mit dem Tie2-Promotor, zeigte keine Aktivierung.

In dieser Studie wurde, wie oben genannt, im weiteren Schritt der Einfluss von Schilddrüsenhormonen auf die Tumorangiogenese im Kontext von MSCs untersucht. MSCs, die mit den oben genannten Reporter-Konstrukten transfiziert wurden, wurden dann mit Schilddrüsenhormonen teilweise in Kombination mit $CoCl_2$ stimuliert.
Dies führte für alle Konstrukte zu einer leichten Erhöhung von *Gaussia*- Luciferase nach Stimulation mit T3, vor allem in Kombination mit $CoCl_2$ (siehe Resultate 4.3.4.). Die Ergebnisse nach Stimulation mit T4 mit oder ohne $CoCl_2$ zeigten kein eindeutiges Bild. (Siehe Resultate 4.3.)

Bei der Implementierung des in vitro Angiogeneseassays zeigten sowohl humane Endothelzellen (HUVECs) als auch mesenchymale Stammzellen (MSCs) eine solide Röhrenbildung *in - vitro*, während die hepatozelluläre Karzinomzelllinie (HUH7) in dieser Studie dies nicht eindeutig tat.
In einem weiteren Schritt testeten wir Schilddrüsenhormone in dem Angiogeneseassay, um den Einfluss auf die Angiogenese und das Verhalten von MSCs zu untersuchen. Eine dosisabhängige Aktivierung konnte für T3 und T4 nach Stimulation der Endothelzellen (HUVECs) im Angiogenese Assay gesehen werden. Zusätzliche Stimulation der HUVECs mit konditioniertem MSC-Medium und T3 und T4 führte auch zu einer Steigerung der Röhrenbildung im Angiogeneseassay. (Siehe Resultate 4.4.)

Die letzte Versuchsreihe kombinierte den Angiogeneseassay und die Untersuchung der gentechnisch veränderten MSCs. Die MSCs, die Hif-1α-, Tie2- und VEGF-Reporter-Plasmide enthielten, wurden im Angiogeneseassay mit und ohne Schilddrüsenhormone getestet. Während die meisten Ergebnisse nicht signifikant waren, war ein signifikantes Ergebnis für MSCs, transfiziert mit Hif-1α-*Gaussia,* nach Stimulation mit T3 zu verzeichnen. Die Ergebnisse unterstützen die Hypothese, dass die

Schilddrüsenhormone T3 und T4 die Tumorangiogenese beeinflussen können und dies eventuell teilweise durch Aktivierung von MSCs vermittelt werden. (Siehe Resultate 4.5)

Mit diesen Fragestellungen und unterstützenden Experimenten war das Ziel der Doktorarbeit eine Grundlage für eine individualisierte Tumorzieltherapie zu schaffen, um das Behandlungsergebnis von Krebspatienten zu verbessern.

Summary

New approaches are slowly improving the outcomes of cancer patients. The use of engineered versions of mesenchymal stem cells (MSCs) is currently under investigation as a platform to deliver therapeutic genes to solid tumors.

This approach makes use of the robust tumor tropism of adoptively applied mesenchymal stem cells to deliver a therapeutic gene such as a suicide gene or cytokine deep into the tumor environment.

This thesis focused on the effect of thyroid hormones in the context of mesenchymal stem cell biology, and specifically, on the influence of thyroid hormones on tumor angiogenesis.

To this end, an *in vitro* angiogenesis assay was established. (See results 4.1.) In parallel, a novel cloning platform was applied for the expression of novel reporter transgenes linked to tumor hypoxia and angiogenesis biology. (See results 4.2.)

Stable transgenes were introduced into primary human mesenchymal stem cells making use of gene promoters activated in the context of the tumor stroma and tumor angiogenesis. These included a synthetic Hif-1α - driven promoter that is responsive to hypoxia, a human VEGF promoter thought to be activated in early angiogenesis, and the Tie2 promoter, the promoter driving expression of the TEK receptor tyrosine kinase receptor that binds angiopoietin and plays an important role in late angiogenesis. The gene promoters were engineered to drive expression of a secreted version of the *Gaussia* luciferase as a reporter gene.

While the synthetic Hif-1α - responsive promoter did show induction following treatment of MSCs with $CoCl_2$, an agent that can mimic a hypoxic milieu, in a significant dosis-dependant way, similar stimulation of MSCs containing the VEGF promoter reporter constructs did show a significant activation, but not in a dosis-depandant way. Stimulation of MSCs, transfected with the Tie2 promoter, with CoCl2 not induce promoter activity.

The study then focused on the influence of thyroid hormones on tumor angiogenesis in context of MSC biology. MSCs containing one of the three reporter constructs Hif-1α, Tie2 and VEGF were then stimulated with thyroid hormones with and without $CoCl_2$. For all constructs a slight enhancement in *Gaussia* light reaction was seen for T3, especially in combination with $CoCl_2$. (See results 4.3.)

When applying the *in vitro* angiogenesis assay, human endothelial cells (HUVECS) and mesenchymal stem cells (MSCs) were both shown to generate solid tube formation *in vitro* while the hepatocellular carcinoma cell line (HUH7) used in this study did not.

Thyroid hormones have been previously shown to influence aspects of MSC biology and angiogenesis. A dose-dependent activation could be seen for T3 and T4 stimulation of endothelial cells in the context of the angiogenesis assay. Stimulation of HUVECs with MSC conditioned media plus T3 or T4 lead to an increase in experimental angiogenesis. (See results 4.4.)

The last series of experiments combined the angiogenesis assay and the genetically modified transgene MSCs. MSCs containing Hif-1α, Tie2 and VEGF reporter plasmids were tested in the angiogenesis assay with and without thyroid hormones. While most of the results were not significant, a significant result was seen for MSCs containing Hif-1α in the angiogenesis after stimulation with T3. (See results 4.5.)

The results suggest that thyroid hormones T3 and T4 influence tumor angiogenesis and partly through activation of MSCs. Next to the goals stated above, the aim was to lay the foundation of future individualized tumor-target therapy to enhance the outcome of cancer patients.

1 Introduction

1.1 Cancer and the Hepatocellular Carcinoma

Cancer is one of the main causes of deaths worldwide. 9.6 million People were estimated to have died from cancer in 2018, according to the GLOBOCAN and WHO (Bray et al., 2018). It is the second largest group of deaths after cardiovascular diseases. 16% of patients with cancer die from it and every fourth person has a lifetime risk of getting cancer. (Roy & Saikia, 2016) The most common cancers in order of frequency are: breast, colorectal, lung, cervix and thyroid cancer among women and lung, prostate, colorectal, stomach and liver cancer among men. (Bray et al., 2018). In the annual WHO report (WHO 2019) it is stated that 30-50 % of cancers could be prevented as they are linked to tobacco and alcohol consumption, a high body weight, lack of physical exercise and a lack of available vaccinations for some infectious diseases.

Liver cancer causes approximately 728,000 deaths per year and represents the fourth leading cause of death worldwide (WHO 2019). Many liver cancers are hepatocellular carcinomas (HCC) (Robert Koch Institute 2019). The main risk factor for liver cancer is cirrhosis occurring after a hepatitis-B or -C infection, chronic alcohol use or consumption of aflatoxin B1 contaminated food (Robert Koch Institute 2019). The high death rate seen is related to the fact that liver cancers are generally diagnosed at relatively advanced stages of the disease. In these instances, a curative resection and liver-transplantation is not always possible. (Raza & Sood, 2014) Better understanding of the underlying tumor biology may assist in the development of novel treatments allowing better recovery even in late stages of the disease. As with all tumors, the development of HCC is a multistep process where hypoxia and angiogenesis play a significant role in carcinogenic process (Raza & Sood, 2014). In the present thesis, we examined the biology surrounding hypoxia and angiogenesis in context of HCC tumor growth and specifically with the interplay of thyroid hormones and mesenchymal stem cells in experimental angiogenesis.

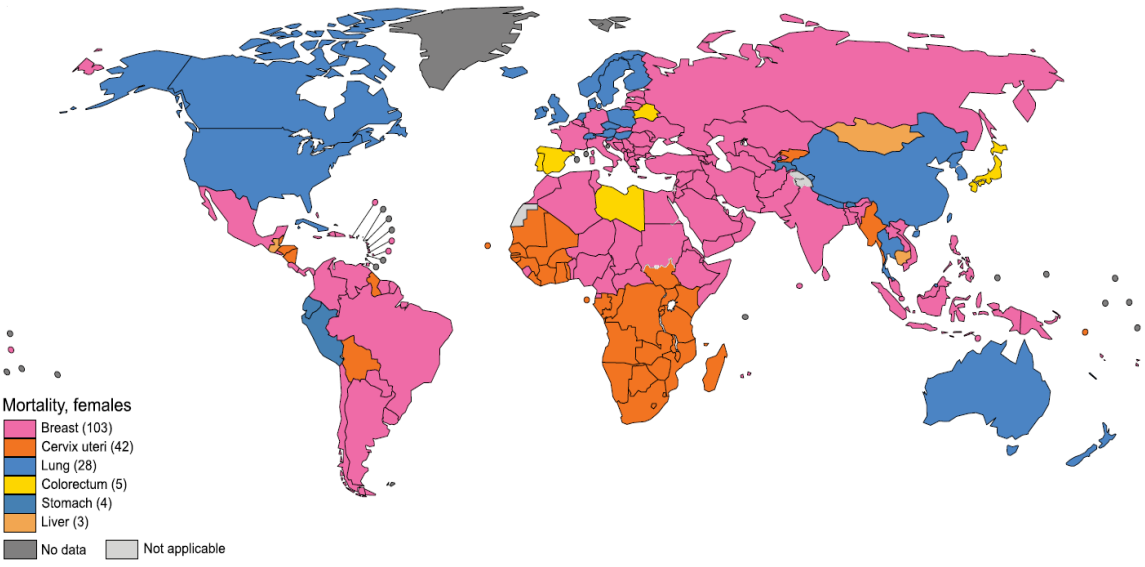


Figure 1: Worldwide map showing the most common type of cancer mortality in women in 2018 (Bray et al., 2018)

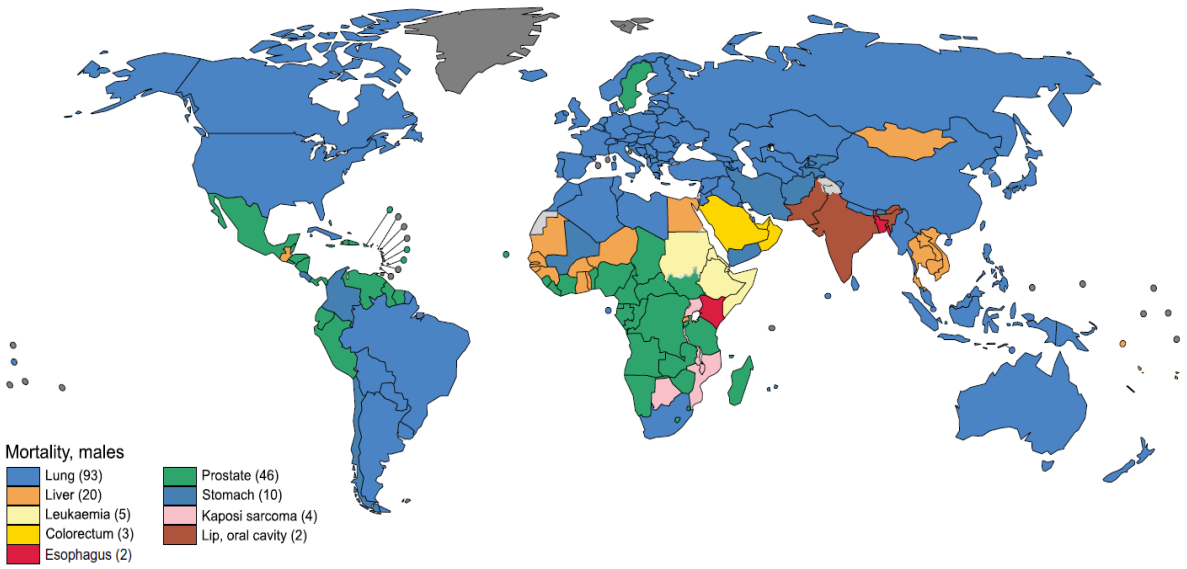


Figure 2: Worldwide map showing the most common type of cancer mortality in men in 2018 (Bray et al., 2018)

1.2 Tumor stroma

1.2.1 Composition of tumor stroma

Normal cells transform into a tumor cell when genes linked to tightly regulated cell growth undergo mutation. (Hanahan & Weinberg, 2011) In this context, the interplay of genetic predisposition and environmentally induced changes result in a series of events leading to a transformed cell. An overview of the process has been provided by Hanahan et. al (Figure 3.)

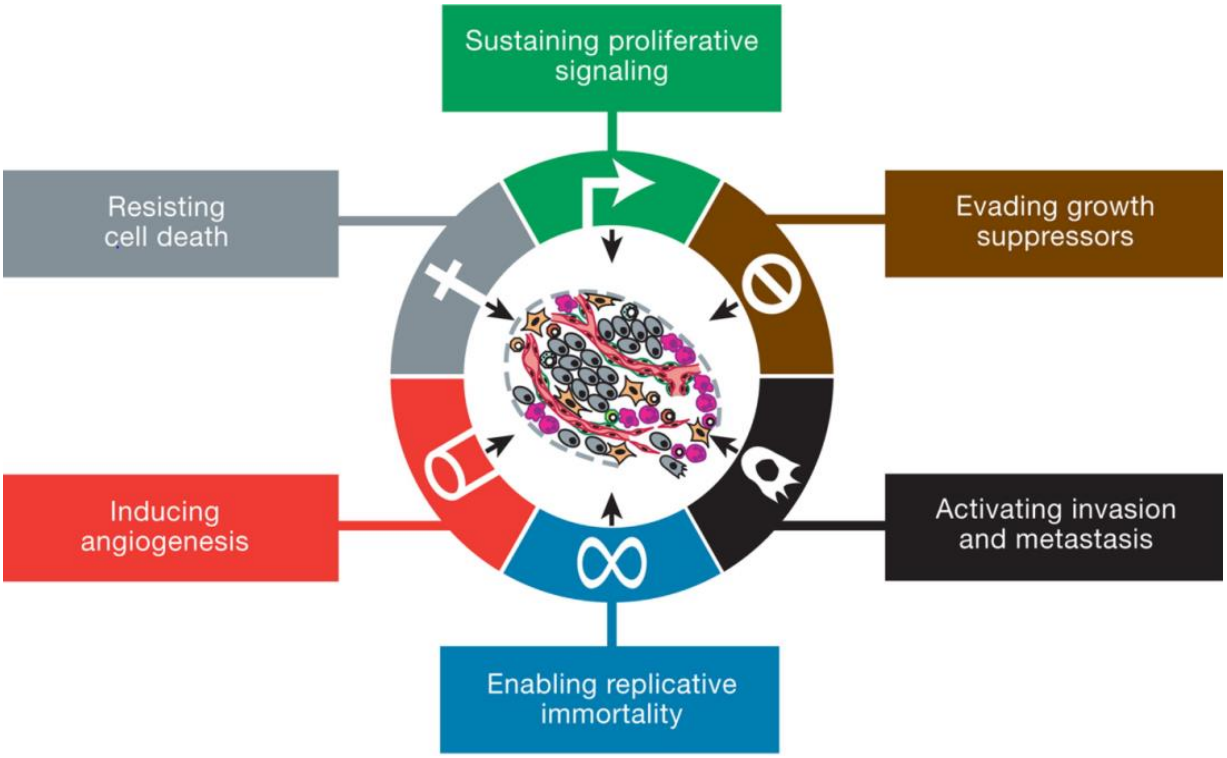


Figure 3: The Hallmarks of Cancer (Hanahan et al.)

When one examines these events in more detail, it is thought that normal cells convert into tumor cells when their growth control systems are affected by endogenic or exogenic mutations and

consequently lose limitation on growth. (Pietras & Ostman, 2010) This can happen through sustained proliferative signaling, suppression of growth suppressors, the acquisition of replicative immortality, a circumvention of cell death mechanisms, as well as the induction of angiogenesis and enabling metastasis and invasion. (Hanahan & Weinberg, 2011)

The resulting tumor cells can be classified as benign or malignant tumor cells. Benign tumors grow expansively locally, but slowly, and are well differentiated. They show a low number of cells, limited cellular changes, and do not build metastases. By comparison, malignant tumor growth is fast, locally infiltrating with consecutive formation of metastasis, the number of cells is high, and the grade of differentiation is low. (Sedlacek, 2013). In addition to tumor cells, the tumor stroma is composed of different cell types including endothelial cells, pericytes, fibroblasts, also called cancer-associated fibroblasts (CAFs), leukocytes, mesenchymal stem cells and extracellular matrix. (Pietras & Ostman, 2010) The non-tumor cell types are recruited to the tumor milieu (Krueger, Thorek, Denmeade, Isaacs, & Brennen, 2018) The various cell types communicate through auto- and paracrine pathways with help of chemokines, cytokines, growth factors and enzymes and promote tumor growth, progression and tumor angiogenesis. (Pietras & Ostman, 2010)

This thesis focuses on better understanding the formation of new vessels – angiogenesis - within the tumor and the role and effect of mesenchymal stem cells (MSCs) and its interaction with endothelial cells (ECs).

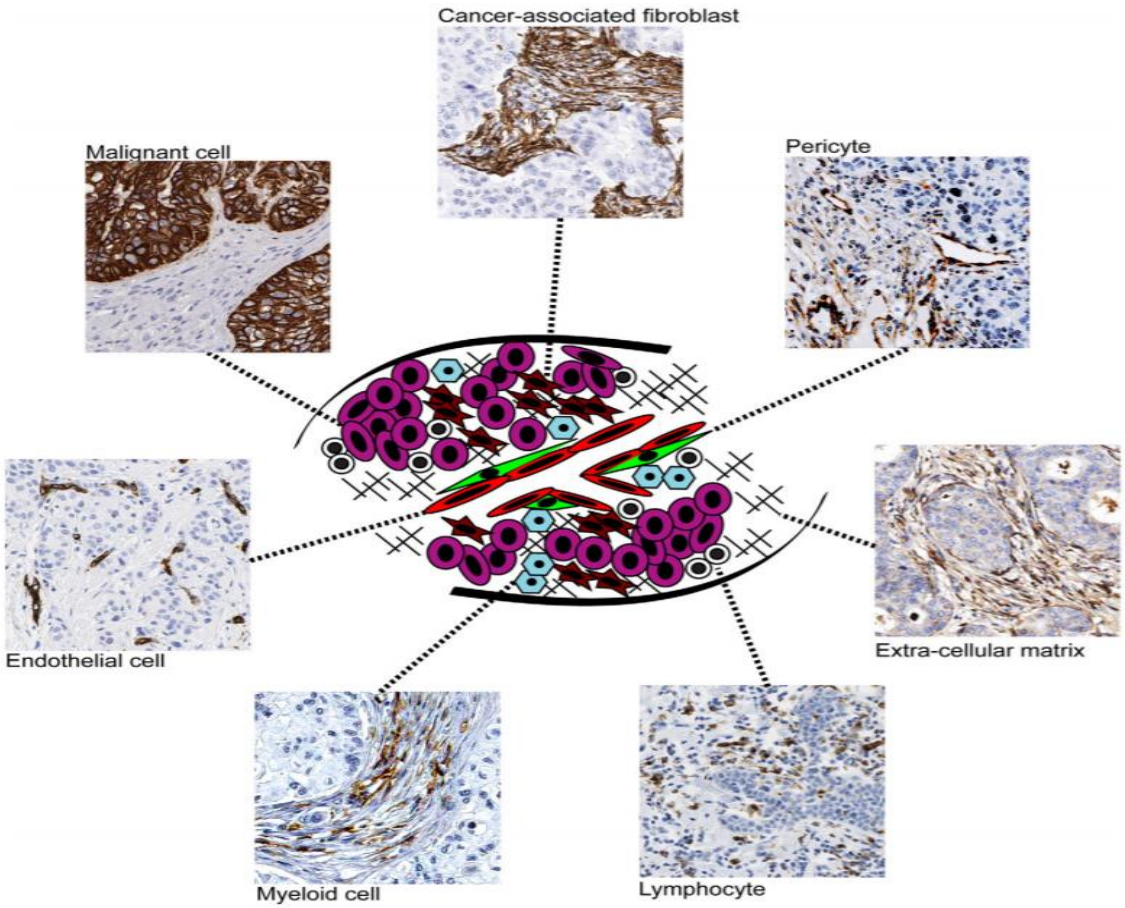


Figure 4: Overview over the different cell types forming the tumor stroma (Pietras & Ostman, 2010): Apart from tumor cells, the tumor stroma consists of endothelial cells, pericytes, fibroblasts (Roy & Saikia, 2016) also so-called cancer-associated fibroblasts (CAFs), leukocytes, mesenchymal stem cells and extracellular matrix (Pietras & Ostman, 2010).

1.2.2 Hypoxia and angiogenesis in tumors

Tumor progression leads to a hypoxic milieu. The diffusion distance for oxygen is 100 to 200 nm, measured from the blood vessel wall. Beyond this point a hypoxic milieu exists (Carmeliet & Jain, 2000). When tumor growth proceeds a hypoxic milieu develops quickly and activates the so-called hypoxia response network in the surrounding cells. An early step in the hypoxia response is the stabilization and activation of the transcription factor Hif1-α that helps drive the transcription of diverse genes including pro-angiogenic factors. This thesis deals with the regulation of two components of the angiogenic response, namely vascular endothelia growth factor (VEGF-A) and the angiopoietin-1 receptor (Tie2). Other factors linked to this biology include PGF, TGF-ß among many others (Ho & Lam, 2013). The secretion and expression of pro-angiogenic factors by tumor cells and stromal cells such as mesenchymal stem cells (MSCs) help foster angiogenesis. (Balkwill, Capasso, & Hagemann, 2012; Bronckaers et al., 2014). Angiogenesis, also called neo-vascularization, is crucial for the growth and survival of tumors (Carmeliet & Jain, 2000). It starts from pre-existing blood vessels. It follows the same rules as seen in early embryonic development and occurs through the interplay between pro-angiogenic and anti-angiogenic factors. There are different aspects of angiogenesis including angiogenic sprouting and intussusceptive growth. (Carmeliet & Jain, 2000) MSCs are thought to help foster angiogenesis by acting first as pericyte-like cells and by their differentiation into the cancer associated fibroblasts (CAFs) linked to tumor angiogenesis. Normally new vessels are formed when the surrounding tissue produces hypoxic and inflammatory signals that lead to a breakdown of the extracellular matrix and thereafter the release of growth factors as well pro-angiogenic factors as e.g. VEGF. (Arroyo & Iruela-Arispe, 2010; Bronckaers et al., 2014) VEGF among other growth factors initiate angiogenesis by activating the endothelial cells to form new branches by differentiating into tip and stalk cells. (Potente, Gerhardt, & Carmeliet, 2011) As a result, newly formed vessels are made, the tip cells form filopodia and grow towards the oxygen deficiency area and the stalk cells form the lumina. (Potente et al., 2011)

In tumor neo-angiogenesis the newly formed vessels are generally not as structurally round as normal vessels. This often leads to fluid and molecule leakage, with chaotic sprouts as well as uneven lumens. This can result in an insufficient oxygen und nutrient supply, and the tumor milieu becomes even more hypoxic and tumor cells start to undergo metastases. (Balkwill et al., 2012)

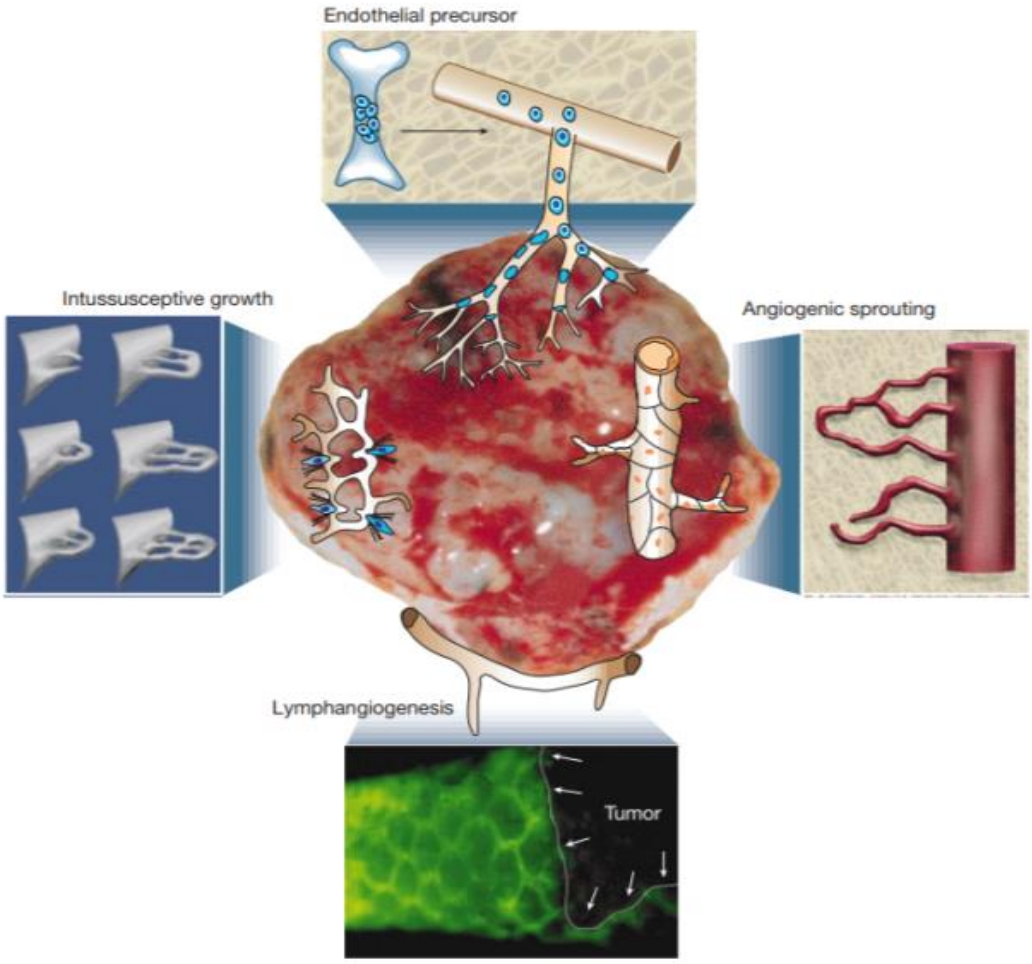


Figure 5: Different mechanisms of tumor angiogenesis (Pandya et al., 2006)

1.2.2.1 Hif-1α, VEGF and the angiopoietin-1-receptor (Tie2)

The hypoxia-inducible-factor transcription factors consists of an α- subunit, Hif-1α, and a β-subunit, Hif-1β. Hif-1β is constitutively expressed, while the α-subunit is oxygen-dependent. (Wang, Jiang, Rue, & Semenza, 1995) In normal situations where a sufficient oxygen supply is present in cells, the constitutively produced Hif-1α gets hydroxylated by propylhydroxylases and is rapidly degraded via the Von-Hippel-Lindau-E3-Ubiquitinligase complex - in cellular proteasomes. Under conditions that lack oxygen, Hif-1α becomes stabilized when degradation is blocked allowing the factor to translocate to the nucleus where it forms a functional unit with Hif-1β to act as a transcriptional factor. (Zimna & Kurpisz, 2015) In this role it binds to specific target gene promoters which contain a functional HRE-sequence. (Pugh, O'Rourke, Nagao, Gleadle, & Ratcliffe, 1997) The HRE-sequence consists of a 5 nucleotide 3'RCGTG 5' sequence. This sequence is found in hundreds of hypoxia and angiogenesis related genes that are broadly involved in the response to low oxygen conditions including angiogenesis, proliferation, glucose metabolism and cancer development. (Schödel et al., 2011; Semenza, 2010) The activation of Hif-1α helps to shift the oxidative cellular metabolism to a glycolytic one in order to produce ATP. It is possible to in part mimic a hypoxic milieu in-vitro using cobalt chloride that leads to blockage of the enzyme that hydroxylates Hif-1α and therefore leads to stabilization of the protein. (Yuan, Hilliard, Ferguson, & Millhorn, 2003)

The pro-angiogenic factors VEGF and angiopoietin-1 receptor (Tie2) were studied in this thesis. The VEGF gene promoter contains a HRE sequence (J. A. Forsythe et al., 1996) and can be directly

activated by the transcription factor Hif-1α. With regards to the Tie2 promoter, there is growing evidence that it may also become activated by Hif-1α but this is not well characterized to date. (Sarkar et al., 2011)(Licht et al., 2006; Willam et al., 2000).

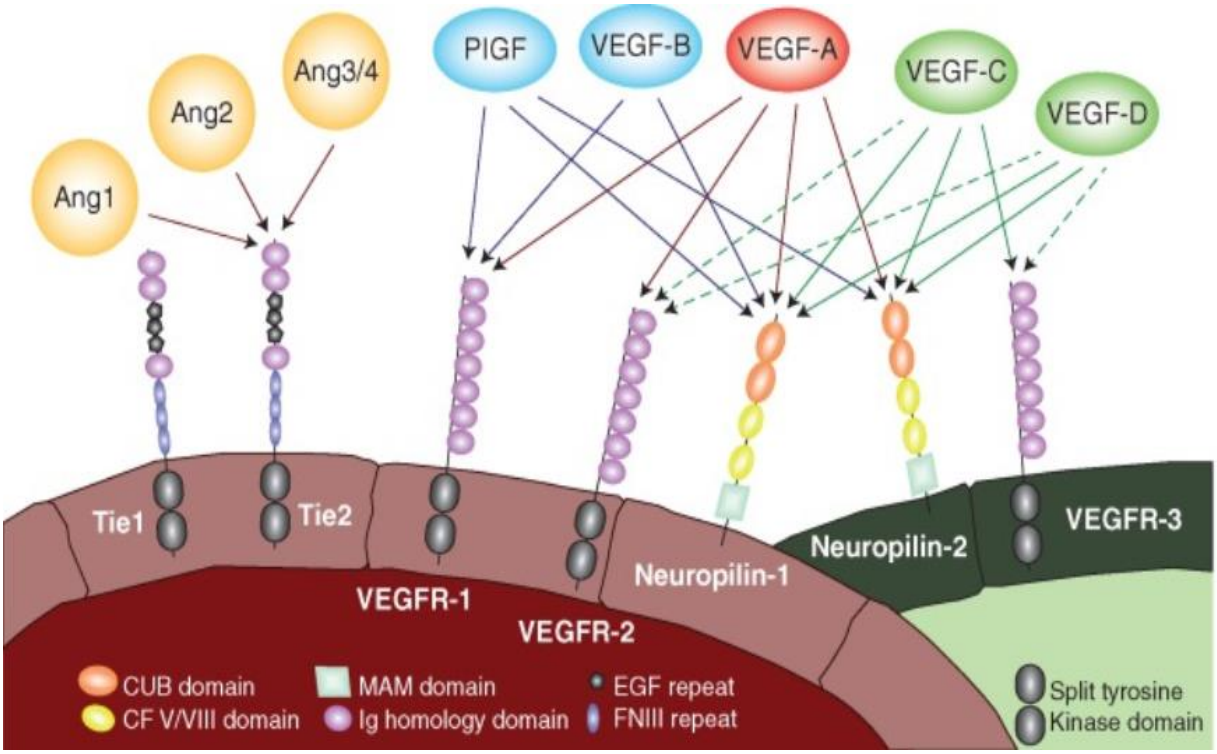


Figure 6: Tie-receptors and VEGF receptors and their binding ligands. (Fagiani & Christofori, 2013)

Both VEGF-R/VEGF as well as the Tie2/Ang pathway are receptor tyrosine kinase signaling pathways that are found in endothelial cells as well as related cells and play a crucial role in initiating angiogenesis. (Jeltsch, Leppanen, Saharinen, & Alitalo, 2013)

The vascular endothelial growth factor A (VEGF-A) becomes activated in early angiogenesis. VEGF-A belongs to the VEGF growth factor group (VEGF-A through F). It is secreted by tumor cells, endothelial and mesenchymal stem cells as a response to reduced oxygen levels. (Holmes, Roberts, Thomas, & Cross, 2007) VEGF-A binds to the VEGFR-1(Flt-1) and VEGFR-2 (KDR/Flk-1) receptors. Important metabolic pathways are mediated by VEGFR-2 and its activation promotes angiogenesis, (Karkkainen & Petrova, 2000) while VEGFR-1 activation is thought to inhibit angiogenesis. (Fagiani & Christofori, 2013) VEGFR-2 signaling leads to activation of the tyrosine kinases pathway, one the most important pathways linked to the initiation of angiogenesis.
VEGF-A plays an important role in normal vascularization. For example, during embryonic development when new vessels form as well as in neo-angiogenesis when VEGF-A is upregulated in response to hypoxic activation of Hif-1α leading to formation of new vessels from pre-existing vessels. (Apte, Chen, & Ferrara, 2019) The angiogenic process induced through VEGF includes enhanced migration of endothelial cells, increased of proliferation of endothelial cells with the formation of new blood vessels. It is thought that endothelial cells and CAFs secrete VEGF and thereafter help drive angiogenesis (Petrova et al., 2018; Pietras & Östman, 2010). It was proposed that VEGF stimulation may drive expression of the angiopoietin-1 receptor (Tie2) which until now could not be validated by experimental studies. By contrast, some studies have suggested that VEGF breaks down Tie2. (Findley et al., 2007)

Angiopoietin-1 receptor (Tie2), a TEK receptor tyrosine kinase, is an important factor in angiogenesis, especially in late stages of angiogenesis. It is a tyrosine kinase receptor that contains an immunoglobulin and epidermal growth factor (EGF) domain. It is largely expressed by endothelial cells but is also found on pericytes, subpopulations of CAFs and myloid cells and is upregulated during tumor angiogenesis. (De Palma et al., 2005) Angiopoietin-1 receptor binds the angiopoetins Ang-1, -2, -4 (Jones & Dumont, 1998). Depending on the state of the endothelial cells after binding Ang-1 and Ang-4. The angiopoietin-1 receptor can either promote or inhibit angiogenesis. In quiescent endothelial cells it gets translocated to the endothelial cell junctions and forms cross junctions with the angiopoietin-1 receptor (Tie2) of the neighboring endothelial cell and thereby plays a role in barrier function. (Fukuhara et al., 2008; Saharinen et al., 2005) In quiescent endothelial cells angiopoietin-1 receptor binding of Ang-1 and Ang-4 leads to activation of the phosphatidyl-inositol-3 kinase (PI3K)/Akt kinase pathway. This pathway helps drive proliferation, growth and cell death. (Vivanco & Sawyers, 2002) In comparison to activated endothelial cells, angiopoietin-1 receptor binding of Ang-1 and -4 activates angiogenesis by activating vessel proliferation, migration and sprouting of the endothelial cells. (Fagiani & Christofori, 2013) In this setting the angiopoietin-1 receptor initiates angiogenesis by predominately activating the PTK2/FAK pathway and the downstream MAPK1/ERK2 and MAPK3/ERK1 pathways after the angiopoietin-1 receptor forms complexes with the endothelial matrix. These pathways lead to endothelial cell survival and stability as well as the activation of migration. (Fukuhara et al., 2008; Saharinen et al., 2005)
The underlying processes linked to the activation of the angiopoietin-1 receptor by Ang-2 are less well understood. In normal circumstances Ang-2 is thought to inhibit angiogenesis through an antagonistic effect. However, Ang-2 in combination with VEGF can drive angiogenesis. (Fagiani & Christofori, 2013) Ang-2 is increased in tumor patients and in this context can support angiogenesis. (Daly et al., 2013) Whereas Ang-2 is expressed by active endothelial cells and Ang-1 by tumor and CAFS. (Augustin et al., 2009; Thomas & Augustin, 2009)

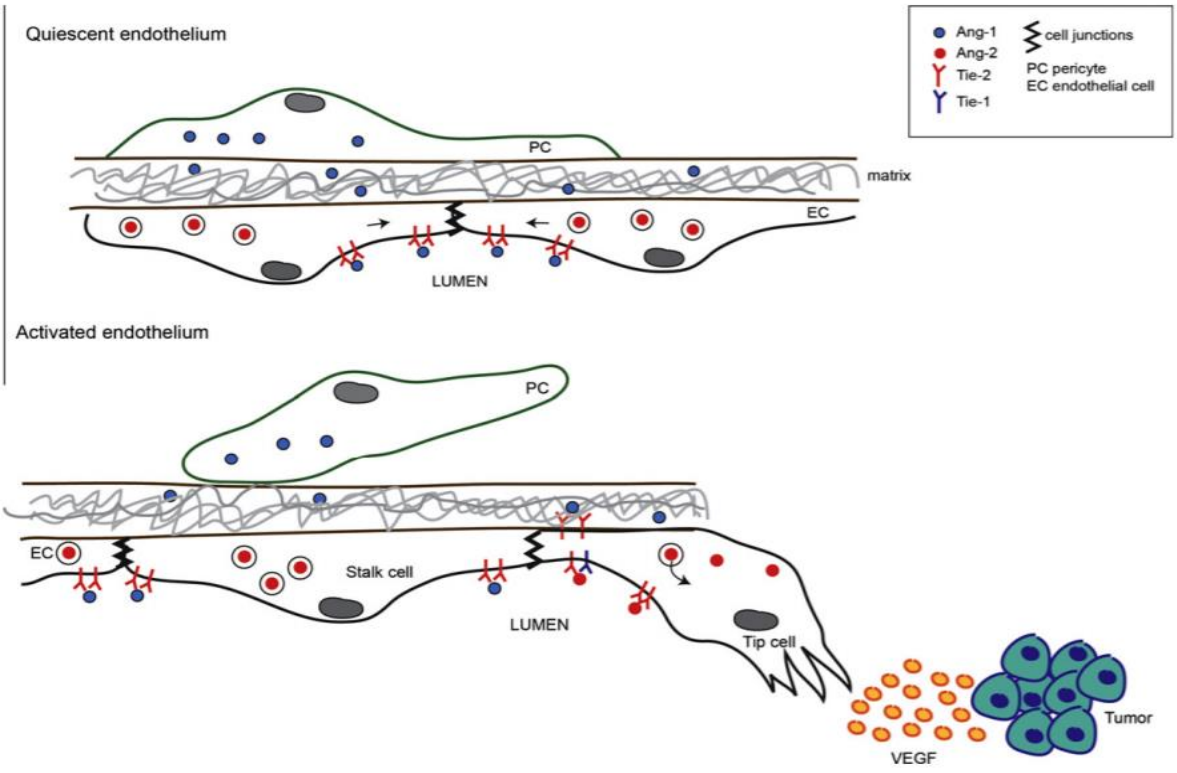


Figure 7: Tie2, angiopoietin and VEGF interplay (Fagiani & Christofori, 2013)

Tie-1 supports Tie-2 as co-receptor while binding angiopoietin itself but does not alone lead to vessel maturation. (Saharinen et al., 2005) There is little known about Tie-2 transcription in endothelial cells. The gene coding for Tie-2 receptor is the TEK gene. (Partanen et al., 1992) There is growing evidence that the TEK promoter is a target gene of Hif-1α. (Sarkar et al., 2012)

1.3 Mesenchymal stem cells (MSCs)

1.3.1 Mesenchymal stem cell (MSC) biology

Mesenchymal stem cells (MSCs) are multipotent non-hematopoietic adult stem cells with the capacity to differentiate in all three forms of the mesodermal lineage: adipocytes, osteocytes and chondrocytes (see also results 4.3.1). MSCs can be derived from different tissues such as bone marrow, peripheral blood, umbilical cord, amniotic fluid, adipose tissue, muscles, liver, lung, heart and dental pulp. The principal sources are peripheral blood, adipose tissue and bone marrow (Hass, Kasper, Böhm, & Jacobs, 2011). Bone marrow derived MSCs (also later in this thesis called hbMSCs) were used for this thesis as they can be isolated in large numbers from the bone marrow even though the percentage is low with 0.001 – 0.01% of total cell amount in the bone marrow (Rastegar et al., 2010). MSCs were first described in the seventies by researchers which had derived cells from bone marrow that appeared fibroblast-like could differentiate into osteoblasts, adipocytes and chondroblasts. They were named mesenchymal stem cells (Friedenstein, Chailakhyan, Latsinik, Panasyuk, & Keiliss-Borok, 1974). In 2006 the International Society for Cellular Therapy (ISCT) postulated three characteristics to define MSCs. These are adherence to plastic, the expression of specific surface antigens and the potential of multipotent differentiation was postulated (Dominici et al., 2006).

1 Adherence to plastic in standard culture conditions

2 Phenotype

Positive (≥ 95% +)	Negative (≤ 2% +)
CD105	CD45
CD73	CD34
CD90	CD14 or CD11b
	CD79α or CD19
	HLA-DR

3 *In vitro* differentiation: osteoblasts, adipocytes, chondroblasts (demonstrated by staining of *in vitro* cell culture)

Figure 8: Main characteristics to define MSCs, (Dominici et al., 2006): The first criterion is the adherence to plastic; the second criterion is the expression of specific surface antigens and the last criterion the potential of multipotent differentiation.

In more detail, the cells must be positive for CD105, CD73, CD90, and negative for CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II, as measured by flow cytometry (Figure 8: Main characteristics to define MSCs (Dominici et al., 2006).).

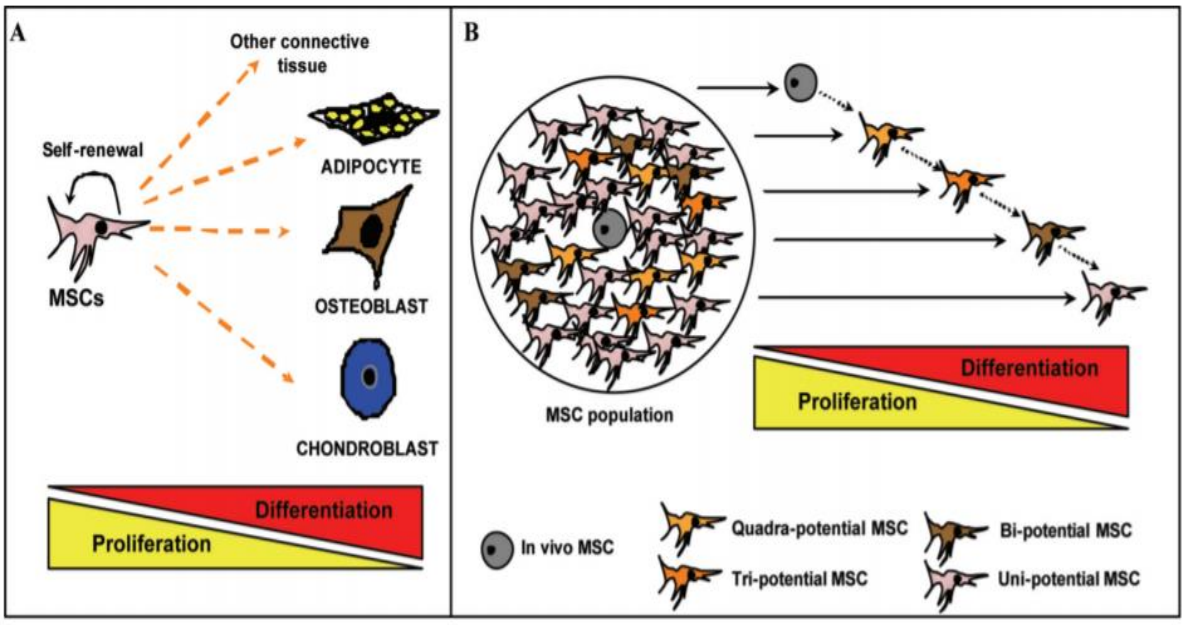


Figure 9: MSCs potential of multipotent differentiation (Baksh et al., 2004): Mesenchymal stem cells (MSCs) have the potential to differentiate into all connective tissue which includes cartilage, tendon, bone, muscle, bone marrow, fat and dermis. Additionally, MSCs have the capacity to self-renew.

1.3.2 MSCs in tumor biology – in general and regarding their role in tumor tropism and angiogenesis and as progenitor cells to form tumor stroma

MSCs are part of the body's response to tissue damage. They are strongly attracted to damaged tissues such as chronic wounds or tumors. Since tumors are basically seen by the body as chronic wounds, they attract MSCs to the tumor environment. This phenomenon is also called tumor tropism and is thought to involve diverse signaling molecules and pathways (Ho & Lam, 2013). Much research, including work from our group has been performed to better understand the underlying mechanisms driving recruitment. Signaling molecules involved and pathways activated in this context are thought to be linked to hypoxia, inflammation and necrosis (Studeny et al., 2002).

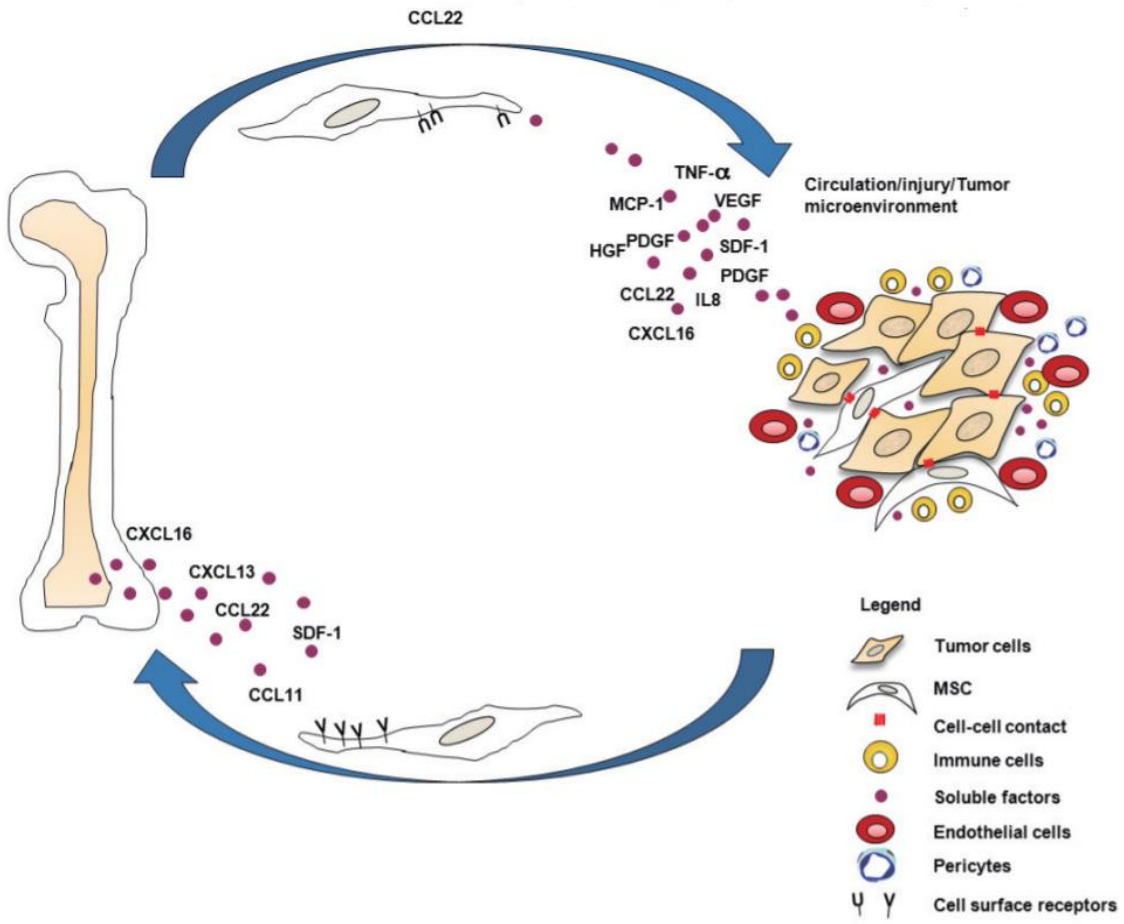


Figure 10: Tumor tropism of MSCs (Ho & Lam, 2013): Divers molecules as integrins, cytokines, chemokines, growth factors and adhesion molecules can attract MSCs to the tumor. Those molecules are molecules which get activated in hypoxic and inflammatory milieu.

Molecules such as integrins, cytokines, chemokines and adhesion molecules act in concert to attract MSCs to the tumor. Some important signaling molecules are linked to the hypoxia response network as tumors can lack oxygen due to their fast growth requiring neoangiogenesis for survival of the tumor. Important hypoxia-regulated genes studied in this thesis are Hif-1α, VEGF and Tie2. Hif-1α becomes stabilized and then functions as a transcriptional factor (Hif-1α) while VEGF becomes secreted and the angiopotien 1/Tie2 receptor is expressed in the hypoxic milieu of the tumor environment. (Ho & Lam, 2013) The production of VEGF has been shown to lead to tumor homing of MSCs. (Kerbel, 2008) Hif-1α can also influence MSC tumor tropism through the activation of hepatocyte growth factor (HGF), tumor necrosis factor (TNF-α) and platelet-derived growth factor (PDGF) in tumor stroma cells resulting in enhanced homing of the MSCs to the tumor. (Rosová, Dao, Capoccia, Link, & Nolta, 2008) Many molecules become activated as a result of the hypoxic inflammatory environment of the tumor. TNF-α, interleukin (IL)-6and the monocyte chemoattractant protein (MCP)-1 are a few examples of important cytokines expressed within the tumor environment. Those factors can work in a paracrine fashion enhancing the tumor tropism of MSCs (Ho & Lam, 2013). Figure 10 provides a general overview of what is understood in this regard. The release of the chemokines CCL2 and CCL5 by endothelial cells from the tumor milieu has also been shown to play a role in the tumor tropism of MSCs. (Hämmerling & Ganss, 2006).

After being incorporation into the tumor stroma, MSCs can secret soluble factors such as VEGF, PDGF and EGF that help promote the so-called angiogenetic switch to a highly effective angiogenesis status. (Hämmerling & Ganss, 2006) MSCs interact with endothelial cells and tumor cells and can

promote tumor growth and metastases. (Bronckaers et al., 2014) Bronckaers et al. provided an overview over angiogenic factors of the secretome of MSCs.

Angiogenic factors secreted by cultured MSCs.

Angiogenic factor	Full-name	Function	MSC source
Angiogenin	Angiogenin	Cell migration, invasion, proliferation and tube formation	hBM-MSC
Ang-1	Angiopoietin-1	Vessel stabilization, EC survival, recruitment of pericytes	mBM-MSC
Ang-2	Angiopoietin-2	EC migration and sprouting	mBM-MSC
Cyr61	Cysteine-rich 61	Cell adhesion and EC migration	mBM-MSC
FGF-2	Fibroblast growth factor-2	EC proliferation, migration, remodeling of the extracellular matrix	mBM-MSC
HGF	Hepatocyte growth factor	EC and SMC proliferation, migration	rBM-MSC hAD-MSC
IGF-1	Insulin-like-growth factor-1	EC proliferation, survival, induction VEGF and plasminogen activators	rBM-MSC
IL6	Interleukin-6	EC proliferation and migration	hBM-MSC hPl-MSC
IL8	Interleukin-8	EC proliferation, survival, migration and tube formation	hBM-MSC
MCP-1	Monocyte chemoattractant protein-1	EC migration	mBM-MSC hBM-MSC
MIG	Monokine induced by interferon-gamma	Chemoattractant of T-cells, inhibition of angiogenesis	mBM-MSC
MIP-1α	Macrophage inflammatory protein-1alpha	Proinflammation, chemoattractants of immune cells	mBM-MSC
MIP-1β	Macrophage inflammatory protein-1beta	Proinflammation, chemoattractants of immune cells	mBM-MSC
NAP-2 (CXCL7)	Neutrophil activating protein 2	Neutrophil recruitment, EC migration, release of VEGF and MMP	hBM-MSC
PLGF	Placental growth factor (PLGF)	Induction of vessel formation	mBM-MSC
TGF-beta	Tumor growth factor-beta	Tube formation, vessel stabilization, ECM synthesis	hAD-MSC
TIMP-1	Tissue inhibitor of metalloproteinase-1	Inhibitor of metalloproteinases	hBM-MSC
TIMP-2	Tissue inhibitor of metalloproteinase-2	Inhibitor of metalloproteinases	hBM-MSC
VEGF	Vascular endothelial growth factor	Increase vessel permeability, ECM degradation, EC proliferation, migration, tube formation and survival	mBM-MSC hBM-MSC hDPSC hAD-MSC

Figure 11: Overview angiogenic factors of the secretome of MSCs (Bronckaers et al., 2014)

MSCs can also function as progenitor cells that help form the tumor stroma. MSCs can differentiate into cancer-associated-fibroblasts (CAFs) and pericytes. (Casazza et al., 2014) CAFs function as support for tumor stroma and can also enhance angiogenesis and inflammation. (Chandler, Liu, Buckanovich, & Coffman, 2019; Spaeth et al., 2009) There is contradictory evidence whether MCSs can differentiate into endothelial cells. (Mishra et al., 2008)

1.4 Gene therapy with genetically modified MSCs

Gene therapy was first established at end of the last century. Various approaches have examined for effective gene therapy. (Friedmann & Roblin, 1972; Ginn, Amaya, Alexander, Edelstein, & Abedi, 2018). It has been used to treat inherited genetically diseases and more recently, for targeting tumor disease. Gene therapy is categorized as viral or non-viral. (Ginn et al., 2018) Viral gene therapy uses viral vectors based on retro- or adenoviruses that either integrate their DNA in the human genome or enter the nucleus. Non-viral gene therapies make use of incorporating naked DNA as exosomes, lipososomes, catatonic polymers, next to the one described here, making use of stem cells by using electroporation of the naked DNA into stem cells. Each approach has its advantage and disadvantage. The advantage of using viral gene vehicles is the high incorporation rate and therewith high transgene expression which lead to a high therapeutic effect but on the other hand they have oncogenic and immunogentic potential and high bystander effect of infecting non-target cells.(Bao et al., 2012; Young, Searle, Onion, & Mautner, 2006) The advantages of using non-viral gene vehicles includes a low immunogenic potential, low mutation risk and simplified multiplication and as stated above exemplary for MSCs, high tissue specific targeting and tissue specific expression of the genes.(Ledley, 1994; Li & Huang, 2006) Besides of that, MSCs lack HLAcomplexes, meaning they are not getting affected by the immune system in case you perform gene therapy with MSCs from

another donor. (Rastegar et al., 2010) The tissue specific homing of MSCs can be used for therapeutic strategies. In comparison to transgene products earlier produced in our lab we used a new cloning platform system, Gateway cloning, and transfection system, Sleeping Beauty, to as first step generate more efficient stable transgene products which then can be stable transfected into the stem cells (see results methods 3.3 and results 4.2).

MSCs have been used as a 'Trojan horse'-like approach to deliver genetically modified MSCs deep into tumors. (Bao et al., 2012; Conrad et al., 2007) MSCs as therapeutic vehicles have been used to deliver therapeutic factors, e.g., suicide genes as ganciclovir or theranostic genes such as the NIS symporter into the tumor environment. (Keung, Nelson, & Conrad, 2013; Niess et al., 2011, Müller et al., 2016) To enhance the selectivity of transgene expression, specific gene promoters that become activated in tumor milieu have been used to limit the potential side effects of transgene expression after application of adoptively applied engineered MSCs. (Bao et al., 2012) In this thesis we focused on genetically engineered MSCs with gene promoters important in context of the hypoxia response network; Hif-1α, Tie2 and VEGF.

Our working group is focused largely on use of the NIS symporter in the context of MSC-based tumor therapy. NIS functions as a theranostic gene that allows monitoring of MSC distribution and enables the uptake of therapeutic radionuclides such as ^{131}I that help target the tumor from the inside.(Müller et al., 2016) When NIS is used as a theranostic gene outside the thyroid, patients are treated beforehand with the thyroid hormone T3. This leads to the downregulation of NIS from the thyroid gland thus protecting the organ from radioiodine as well as ensuring higher circulating levels of the nucleotide available for therapy. Thyroid hormones can have an influence on Hif-1α, angiogenesis, and have been shown to influence the emigration of MSCs into tumor environments (paper from Kati). (Davis et al., 2009; Müller, 2017; Müller et al., 2016)

1.5 Thyroid hormones T3 & T4 and their genomic and non-genomic effects in MSCs, tumor hypoxia and angiogenesis

Thyroid hormones 3,5,3'-triiodo-L-thyronine, T3, and L-thyroxine, T4, play a role in cell differentiation, -growth and metabolism. (Köhrle, 2018) Thyroid hormones have an impact on angiogenesis, proliferation and inflammation in tumor formation. (Schmohl et al., 2015) Thyroid hormones act and stimulate different pathways via a genomic pathway through the nuclear thyroid hormone receptor TRα and TRβ, and by a non-genomic pathway acting through the integrin αvβ3 that contains binding sites for T3 and T4. Thyroid hormones are produced in the thyroid gland via a hypothalamic-pituitary-feedback-mechanism with release of thyrotropin-releasing hormone, TRH, through the hypothalamus, and thyroid stimulating hormone, TSH, through the pituitary. The production of T4 is higher than T3; however, after secretion in the blood stream de-iodination of T4 takes place with transformation into T3. T3 was earlier thought to have the higher metabolic impact. This is true for the effect transmitted by the TRα and TRβ signal transduction pathways - the genomic pathways - which play a role in the growth, metabolism and differentiation pathways. Both receptors act as transcriptional factors after binding T3 and initiate the transcriptional cascade through binding on thyroid-hormone-responsive elements (TRE) on the promoter region of genes important for the pathways stated above. With the growing interest in thyroid hormones regarding cancer metabolism, there is now evidence suggesting that T4 primarily activates the non-genomic pathway through the integrin αvβ3 receptor. After binding thyroid hormones, αvβ3 can activate the PI3K, MAPK/ERK1/2 and STAT1 pathways. These pathways play a role in cancer metabolism and can help drive angiogenesis. In this regard, we have shown that thyroid hormones can lead to angiogenesis by

activation of the S1 side of αvβ3 and thereafter PI3K pathways which lead to stabilization of the transcriptional factor Hif-1α. (P. Davis et al., 2009; Lin et al., 2009; Schmohl et al., 2015) After binding of the S2 side, thyroid hormones can activate the ERK1/2 (MAPK) pathway which can lead to transcription of pro-angiogenic factors like VEGF and to cell proliferation.(Pinto, Soares, & Ribatti, 2011, see Figure 12). An interaction with Tie2 has not be shown so far.

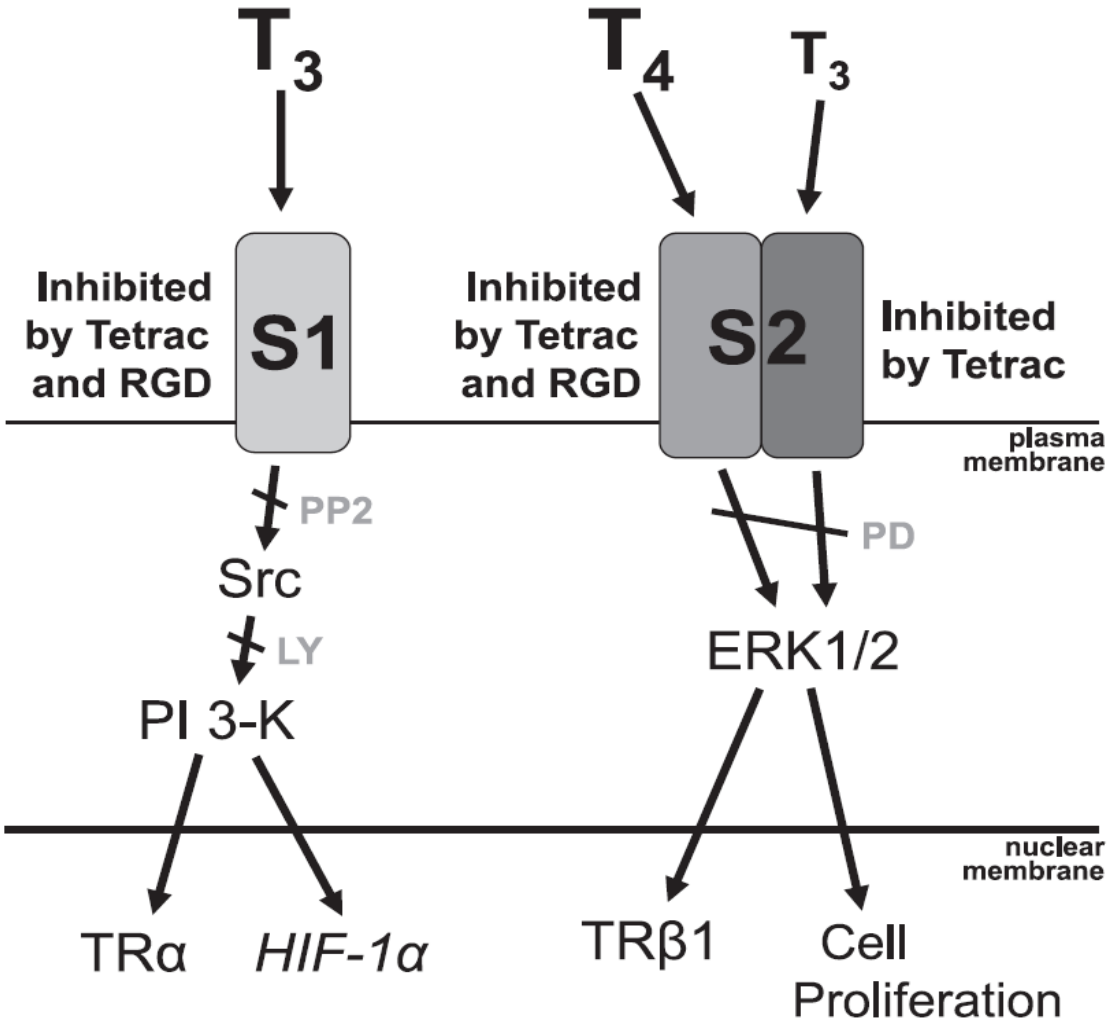


Figure 12: Thyroid hormone signaling mediated through αvβ3 (Davis et al., 2009): After binding thyroid hormones αvβ3 integrin can activate the PI3K, ERK1/2 and STAT1 pathway.

Integrins are primarily known for their role in cell-cell interactions and more recently in transducing signaling cascades. (Bergh et al., 2005) Integrin αvβ3 has been studied with regards to its role in cancer metabolism through the activation of regulatory pathways and in hypoxia and angiogenesis after binding of T3 and T4. (Schmohl et al., 2015) These pathways can be blocked by tetrac, a deaminated antagonistic T4 derivate. (Schmohl, Mueller, et al., 2019) Integrin αvβ3 is found on activated, but not quiescent endothelial, tumor and tumor stroma cell as well as mesenchymal stem cells. (Cai & Chen, 2006; Schmohl et al., 2015; see own results 4.4.1.) In addition to an impact on tumor hypoxia and angiogenesis network activation of αvβ3 by thyroid hormone can also lead to tumor cell proliferation. (P. J. Davis, Leonard, Lin, Leinung, & Mousa, 2018)

1.6 Aims of this study

Thyroid hormones play a role in cancer progression. Potential effects of thyroid hormones include direct activation of tumor cells as well as modulation of tumor vessel growth through a stimulation of neoangiogenesis. While most thyroid hormone effects were thought to be mediated through their binding to nuclear receptors with subsequent downstream effects on gene transcription, the recent identification of thyroid hormone binding sites on the integrin αvβ3 have expanded the potential mode of action of these hormones. The αvβ3 integrin has been shown to be expressed on tumor cells suggesting a potential mechanism for direct activation of tumor cells, and it is also expressed by cells important to the growth of new blood vessels including mesenchymal stem cells (MSCs) that are actively recruited to the tumor environment. MSCs have been shown to help foster angiogenesis through their contribution to the fibrovascular network of growing tumors, either way as CAFs or by transforming into endothelial cells. Because of their expression of αvβ3, it was proposed that thyroid hormones may influence tumor angiogenesis in part through activation of MSCs.

The goals include: **(1)** Testing the effects of the thyroid hormones thyroxine (T4) and triiodothyronine (T3) in *in vitro* models of angiogenesis. This includes potential effects on the biology of the MSC response to tumor environment-derived signals, specifically, the response of these cells to hypoxia may be modulated by the presence of T3 or T4. **(2)** Study the MSC-response to hypoxia and angiogenesis using a vector platform established for the efficient engineering of primary human MSCs with synthetic hypoxia -responsive gene (Hif-1α) promoter, VEGF promoter and Tie2 promoter driving expression of a secreted version of *Gaussia* luciferase. (See results in 4.2, 4.3 and 4.5). **(3)** Evaluate the potential effect of thyroid hormones on the response of these cells to experimental hypoxia using the resultant engineered MSCs in the angiogenesis assay. (See results in 4.5)

2 Materials

2.1 Cell culture

2.1.1 Cell lines and primary cells

Cell line	Description	Media	Supplements	Source
hBMSCSAp172.1 (MSCS)	Primary mesenchymal stem cells (derived from human bone marrow of 37-year old man)	DMEM	-10% FCS -1% PS -5% platelet concentrate -2IU Natrium-Heparin/ml	Deutsche Knochenmarkspende Bayern (DKB), Gauting Apceth GmbH & Co
HUVEC	Endothelial cells	Endothelial cell growth media	-0,02ml/ml FCS -0,004ml/ml Endothelial Cell Growth Supplement -0.1ng/ml Epidermal Growth Factor -1ng/ml Basic Fibroblast Growth Factor -90µg/ml Heparin - 1µg/ml Hydrocortison	Promocell
HUH7	Human hepatocellular carcinoma cells (derived from a 57-year-old man)	DMEM/HAM-F12 (1:1)	-10% FCS -1% PS	C. Spitzweg JCRB Cell Bank, Osaka, Japan

2.1.2 Cell culture media

Media	Manufacturer
DMEM	Gibco, Thermo Fisher Scientific, Waltham, USA
RPMI 1640	Gibco, Thermo Fisher Scientific, Waltham, USA
HAM-F12	Life Technology, Carlsbad
OptiMEM	Life Technology, Carlsbad
Endothelial cell growth medium	Promocell

2.1.3 Cell culture supplements and chemicals

Supplements/ chemicals	Manufacturer
3,3', 5,5'-Tetraiodothyroacetic acid Tetrac	Sigma-Aldrich, St. Louis, USA
3,3', 5,5'-Triiodo-L-Thyronin-sodium acid (T3)	Sigma-Aldrich, St. Louis, USA
7-Aminoactinomycin D (7-AAD)	Sigma-Aldrich, St. Louis, Germany
Blasticidin	Invitrogen, San Diego, USA
Bovine serum album (BSA)	Invitrogen, Carlsbad, Germany
Cobalt (II) Chlorid Hexahydrate (CoCl2)	Sigma-Aldrich, St. Louis, USA
Dimethyl sulfoxide	Merck, Darmstadt, Germany
Doxycyclin Hyclate	Santa Cruz Bioechnology, Dallas, USA
Dulbeccos phosphate buffered saline (DPBS)	Pan-Biotech GmbH, Aidenbach, Germany
Ethylene diamine tetraacetic acid (EDTA)	Sigma-Aldrich, St. Louis, USA
Fetal calf serum	Merck/Biochrom GmbH, Berlin, Germany
Hepes, 1M	Invitrogen, Carlsbad, Germany
Hygromycin B	Roche Diagnostics, Mannheim, Germany
Lipofectamine Transfection Reagent	Thermo Fisher Scientific, Waltham, USA
L-Thyroxin (T4)	Sigma-Aldrich, St. Louis, USA
Natrium-Heparin	Ratiopharm, Ulm
Platelet concentrate	Blood bank, Klinikum Schwabing
Puromycin	Invitrogen, San Diego, USA
Trypsin/EDTA solution (T/E)	Pan-Biotech GmbH, Aidenbach, Germany
Tryptan blue 0,4% solution	Lonza AG, Basel, Switzerland
Matrigel®	Corning®, Amsterdam, Niederlande
Matrigel® Growth Factor Reduced	Corning® , Amsterdam, Niederlande

2.2 Bacteria

2.2.1 Bacterial strains

Strain	Manufacturer
Mach1- T1R	Invitrogen, Thermo Fisher Scientific, Waltham, USA
DB3.1	Invitrogen, Thermo Fisher Scientific, Waltham, USA
DH5α	Addgene, Cambridge, USA

2.2.2 Bacterial media

Medium	Composition/ Manufacturer
Freezing medium per liter	132.3mM KH_2PO_4 (Roth, Karlsruhe, Germany) 21mM Sodium citrate x 2 H_20 3,7mM $MgSo_4$ x7 H_20 (Roth, Karlsruhe, Germany) 68.1mM (NH4)2SO4 (Roth, Karlsruhe, Germany) 459.3mM K_2HPO_4 x 3 H_20 35.2% Glycerol (Roth, Karlsruhe, Germany) H_20
Lysogeny broth (LB)- medium per liter	10g Bacto Tryptone (BD,Franklin Lakes, USA) 10g NaCl (Roth, Karlsruhe, Germany) 5g Yeast Extract (BD,Franklin Lakes, USA)
Lysogeny broth (LB)- plates per liter	10g Bacto Tryptone (BD,Franklin Lakes, USA) 10g NaCl (Roth, Karlsruhe, Germany) 5g Yeast Extract (BD,Franklin Lakes, USA) 15g Bacto Agar (BD,Franklin Lakes, USA)
Super optimal broth with catabolite (SOC Medium) per liter	5g Yeast Extract (BD,Franklin Lakes, USA) 20g Bacto Tryptone (BD,Franklin Lakes, USA) 10mM NaCl (Roth, Karlsruhe, Germany) 2,5mM KCL (Roth, Karlsruhe, Germany) 10mM MgCl (Roth, Karlsruhe, Germany) 10mM $MgSO_4$ (Roth, Karlsruhe, Germany) 20mM Glucose (Roth, Karlsruhe, Germany)

2.2.3 Microbiology solutions

Solution	Composition/ Manufacturer
Ampicillin solution	50mg/ml Ampicillin (Roth, Karlsruhe, Germany) 70% Ethanol (Merck, Darmstadt, Germany) 30% H_20

Kanamycin stock solution	10mg/ml Kanamycin (Roth, Karlsruhe, Germany) 100% H_20
Chloramphenicol stock solution	34mg/ml Chloramphenicol (Serva, Heidelberg, Germany) 100% Ethanol (Merck, Darmstadt, Germany)
Spectinomycin stock solution	100mg/ml Spectinomycin (Merck, Darmstadt, Germany) 50% DMSO (Merck, Darmstadt, Germany) 50% H_20
$CaCl_2$ solution	60mM $CaCl_2$(Merck, Darmstadt, Germany) 15% Glycerol (Roth, Karlsruhe, Germany) 10mM PIPES, pH7 (Merck, Darmstadt, Germany) 65% H_20

2.3 Buffer and solution for molecular biology

Solution	**Composition/Manufacturer**
1 kb DNA ladder	Invitrogen, Carlsbad
Buffer TE (endotoxin-free)	Qiagen, Hilden
DNA restriction enzymes buffer, 10x	NEB, Frankfurt, Germany
dNTP Set (dATP, dGTP dCTP dTTP)	Thermo Fisher Scientific, Germany
Loading buffer for agarose gels, 6x (per liter)	0.25% Bromphnol blue (Roth, Karlsruhe, Germany) 0.25% Xylen-Cyanol FF (Roth, Karlsruhe, Germany) 30% Glycerin (Merck, Darmstadt, Germany) H_20
Phusion HF buffer for phusion polymerase, 5x	NEB, Frankfurt, Germany
Phusion GC buffer for phusion polymerase, 5x	NEB, Frankfurt, Germany
Thermo pol buffer	NEB, Frankfurt, Germany
Tris - Borate- EDTA (TBE) buffer, 5x (per liter)	90mM Tris (Roth, Karlsruhe, Germany) 2mM boric acid (Merck, Darmstadt, Germany) 0.01M EDTA (Roth, Karlsruhe, Germany) pH8
T4 DNA ligase buffer	NEB, Frankfurt, Germany
Antartic phosphatase buffer	NEB, Frankfurt, Germany
Electroporation buffer 1M	5mM (Merck, Darmstadt, Germany) 15mM MgCl (Roth, Karlsruhe, Germany)

	120mM Na_2HO_4/NaH_2PO_4 (Merck, Darmstadt, Germany) pH7.2 (Merck, Darmstadt, Germany) 50mM Mannitol

2.4 Size standards for electrophoresis

Name	**Manufacturer**
1 kb ladder	Thermo Fisher Scientifc, Waltham, USA
2 log ladder	NEB, Iswich, USA

2.5 Antibodies

Name	**Manufacturer**
Anti - mIg1 (Sigma M9264)	Sigma-Aldrich, St. Louis, USA
Anti - αvβ3 (Abcam ab78289)	Abcam, Cambridge, USA
Anti - α-mouse Ig-PE (R0439)	USBio, USA

2.6 Enzymes

Name	**Manufacturer**
DNAse I	Qiagen, Hilden, Germay
RNase A	Roche, Basel, Switzerland
T4 DNA Ligase	NEB, Ipswich, USA
Taq DNA-Polymerase (5U/µl)	NEB, Ipswich, USA
Phusion DNA-Polymerase	NEB, Ipswich, USA
Calf Intestinal Phosphatase	NEB, Ipswich, USA
Antartic Phosphatase	NEB, Ipswich, USA
EcoRI	Roche, Mannheim, Germany
Gateway BP Clonase II	Invitrogen, Carlsbad, Germany
Gateway LR Clonase II Plus	Invitrogen, Carlsbad, Germany
Restriction Enzymes AgeI, BsrGI, BamHI, EcoRI, HindIII-HF, NcoI, PstI, SacII, SalI, XbaI, XhoI, FspI, SapI	NEB, Ipswich, USA

2.7 PCR Primers

Application	Primer sequence (5' to 3')
GatewayKass_fw	TACGAAAGCTTCCACTGCTTACTGGCTTATCG
GatewayKass_rv	TCAGTAAGCTTGGGCCCTCTAGATCAACCAC
GW_Kassette_fw_v2_HindIII	TGGGTAAAAGCTTACCGACAATTCTCTGGCTAAC
GW_Kassette_rv_v2_XhoI	GGTCAGACTCGAGAGATCTGCTATGGCAGGGC
GW_Kassette_fw_v3_HindIII	ATCGGTAAAGCTTACAACAAGGCAAGGCTTGAC
GW_Kassette_rv_v3_XhoI	ATCGTTCTCGAGTGAACAAACGACCCAACACC
GW_Kassette_fw_v4_HindIII	AGTGATCCGGAAAGCTTGCTTGACCGACAATTCTCTG
GW_Kassette_rev_v4_XhoI	GATCCATGCTCGAGGGGGATACCCCCTAGAGC
GW_Kassette_rev_v4_HindIII	TGCATGAGAAGCTTGGGGATACCCCCTAGAGC
Emptyclone_fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTTATCGATAGGTACCGAGCTCTT
Emptyclone_rv	GGGGACAACTTTTGTATACAAAGTTGTGCTTTACCAACAGTACC
Hif-1A1A_P402A-P564A-fw-GW	GGGGACAACTTTGTATACAAAAGTTGTATAGGGAGACCCAAGCTTAC
Hif-1A1A_P402A-P564A-rv-GW	GGGGACCACTTTGTACAAGAAAGCTGGGTTGCGAGCTCTAGCTCTAGCATTTAGG
Hif-1A1A_fw_v2	GGGGACAACTTTGTATACAAAAGTTGTAAAGCTAAAGCTTACCATGGCCTACC
Hif-1A1A_rv_v2	GGGGACCACTTTGTACAAGAAAGCTGGGTTGCCTCAGAAGCCATAGAGC
VEGF2.1kb-FW	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGCCCAAGCTACCATGATAAG
VEGF2.1kb-RV	GGGGACAACTTTTGTATACAAAGTTGTAACAGTACCGGAATGCCAA

2.8 Plasmid and vectors

Plasmid	Background/Comments	Antibiotic resistance gene	Source	Application
pcDNA6.2PLITR-Blasti-VEGF2.1Pro-Cherry	Constructed with 2 Fragment Gateway Reaction out of backbone Gateway Destination Vector pcDNA6.2PLITRBlasti-	Ampicillin Blasticidin	Maike Dohmann/Carsten Jäckel (11.05.2015) Department	-Cherry Reporter vector with VEGF promoter -Vector can be stable integrated via Sleeping

	Dest and 2 Entry Clones pEntr221-Cherry, pEntr221-VEGF2.1Pro		of Clinical Biochemistry, LMU Munich, AG Nelson	Beauty Cloning Sides
pcDNA6.2PL/ITRBlasti-Dest	Backbone Gateway Destination Vector	Blasticidin	Carsten Jäckel (12.2015), Department of Clinical Biochemistry, LMU Munich, AG Nelson	-Gateway Destination Vector -used to build
pcDNA6.2ITR-Blasti-VEGF2.1Pro-Gaussia Luc	Constructed with 2 Fragment Gateway Reaction out of backbone Gateway Destination Vector pcDNA6.2PLITRBlasti-Dest and 2 Entry Clones pEntr221-Gaussia Luc, pEntr221-VEGF2.1Pro	Ampicillin Blasticidin	Maike Dohmann/ Carsten Jäckel (11.05.2015) Department of Clinical Biochemistry, LMU Munich, AG Nelson	-Gaussia Luciferase Reporter vector with VEGF promoter -Vector can be stable integrated via Sleeping Beauty Cloning Sides
pcDNA6.2ITR-Blasti-VEGF2.1Pro-sGFP	Constructed with 2 Fragment Gateway Reaction out of backbone Gateway Destination Vector pcDNA6.2PLITRBlasti-Dest and 2 Entry Clones pEntr221-sGFP, pEntr221-VEGF2.1Pro	Ampicillin Blasticidin	Maike Dohmann/ Carsten Jäckel (10.08.2015), Department of Clinical Biochemistry, LMU Munich, AG Nelson	-sGFP Reporter vector with VEGF promoter -Vector can be stable integrated via Sleeping Beauty Cloning Sides
pcDNA6.2ITR-Hygro-VEGF2.1Pro-Cherry	Constructed with 2 Fragment Gateway Reaction out of backbone Gateway Destination Vector pcDNA6.2PLITRHygro-Dest and 2 Entry Clones pEntr221-Cherry, pEntr221-VEGF2.1Pro	Ampicillin Hygromycin	Maike Dohmann/ Carsten Jäckel (11.05.2015) Department of Clinical Biochemistry, LMU Munich, AG Nelson	-Cherry Reporter vector with VEGF promoter -Vector can be stable integrated via Sleeping Beauty Cloning Sides

pcDNA6.2.PL/ITR-Blasti-TIE2-Dest	-Multi-step cloning performed between pSPTg.T2pAXK, pCDNA6TR/ITR and a Gateway-cassette - Gateway cassette was inserted in anti-sense direction -Gateway Dest Vector with Blasticidin/Hygromycin selection and Sleeping Beauty ITRs	Ampicillin Blasticidin	Maike Dohmann/ Carsten Jäckel (17.07.2015) Department of Clinical Biochemistry, LMU Munich, AG Nelson	- Gateway vector for insertion of divers reporter genes under the control of Tie2 promoter and enhancer. - Vector can be stable integrated via Sleeping Beauty Cloning Sides
pcDNA6.2.PL/ITR-Blasti--TIE2-Gaussia	Constructed with 2 Fragment Gateway Reaction out of backbone Gateway Destination Vector pcDNA6.2PLITRBlasti-Tie2 and 2 Entry Clones pEntr221-Gaussia, pEntr221-Emptyclone	Ampicillin Blasticidin	Maike Dohmann/ Carsten Jäckel (10.08.2015) Department of Clinical Biochemistry, LMU Munich, AG Nelson	-Gaussia Reporter vector with Tie2 Promoter/Enhancer, -Vector can be stable integrated via Sleeping Beauty Cloning Sides
pcDNA6.2.PL/ITR-Blasti--TIE2-sGFP	Constructed with 2 Fragment Gateway Reaction out of backbone Gateway Destination Vector pcDNA6.2PLITRBlasti-Tie2 and 2 Entry Clones pEntr221-sGFP, pEntr221-Emptyclone	Ampicillin Blasticidin	Maike Dohmann/ Carsten Jäckel (10.08.2015), Department of Clinical Biochemistry, LMU Munich, AG Nelson	-sGFP Reporter vector with Tie2 Promoter/Enhancer, -Vector can be stable integrated via Sleeping Beauty Cloning Sides
HA-Hif-1A1alpha P402A/P564A-pcDNA3	- Gateway Expression vector -Constitutive active Hif-1A -inducible Tet repressor, Tirex system		Plasmid #18955 Addgene, Cambridge, USA	Gateway Expression vector, transcribes Hif-1A1α protein constitutive
pcDNA6.2PLITR.Hygro.CMV/TO-Dest	Backbone Gateway Destination Vector	Hygromycin	Carsten Jäckel (01.05.2015) Department of Clinical	-Gateway Destination Vector

			Biochemistry, LMU Munich, AG Nelson	
pcDNA6.2PLITR.Hygro.CMV/TO-Hif-1A1A.CA	Constructed with 2 Fragment Gateway Reaction out of backbone Gateway Destination Vector pcDNA6.2PLITR.Hygro. CMV/TO-Dest -Dest and 2 Entry Clones pEntr221-Gaussia, HA-Hif-1A1alpha P402A/P564A-pcDNA3	Ampicillin Hygromycin	Maike Dohmann/ Carsten Jäckel (11.05.2015) ,Department of Clinical Biochemistry, LMU Munich, AG Nelson	-Cherry Reporter vector with VEGF promoter -Vector can be stable integrated via Sleeping Beauty Cloning Sides
pcDNA-Gluc3-Hif-1A(Mini-TK)	-pcDNA-Gluc3 backbone (fusion of pGL3 and pcDNA6TR/ITR) -Insert (Hif-1A-responsive + MiniTK promoter) cut by NotI and BsrGI and ligate with pcDNA-Gluc3 backbone	Ampicillin Blasticidin	Melanie Schmitt Nogueira/ Carsten Jäckel (05.2015), Department of Clinical Biochemistry, LMU Munich, AG Nelson	-Gaussia reporter vector for Hif-1Aactivation driven by Mini Tk promoter? -Vector can be stable integrated via Sleeping Beauty Cloning Sides -Vector stable expresses TET-Repressor
pEntr221-Cherry	-Per Gateway reaktion in pDONR221 P5P2 **-Insert cloned from pCAG-Kosak-Cherry Vektor**	Kanamycin	Anna Hagenhoff (12.2014), Department of Clinical Biochemistry, LMU Munich, AG Nelson	Entry Clone for 2 fragment Gateway (2^{nd} fragment) Cherry CDS
pEntr221-Firefly	-Per Gateway reaktion in pDONR221 P5P2 **-Insert cloned from Luciferase-pCDNA3 (Addgene)**	Kanamycin	Carsten Jäckel (12.2015), Department of Clinical Biochemistry, LMU Munich, AG Nelson	Entry Clone for 2 fragment Gateway (2^{nd} fragment) Firefly Luciferase CDS

pEntr221-Gaussia	-Per Gateway reaktion in pDONR221 P5P2 - **Insert cloned from pcDNA-Gluc3-TGF**	Kanamycin	Anna Hagenhoff (12.2014), Department of Clinical Biochemistry, LMU Munich, AG Nelson	Entry Clone for 2 fragment Gateway (2nd fragment) *Gaussia* Luciferase CDS
pEntr221-Renilla Luc	-Per Gateway reaktion in pDONR221 P5P2 - Insert cloned from pRL-TK	Kanamycin	Carsten Jäckel (12.2015), Department of Clinical Biochemistry, LMU Munich, AG Nelson	Entry Clone for 2 fragment Gateway (2nd fragment) Renilla Luciferase CDS
pEntr221-VEGF2.1 Promoter	-Per Gateway Reaktion in pDONR221 P1P5r **-Insert cloned from VEGF2.1kb-Luc (Addgene)**	Kanamycin	Carsten Jäckel (12.2015), Department of Clinical Biochemistry, LMU Munich, AG Nelson	Entry Clone for 2 Fragment Gateway (1st Fragment) VEGF2.1 kb Promoter
pEntr221-sGFP	-Per Gateway reaktion in pDONR221 P5P2 - **Insert cloned from pEntr221**	Kanamycin	Carsten Jäckel (12.2015), Department of Clinical Biochemistry, LMU Munich, AG Nelson	Entry Clone for 2 fragment Gateway (2nd fragment) sGFP CDS

2.9 Recombinant proteins and pathway modulators/cytokines

Name	**Manufacturer**
VEGF	PeproTech, Hamburg, Germany
3,3', 5,5'-Tetraiodothyroacetic acid Tetrac	Sigma-Aldrich, St. Louis, USA
3,3', 5,5'-Triiodo-L-Thyronin-sodium acid (T3)	Sigma-Aldrich, St. Louis, USA
L- Thyroxin (T4)	Sigma-Aldrich, St. Louis, USA

2.10 Chemicals, (cytokines, stimulants, dyes)

Name	Manufacturer
Acetic acid	Merck, Darmstadt, Germany
Agarose ultrapure	Thermo Fisher Scientific, Waltham, USA
Alizarin-Red	Sigma-Aldrich, St. Louis, USA
Aqua ad injectabilia	Braun, Melsungen, Germany
Bromphenolblue	Sigma Aldrich, Taufkirchen, Germany
Calciumchloride ($CaCl_2$)	Merck, Darmstadt, Germany
Ethanol	Merck, Darmstadt, Germany
Ethidium bromide 1%	Merck, Darmstadt, Germany
Ethylenediaminetetraacetic acid (EDTA)	Merck, Darmstadt, Germany
Glycerin	AppliChem, Darmstadt, Germany
Isopropanol	Merck, Darmstadt, Germany
Magnesiumchloride	Merck, Darmstadt, Germany
Methanol	Merck, Darmstadt, Germany
Oil-Red-O	Sigma-Aldrich, St. Louis, USA
β-Mercaptoethanol	Roth, Karlsruhe, Germany

2.11 Kits

Kit	Manufacturer
BioLux Gaussia Luciferase Assay Kit	NEB, Ipswich, USA
Gateway BP Clonase ll	Thermo Fisher Scientific, Waltham, USA
Gateway LR Clonase ll Plus	Thermo Fisher Scientific, Waltham, USA
Innprep Plasmid Midi Prep	Analytik Jena AG, Jena, Germany
Innprep Plasmid Mini Prep	Analytik Jena AG, Jena, Germany
Lipofectamine ®2000 Transfection Reagent	Thermo Fisher Scientific, Waltham, USA
MultiSite Gateway Pro Plus	Thermo Fisher Scientific, Waltham, USA
Neon Transfection System 10µl Kit	Invitrogen, Carlsbad, Germany
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific, Waltham, USA

Purelink RNA Mini Kit	Thermo Fisher Scientific, Waltham, USA
Qiagen PCR Cleanup Kit	Qiagen, Hilden, Germany
QIAquick Gel Extraction Kit	Qiagen, Hilden, Germany
Taqman Universal PCR Master mix	Applied Biosystems, Darmstadt, Germay
ZymoPURE Plasmid Maxiprep Kit	Zymo Research Corp, Irvine, USA

2.12 Other laboratory equipment

Device	**Manufacturer**
Biofuge pico	ThermoFisher, Waltham, Germany
Co_2 incubator New Brunswick™ Galaxy® 48 R	Eppendorf, Hamburg, Germany
Fluorescence activated cell scanner Facscalibur	BD Bioscience, San Jose, USA
GENios plate reader	Tecan, Männedorf, Switzerland
Inverted fluorescence microscope DMIL	Leica, Wetzler, Germany
L70 Ultracentrifuge	Beckman, Brea, USA
Lumat LB9507	Berthold, Bad Wildbad, Germany
Megafuge 1.0R	ThermoFisher, Waltham, Germany
Nano Drop ND-100 Spectrophotometer	PEQLAB Biotechnology GmbH, Erlangen, Germany
Neon® Transfection System	ThermoFisher, Waltham, Germany
PCR maschine Mastercycler Pro	Eppendorf, Hamburg, Germany
Photo Camera for Leica DMIL	Jenoptik, Jena, Germany
Photometer Lumat 9507	Berthold, Bad Wildbad, Germany
Rotanta 460R	Hettich, Tuttingen, Germany
Thermomixer comfort	Eppendorf, Hamburg, Germany
Vapo.protect thermocycler	Eppendorf, Hamburg, Germany
Multichannel pipette	ThermoFisher, Waltham, Germany

2.13 Consumables

Consumable	Manufacturer
FACS tubes (1,5ml, polypropylen)	BD Bioscience, San Jose, USA
Tubes, 5ml 75x12mm, PS (for luminometer)	Sarstedt, Nürnberg, Germany
PCR tubes Eppendorf 0.2ml	Eppendorf, Hamburg, Germany
µ-Slide Angiogenesis Assay, ibidi	Ibidi, Martinsried, Germany
µ-Plate Angiogenesis 96 well, ibidi	Ibidi, Martinsried, Germany
Cell culture plates (6,12, 24, 96 well plate)	TPP, Trasadingen, Switzerland
Cell culture flask (75/175cm^2)	TPP, Trasadingen, Switzerland
Conical centrifuge tubes 15ml/50ml	BD Bioscience, Franklin Lakes, USA
Eppendorf tubes (0,5/1,5/2ml)	Eppendorf, Hamburg, Germany
Vortexer	Neo-Lab, Munich, Germany
Pipette tips	Eppendorf, Hamburg, Germany

2.14 Software

Software	Manufacturer	Application
ImageJ	National Institute of Health, USA	Analysis of icroscopy pictures in angiogenesis assay
Prism 6	GraphPad Software, La Jolla, USA	Graphic program, statistical tests
Clone Manager	Scientific an Educational Software, Morrisville, USA	PCR promoter design
CellQuest	BDBioscience, San Jose, USA	Facs data analysis
SnapGene Viewer	GSL Biotech LLC, Chicago	Plasmid map design
pDraw32	Kjeld, Olsen	Generation of plasmid maps
ProgRes Capture software	Jenoptik, Jena, Germany	Automatized photography

3 Methods

3.1 Cell culture

3.1.1 General cell culture

All primary cells and cell lines were incubated at 37°C, 21% O_2, 5% CO_2 with 95% air humidity. Sub-culturing of cells was performed in a laminar flow hood under sterile conditions to avoid contamination. The reagents necessarily for sub-culturing of cells were warmed to 37°C in a water bath.

As a first step, adherent cells were rinsed with 1 x PBS to remove all traces of FCS and media to allow EDTA-Trypsin to fully work to detach the cells from culture flasks or plates. Depending on the cell line, the digestion step was performed with 3-5ml EDTA-Trypsin for 3 to 10min. A two times volume of media containing FCS was added to stop the reaction. The suspension was slowly mixed while pipetting up and down and eventually centrifuged in a 15-50ml plastic tube at 200G for 3min. As a last step, the supernatant was removed, and the cell pellet was carefully re-suspended in fresh media. Cells were then used for experiments and/or for further culture.

3.1.2 Freezing and thawing cells

Cells were frozen in 2ml freezing tubes in a 1:1 ratio of their respective media with freezing media. The freezing media contained 55% of media, 35% FCS and 10% DMSO. All steps were performed quickly since DMSO is toxic for cells. The cells were stored in an isopropanol container at -80°C for 24 - 48h before transferring to a liquid nitrogen storage at -196°C for long-term storage. For thawing cells, the freezing tubes were placed in a 37°C water bath and immediately transferred into a cell culture vessel with pre-warmed media plus FSC to reduce the toxic effects of DMSO. Media was replaced after 12-18h.

3.1.3 Counting cells

Cells were counted using a Neubauer Counting chamber. Cell suspensions were pre-diluted to approximately 10^6/ml if necessary and mixed in a 1:3 - 1:5 ratio with Tryptan Blue to stain for dead cells to distinguish them from viable cells. A drop of this suspension was placed on the Neubauer chamber mounted with a cover slip to fill the chamber by capillary force. Cells were counted under a microscope in all four quadrants. The cell count was calculated taking the dilution factor in consideration, calculating the mean cell amount of all four quadrants and multiplying this number with 10^4. Cells /ml = Cell mean quadrant x 10^4 x dilutorfactor predilution x dilutionfactor TB

3.1.4 Cultivation of HUVEC

HUVECS used in this study were obtained from the company Promocell. The cells were cultured in dedicated endothelial cell growth media, enriched with endothelial cell growth supplement (0,004ml/ml), Epidermal Growth Factor (0.1ng/ml), Basic Fibroblast Growth Factor (1ng/ml), Heparin (90µg/ml), Hydrocortison (1µg/ml) as obtained from Promocell. The cells were used in the angiogenesis assay until 3th till 8th passage. Medium was renewed every second day.

3.1.5 Cultivation of MSCS

MSCs used in this study were hbMSCSAp172_1 donated by Acpeth GmbH & Co. The cells were cultured in DMEM, 10%FCS, 1%PS, 5% platelet concentrate and 2IU/ml of Sodium-Heparin. The cells were used until 5th till 8th passage. Medium was renewed every second day.

3.1.6 Cultivation of HUH7 and cultivation of HUH7 medium

To produce HUH7 conditioned medium, 2,5×10^6 HUH7 tumour cells were plated into 15cm2 culture flasks in 30ml DMEM +hamF12 (ratio 1:1) medium with 10% sFCS and 1% P/S. After 48h, the

conditioned medium was removed, centrifuged at 330xg for 5 min to remove cell debris and stored at -20°C.

3.2 Molecular biology

3.2.1 Freezing and thawing of bacteria

For long-term storage of E.coli, the bacteria were frozen as a bacterial glycerol stock at -80°C. The stocks were made from 900µl of an overnight culture and 100µl freezing solution. Different types of bacteria strains were used, with Mach1 or DB3.1 containing plasmids of interest. To restart cultures from the frozen bacteria, a small amount of sample was spread on an agar plate and incubated at 37°C. After 24h a single colony was picked, and a liquid LB culture was established to start a DNA extraction process.

3.2.2 Preparation of agar plates

Agar plates were made by adding 15g/L agar to prepared LB medium (containing 950ml ionized water, 10g of tryptone, 5g of yeast extract, 10g of NaCl). After autoclaving and adding the required amount of antibiotics at a temperature of approximately 60°C, the liquid agar was poured on to petri dishes and stored in a plastic bag at 4°C. The finale concentrations of antibiotics were Ampicillin 100mg/ml, Kanamycin 50mg/ml and/or Chloramphenicol 25mg/ml.

3.2.3 Test digestion of DNA

To verify the correct sequence of the constructed plasmids, or to cut out fragments of interest, DNA was cut by suitable restriction enzymes. 1µg of DNA was mixed with 1U of restriction enzyme, a suitable buffer and digested at a temperature (37-65°C) for an hour. If larger amounts of DNA was needed higher amounts of restriction enzymes were used but at no more than 10% enzyme in total volume. Possible digestion tests were planed using CloneManager, the correct buffer and temperature were identified with the double digest finder from NEB.

3.2.4 Separation of DNA fragments by electrophoresis

Separation of DNA fragments were performed by agarose gel electrophoresis for diagnostic reasons or for further isolation and preparation. The gel was made out of 50ml 1x TBE Buffer, 4µl ethidium bromide (100ng/ml) and a varied amount of agarose to produce 0,6% to 2% gels depending on the size of the DNA fragment. 0,6% gels were used for fragments larger than 1000bp and 2% gels for fragments smaller 1000bp. After heating the gel, it was poured into an electrophoresis chamber where the restriction digest were loaded into little slots after mixed with a blue loading buffer (6x) in a 5:1 ratio. For interpretation a size standard (2 log ladder, NEB) was loaded as well. The gels were usually run using 0,5x TBE buffer at 170 V. As final steps bands could directly be identified under an UV lamp and photographed.

3.2.5 Gel extraction of DNA fragments

For further preparation of the DNA fragments they were separated via gel electrophoresis and the appropriate bands were cut out under UV light. Gel fragments were extracted using a commercial kit (GIAquick Gel Extraction Kit) using the manufacturer's protocol.

3.2.6 Determination of DNA concentration

DNA concentration was measured by Nanodrop spectrophotometer. As a first step, the Nanodrop was calibrated with 2µl RNAse free water and subsequently 2µl of the DNA solution was measured.

3.2.7 Dephosphorylation of DNA ends

Dephosphorylation of the DNA ends was sometimes necessary to avoid re-ligation of the plasmids after restriction digest led to compatible overhangs on both ends. The 5' DNA ends were

dephosphorylated by incubating the DNA sample with 5U of Antarctic phosphatase per pmol at 37°C for 1h. The suspension was then heat shocked at 65°C for 15min to stop the reaction and purified with the Qiagen PCR Cleanup Kit according to the manufacturer's instruction. The next cloning step could was then performed.

3.2.8 Assembly of synthetic DNA elements for reporter constructs

Short single stranded synthetic DNA elements were ordered by Thermo Fisher to generate mini-inserts (up to 200bp) for reporter plasmids. These Oligos were comprised of two strands created to be compatible for annealing. The designed Oligos contained overhangs which allowed ligation of the insert into the desired backbone.

3.2.9 Ligation of DNA fragments

The ligation of DNA inserts into vector backbones was performed at a 10:1, 3:1 and 0:1 (as negative control) ratio in a total volume of 20µl with 1µl T4 DNA ligase and 2µl T4 DNA ligase buffer for 24h at room temperature.

3.2.10 Isolation and analysis of plasmid DNA from transformed bacteria

For analyzing, and possible further usage of the plasmid DNA, it was isolated from transformed bacteria. Depending on the size of the bacteria culture a mini (Innuprep Plasmid Mini Kit, cultures up to 1,5ml) or a maxi (ZymoPURE Plasmid Maxi, cultures up to 100ml) DNA prep was performed to isolate the DNA. All steps were performed according to the manufacturer's instructions. Subsequently, the isolated DNA was analyzed using diagnostic restriction digests and gel electrophoresis.

3.2.11 Preparation and Transformation of competent E.coli. Mach 1 bacteria

E.coli Mach 1 bacteria was made heat competent before being used for propagation and selection of DNA fragments of interest. 4ml from an existing bacterial suspension was mixed with 400ml fresh LB medium and incubated at 37°C and 250rpm. Incubation was stopped when OD_{590} was 0.375. Afterwards, the suspension was distributed into 50ml falcons, incubated for 5min on ice and then centrifuged for 10min at 1600G at 4°C. The supernatant was discharged and the bacterial suspension was resuspended in 10ml of ice-cold $CaCl_2$ solution. This step was repeated twice. As penultimate step the suspension was incubated on ice for 30min and again centrifugation and the supernatant discharged. The bacterial pellet was resuspended in 2ml $CaCl_2$ and subsequently aliquoted in 50µl vials and frozen at 80°C.

For propagation of the DNA fragment of interest, the plasmids were transformed into E.coli Mach1 bacteria. After thawing the bacteria from -80°C 10-100ng DNA of the ligation reaction was mixed with 50µl of the bacteria culture and incubated for 10min on ice. As next step the vial was heat shocked for 45s at 42°C and immediately transferred to ice. The suspension was then mixed with 250µl SOC medium before further incubation at 37°C for 1 hour at 300rpm. Afterwards the transformed bacteria were poured onto an agar plate containing the proper antibiotic selection and incubated over night at 37°C. As last step three to five singe colonies were chosen for a new liquid culture.

3.2.12 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was performed for the amplification of diverse inserts by including the attB recombination sides needed for the Multiside Gateway Cloning to the ends of the primers. The primers were designed using CloneManager and ordered from Invitrogen Life Technology.

The same PCR protocol using the standard reaction mixture was followed (see table1). Only the annealing temperature and elongation time (30s per 1000bp) varied depending on the performed reaction (see table2). PCR products were evaluated via gel electrophoresis.

Standard PCR Mastermix	Amount (per 25µl PCR tube)
dNTPS	4 µl
Fw-primer	1 µl
Rv-primer	1 µl
Phusion HF Reaction Buffer (5x)	5 µl
Phusion High-Fidelity DNA Polymerase	1 µl
Target DNA	1 µl
H_2O	12 µl

Table 1: Standard mixture for PCR

Step	Temperature	Time (Cycles)
Initial Denaturation	98°C	5min (1)
Denaturation	98°C	30s (1)
Annealing	58°C -61°C	30s (30)
Extension	72°C	30s/kbase (1)
Final Extension	72°C	10min (1)

Table 2: PCR settingsSequencing of DNA

Purified DNA samples were sent to GATC Biotech (Konstanz) to verify sequences suing the Sanger methods. Standard primers as well as the primers listed in 2.8 were used.

3.2.13 Stem cell typification

Stem cell characterization was performed with Alizarin Red to detect osteogenic and Red Oil to detect adipogentic differentiation of MSCs.

3.3 Cloning Strategies

Cloning steps were performed using the Gateway Cloning Technology by Invitrogen and supervised by Carsten Jäckel, a former biology PhD student at AG Nelson (Jäckel et al., 2016).

3.1.1. Gateway Cloning Technology

Gateway Cloning is an efficient cloning technology that enables the rapid exchange of DNA fragments between plasmids. It makes use of a natural vector system based on bacteriophage λ which transduces its DNA into the genome of E.coli bacteria by using specific recombination sequences. Those recombination sides were modified by invitrogen and two specific enzyme mixes were produced. BP clonase and LR clonase mix, respectively used for two different steps to subsequently create a final 'Gateway Expression vector' containing divers DNA fragments of interest. (See MultiSite Gateway®Pro user guide by invitrogen for nomenclature.)

A short overview over of the technology using a 1-fragment Gateway reaction is provided below. The first step in Gateway Cloning Technology is to flank the gene of interest with an attB1 and attB2

recombination site at their respective 5' and 3' ends. This step is performed by adding the recombination sites to PCR primers and performing PCR. The PCR product is then mixed with a 'Gateway Donor vector' with matching attP1 and attP2 recombination sides and the BP clonase mix to create a 'Gateway Entry Clone'. After recombination and insertion of the gene of interest the newly formed recombination sides are called attL1 and attL2. The 'Gateway Entry Clone' can now recombine with a 'Gateway Destination vector' that contains matching attR1 and attR2 recombination sides as well as relevant DNA fragments such as gene promoters. The reaction is catalyzed by LR clonase mix; a 'Gateway Expression vector' is made.

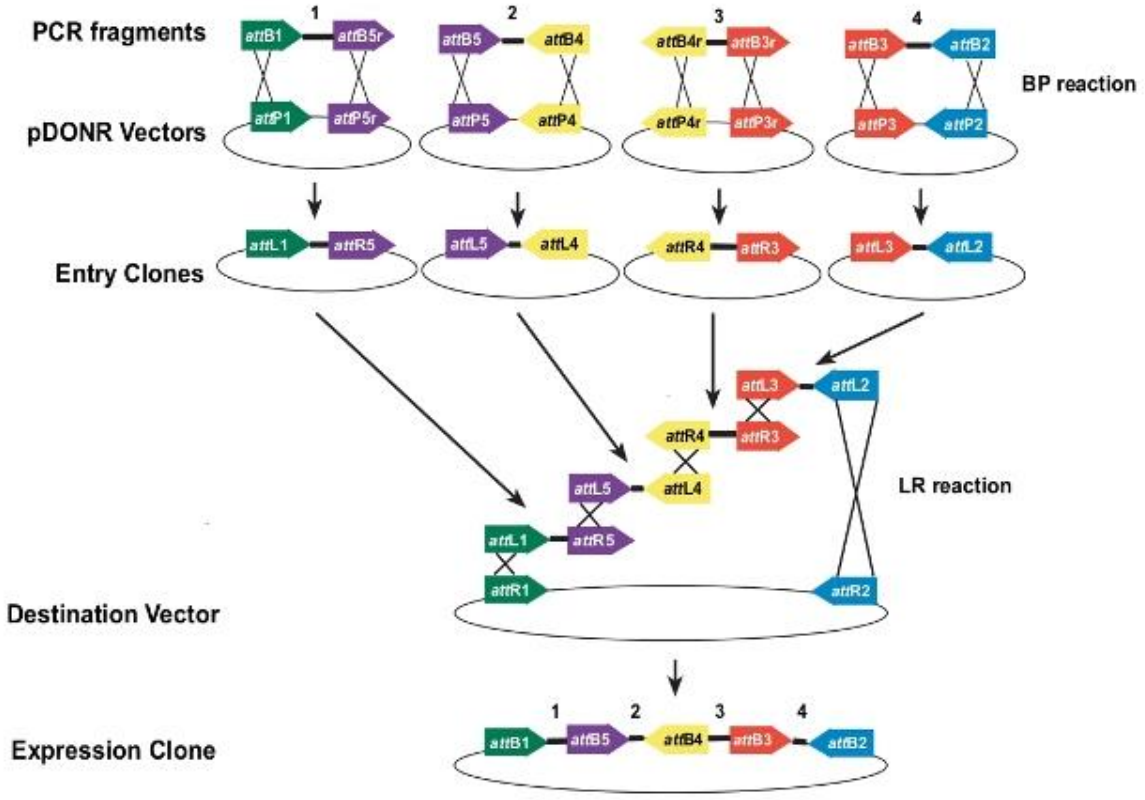


Figure 13: Gateway Cloning Technology in General, (MultiSite Gateway Pro® User Guide)

3.3.1 Production of PCR product with attB1 and attB2 recombination sequences

The first step in the Gateway Cloning Technology is to produce a DNA fragment (PCR template generally ordered from Addgene or DNASU) which contains the gene of interest and the attB recombination sides. For a 1- fragment Gateway Cloning reaction attB1 and attB2 flanks were put on both ends. For a 2 - fragment Gateway Cloning reaction the first PCR products were flanked by attB1 and attB5r sides and the second fragment with attB5 and attB2 sides. This step was performed by adding the recombination sides to PCR primers and consequential do a PCR. Afterwards the PCR product must be purified with gel electrophoresis and gel extraction.

3.3.1.1 BP Reaction to create an Entry Clone

The PCR product (15- 150ng, respectively 1- 7µl) was then mixed with a 'Gateway Donor vector' pDONR221 (150ng/µl) with matching attP1 and attP2 recombination sides (1-fragment Gateway Cloning reaction) or attP1 and attP5 recombination sides flanking the first fragment and attP5r attP2 recombination sides flanking the second fragment (2-fragment Gateway Cloning reaction). To this suspension 2µl BP clonase mix as well as 1-6µl (up to total volume of 10 µl) TE Buffer were added to

create a ‘Gateway Entry Clone’. The reaction was incubated at 25°C for 1h and stopped afterwards by adding 1µl of 2 µg/µl Proteinase K and incubation at 37°C for 10min. 2 µl of this solution was transformed into E.coli Mach1 bacteria and screened for a kanamycin-resistant ‘Gateway Entry Clone’. Two to three of those clones were verified through restriction enzymes digestion, gel electrophoresis and Sanger sequencing.
After recombination, and insertion of the gene of interest, the new formed recombination sides in the ‘Gateway Entry Clone’ are called attL1 and attL2 for a 1-fragment Gateway reaction and attL1 and attR5 for the first vector and attL5 and attL2 for the second vector in a 2- fragment reaction.

3.3.1.2 LR Reaction to create an Expression Clone

As a next step, one to four ‘Gateway Entry Clone(s) (supercoiled, 10fmoles each, not exceeding 7µl in total) were recombined with 1µl of a ‘Gateway Destination vector’ (supercoiled, 20fmoles) containing matching attR1 and attR2 recombination sides regardless of performing a 1 or 2-fragment Gateway Cloning reaction, to create an ‘Gateway Expression vector’. The reaction was catalyzed by 2µl LR clonase mix and 1-6 µl (up to total volume of 10 µl) TE Buffer at 25°C for 16h and stopped by adding 1 µl of 2 µg/µl Proteinase K and incubation at 37°C for 10min. 2 µl of the suspension was transformed into E.coli Mach1 bacteria but this time contrary to the BP reaction it was selected for an ampicillin-resistant ‘Gateway Expression Clone’.
In the scope of this thesis, mainly 2-fragment Gateway Cloning was performed. For this reaction two kinds of ‘Gateway Entry Clones’ were used, one for example coding for a promoter and the other one for a coding DNA sequence (CDS) and one kind of ‘Gateway Destination vector’. Only pSBDEST vector variants were used as ‘Gateway Destination vector’ (Jäckel et al., 2016). Those vectors were built by fusion of the commercially available pcDNA6.2V5Pl-Dest vector, already incorporating the Gateway cassette, and the pcDNA6/TR/ITR plasmid. The last vector was built in our lab after incorporating Sleeping Beauty compatible ITRs/DRs sides into the commercially available pcDNA6/TR vector which originally expressed a blasticidine selection and a Tet-repressor. Last one was cut out after ligation with the pcDNA6.2V5Pl-Dest vector. Blasticidine in the pSBDEST vector, then called pSBDEST.B, could be exchanged in regard of a different question by hygromycine, then called pSBDEST.H for a higher selection variety.

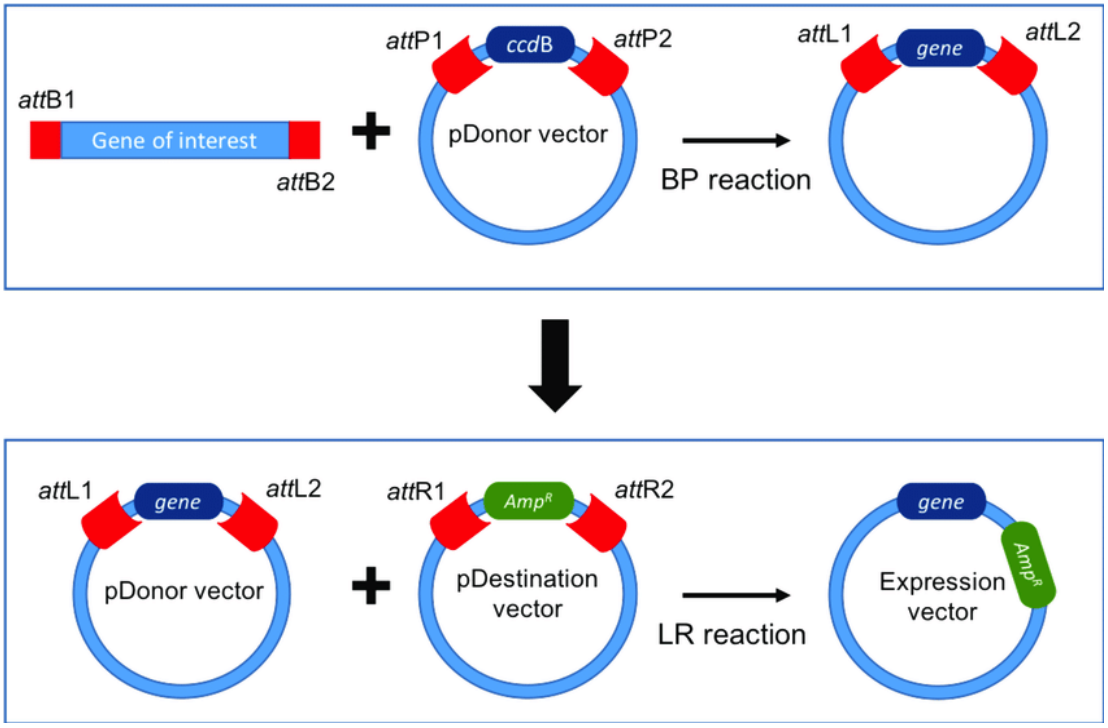


Figure 14: Gateway Cloning Technology (Gamper, 2019)

3.3.2 Tetracycline-controlled transcriptional activation

Some experiments were performed making use of doxycycline (a tetracycline derivate) inducible gene expression. Two different vectors, one reporter plasmid and one expression vector were co-transfected into the desired cells. The first plasmid transcribed a tetracycline- repressor gene, a *Gaussia*-luciferase reporter construct and a blasticidine selection cassette. The second plasmid contains the respective doxycycline inducible gene. Adding doxycycline to the respective cell culture, leads to bind to the tet-repressor protein which blocking expression of the gene of interest driven by the CMVTO promoter.

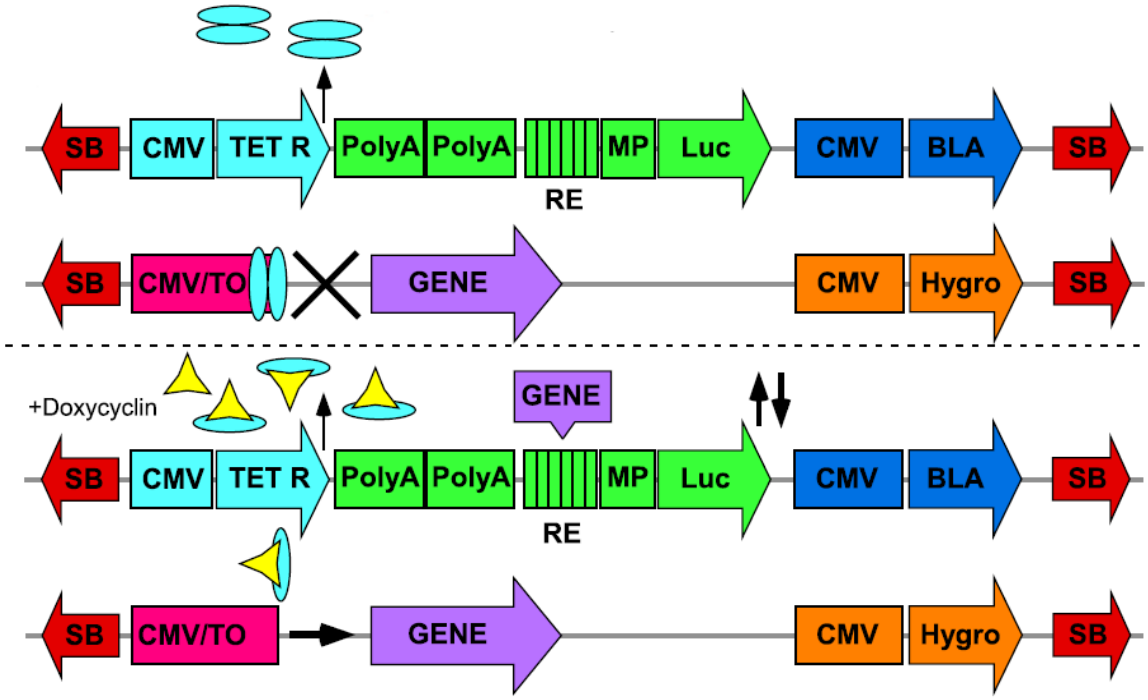


Figure 15: Tetracycline- controlled transcriptional activation (Jäckel et al., 2016)

3.3.3 Generation of Reporter plasmids

Different sets of reporter plasmids (pSBTET.Reporter) were generated by fusion of the genetically altered commercially available pGL3 –promoter vector and the pcDNA6/ITR vector (see 3.3.2.). The pGL3- vector was used as is, or it was modified by replacement of the *Firefly* Luciferase with *Gaussia* Luciferase as well as the exchange of a Simian virus 40 (SV 40) promoter with a CMV Min promoter to reduce background activity, performed by C. Jäckel. After replacement of the *Firefly* Luciferase the vector was named pGL3-CMVMin-GLuc. Every pSBTET.reporter plasmid then contained a Tet-repressor, a *Gaussia* luciferase reporter gene, blasticidine for antibiotic selection and a Sleeping Beauty recognition side allowing stable transfection into the desired target cells.

To investigate different pathways, multimers of transcriptional factor binding sites were cloned upstream of the CMV Min promoter in the pSBTET.reporter of the *Gaussia* luciferase reporter gene, using the restriction sites XhoI and PstI.

The *Gaussia* Luciferase is measured in the supernatant after adding a substrate enzymatic catalyzed by the luciferase and producing light quantified by a luminometer.

3.3.3.1 Generation of the Tie2 Destination Vector and Tie2 reporter plasmids

Generation of the Tie2 Destination vector, the pcDNA6ITR-TIE2-*Gaussia* and pcDNA6ITR-TIE2-s*GFP* were performed in multiple steps. All intermediate and final cloning steps were tested by control restriction digests and sequencing by GATC. The activation of the reporter was established in further

experiments was measured by quantification of *Gaussia* luciferase or the fluorescent marker s*GFP* (see in detail in results 4.2.1.3).

3.3.3.2 Generation of the Hif-1α- constitutive active (CA) Expression vector

The generation of the Hif-1α-CA Expression vector was performed in multiple steps using Gateway Cloning Technology. All intermediate and final cloning steps were tested by control restriction digests and sequencing by GATC. The activation ability of the construct was determined in further experiments by quantification of *Gaussia* luciferase (see in detail in results 4.2.1.1.).

3.3.3.3 Generation of the VEGF reporter plasmids

Generation of the VEGF reporter plamids (pcDNA6.2ITR_BLASTI_Vegf2.1kb.*Gaussia*Luc, pcDNA6.2ITR_BLASTI_Vegf2.1kb. *sGFP*, pcDNA6.2ITR_BLASTI_Vegf2.1kb.*Cherry* as and pcDNA6.2ITR_HYGRO_Vegf2.1kb.*Cherry*) were performed in multiple steps using the Gateway Cloning Technology. All intermediate and final cloning steps were tested by control restriction digests and sequencing by GATC. The activation in further experiments was measured by quantification of *Gaussia* luciferase or the fluorescent marker *sGFP*, *Gaussia* and *Cherry* (see in detail in results 4.2.1.2)

3.3.3.4 Control reporter plasmids

pcDNA-Gluc-CMVMin-Control functioned as a negative control vector (Jäckel et al., 2016). For pathway studies, multimers of specific binding sites were cloned upstream of the CMVMin promoter.

3.4 Transient and stable infection of cells

3.4.1 Lipofectamine 2000

For transient transfection the Lipofectamine 2000 reagent was used. Transfection with Lipofectamine makes use of cationic liposomes that incorporate the negatively charged plasmids. As next step the DNA-containing micelles fuses with the cell membrane by endocytosis. Different concentrations of DNA and Lipofectamine reagents were tested. To transient transfect hbMSCS, L87 and HEK293, we used 0.2µl Lipofectamine, 200ng DNA and 25µl Opti-MEM medium per 96-Well plate. As first step 200ng DNA was diluted in 25µl Opti-MEM for 5min at room temperature. After adding 0.2µl Lipofectamine the suspension was incubated for another 20min at room temperature and subsequently added to the 75% confluent cells in 50µl FCS in the 96 Well-plate. Since lipofectamine has a toxic influence on cells, the medium was changed after 6-8h as well as on the next day.

3.4.2 Electroporation

Stable transfection was conducted with electroporation. Electroporation is a method which enables transfection of cell lines by increasing the cell membrane permeability allowing incorporation of DNA molecules into the desired cell line. Electroporation was performed with the NEON transfection system (Thermo Fisher, Waltham (USA)).

As a first step, the roughly 80% confluent cell were detached, centrifuged and counted. The defined number of cells was then again centrifuged for 1min at 7000G. The cell pellet was resuspended in 10µl Buffer R. The plasmids were added, maximum 3µg in total, usually 1µg pCMV(CAT)T7-SB100 and 2µg of the respective plasmid were transfected. Depending on which cell line was used, different NEON electroporation protocols were chosen (see Table 3). 3ml of Buffer E was added to the container. Electroporation was performed with a 10µl volume tip and afterwards transferred into a 25cm^2 flask with 5ml medium. This medium was changed after 24 hours and antibiotic selection started. The choice and amount of antibiotic selection depends on the plasmids and cell line (see Table 4). Antibiotic was used for 10 days (Blasticidin and Zeocin) or 14 days (Hygromycin).

Cell line	Cells per 10 µl Puffer R	Voltage	Time	Pulses
hBMSCS	1x 10^6	1050V	30s	2
HEK293	0.5x 10^6	950V	30s	1
L87 (iMSCS)	0.5x 10^6	900V	30s	1

Table 3: NEON Electroporation Protocol

Cell line	Blasticidin (µl/ml)	Hygromycin (µl/ml)
hBMSCS	2	40
HEK293	8	150
L87 (iMSCS)	6	200

Table 4: Antibiotic concentration for selection after transfection of different cell lines. Stable transfection using Sleeping Beauty transposase

For stable transfection of cells, the Sleeping Beauty transposase system was used. Transposons are DNA segments in the genome that can translocate. The Sleeping Beauty transposon system can integrate the desired DNA fragment after flanking it with Sleeping Beauty recognition sides, inverted terminal repeats (ITRs) recognized by the transposase. The gene of interest is integrated almost randomly into the target genome using ubiquitous TA dinucleotides as incorporation sides. Transfection of the desired cells was performed as described above with co-transfection with the pCMV (CAT)T7-SB100 transposase expression plasmid to drive integration of the gene of interest into the target genome.

3.5 *In vitro* experiments

3.5.1 Stimulation and validation of reporter cell lines

After transfection by electroporation, selection pressure was applied using the appropriate agent(s). A 96-Well format with 20,000 cells/ well was generally used. First, the cells were detached, centrifuged, counted and seeded in either 50µl or 100µl medium depending on the added amount of the selection agent (1µl or 50µl). The selection agent was added 1-2h after seeding. Depending on the experiment, measurement of the respective reporter element was conducted after 24, 48, 72hours.

3.5.2 Overexpression experiments

For overexpression of a transgene we made use of the tet-repressor system described above. Overexpression experiments were performed by adding 1µg/ml doxycycline 2hours after seeding the cells in the 96-well plate. As described in 3.3.2.

3.5.3 Co-culture experiments

For co-culture experiments 5,000-20,0000 reporter cells were seeded in 50µl medium per 96-well plate. After 2 hours and attachment of the reporter cells to the well, 5,000-20,000 of co-culture cells in 50µl of the same medium were added. 24 to 48 hours later reporter activity was measured.

3.5.4 Supernatant experiments

To investigate whether factors produced during cell could influence a pathway of interest, supernatant experiments were performed. Tumor cells (in scope of this thesis HUH7) were grown to 100% confluency in csFCS medium (see 3.5.10.2.) and after washing twice with PBS, the growth media was allowed to condition the media for 72 hours. The supernatant was then harvested, centrifuged twice at 350G for 5min, frozen and stored at -20°C. Concentrated supernatant was harvested with Amicon Ultra-15 10kDA Spinfilters and centrifugated for 20min at 2000G. Depending on the experiment, supernatant was concentrated 1 - 10x. Supernatant experiments were conducted in a 96-well plate. Cells were seeded in their normal medium. After 24hours medium was changed to 100µl conditioned supernatant and subsequently *Gaussia* Luciferase was measured after 24-48 hours.

3.5.5 *Gaussia* Luciferase Assay

Gaussia luciferase was generally used as the reporter gene to investigate promoter or transcriptional using a *Gaussia* Luciferase Assay. 20µl cell culture supernatant was mixed with 50µl buffer/substrate solution, which was prepared out of 50µl buffer, 8µl stabilisator and 0.5µl coelenterazine substrate. *Gaussia* Luciferase catalyzes the oxidation of coelenterazine to coelenteramide which produces light and can be quantified after 45s incubation via a luminometer.

3.5.6 Dual Light Luciferase Assay (Firefly and *Renilla* Luciferase Assay)

A dual light Luciferase assay was chosen to quantify the activity of the specific reporter systems after normalization of the transfection efficiency by comparing two types of luciferases. *Renilla* Luciferase functioned as normalization of the transfection efficiency and *Firefly* Luciferase as quantification of the reporter activity. The preparation of reagents as well as the needed cell lysis and measurement of the different luciferases were performed as described in the Dual-Luciferase®Reporter Assay Quick Protocol.

3.5.7 Fluorescence microscopy of cells

Fluorescent microscopy was used to measure fluorescent markers as CMFDA and CMTMR. To assess fluorescence an inverted microscope as well as the right fluorescent filter were needed.

3.5.8 Fluorescence activated cell scanning (FACS) analysis

Fluorescent activated cell sorting was used to evaluate surface expression of integrin αvβ3, a non-genomic T3/T4 thyroid receptor, on different cell types used in scope of this thesis. HUVEC, HDBEC, HMECS, HT1080, hBMSCS, V54, L87 and HUH7 were screened for the integrin. IgG functioned as positive control, the cells were incubated without antibody as a negative control. As a first step, 50,000 to 200,000 cells in 100µl were stained with respectively m-Ig1 (SigmaM9269, 20µg/ml), αvβ3 (Abcam ab78289, 20µg/ml) or nothing. After an incubation time of 1h on ice, the cells were washed twice with PBS and centrifuged for 3min at 1600rpm. As a next step, the cells were incubated with the second anti-mouse antibody R0349. Afterwards, the suspension was incubated for 30min on ice and again washed twice with PBS. FACS data were generated as described in the manufacturer's manual of LSR ll-Flow and evaluated with the FlowJo Software.

3.5.9 Angiogenesis Assay

Implementation as well as optimization of the Angiogenesis Assay was performed using the µ-Slide Angiogenesis system by ibidi (Martinsried, Germany) with various factors: Matrigel® or Matrigel® Growth Factor Reduced by Corning (see details in results 4.1.). While implementing the assay, different kind of basal matrixes, cells (HUVECs, hBMSCS and/or HUH7), co-cultures, number of cells and best time of evaluation of the assay were tested. The assessment was conducted with an

inverted microscope and evaluated with ImageJ. Some experiments were performed in collaboration with the workgroup Prof. Spitzweg.
As preparation, the Matrigel® had to be defrosted and incubated on ice (4°C) for 24hours to get the Matrigel® liquid. It was then pipetted onto a 96-well µ-Slide Angiogenesis-plates. While transforming from a liquid phase into gel at 37°C for 1 hour the needed cells (HUVECs, hBMSCS and/or HUH7) were detached, centrifugated and counted and the needed amount diluted in different medium enriched with different stimulants as thyroid hormones, Tetrac or $CoCl_2$ seeded onto the µ-Slide Angiogenesis on top of the Matrigel®. The tube formation was analyzed with Image J plug-in Angiogenesis Analyzer for three different evaluable data as total tube length, number of junction and number of meshes (see Fig.9).

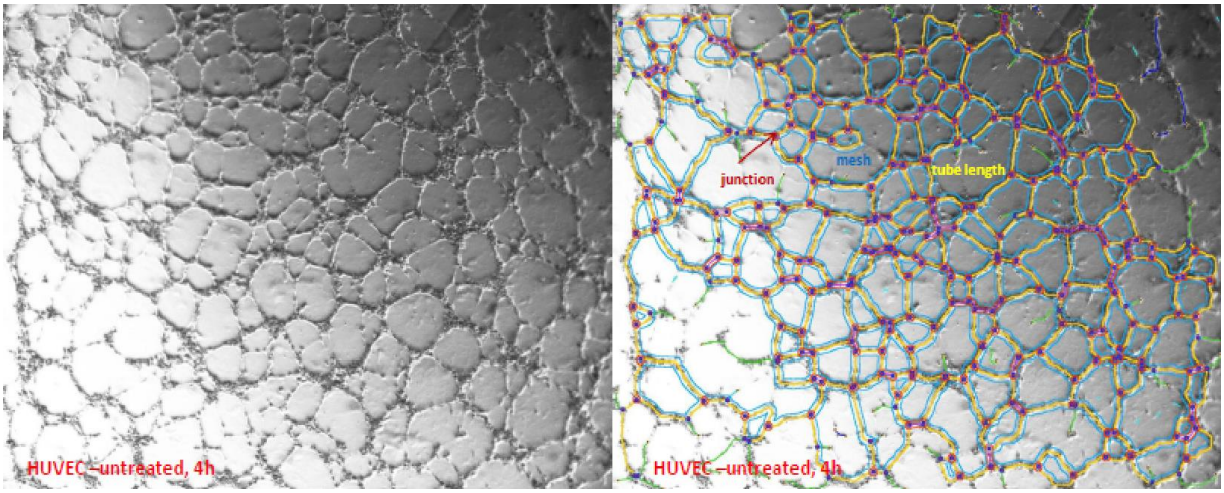


Figure 16: ImageJ plug-in Angiogenesis Assay: Analysis of three different evaluable data: total tube length, number of junction and number of meshes (in collaboration with group of Spitzweg), *Example from an Angiogenesis Assay run on 2015-06-16 (10000 HUVECS, untreated, picture taken after 4h after seeding).*

3.5.10 Generation of hormone reduced FBS (csFBS)

To generate hormone-reduced serum, 1l FBS was incubated with 25g activated carbon covered in 2.5g dextrane and under stirring incubated for 24h at 4°C. Subsequently, the FBS was separated of dextrane-covered activated carbon by filtration and centrifugation. This step was performed twice. As a last step the csFBC was aliquoted and stored at 20°C. Concentration measurements of T3 and T4 in the csFBS was conducted by the Institut für Laboratoriumsmedizin des Klinikums der Universität München. The test showed a T3 concentration of approximately 0.2pg/ml and a T4 concentration of 0.2 – 0.6ng/dl in the depleted FBS.

3.5.11 Generation of conditioned HUH7 medium (cHUH7M)

Conditioned HUH7 medium was generated as described in 3.5.4.

3.5.12 Stimulation of cells with thyroid hormones T3/T4

The thyroid hormones have the following concentrations in the human body: T3 = 1nM (ranges from 1.1-2.9nm in humans) and T4 = 100Nm (ranges from 64-154nm in humans). Commercial acquired T3 and T4 as well as the thyroid hormone inhibitor Tetrac were dissolved into 0,1M NaOH and diluted in normal cell culture medium until a concentration of 25µM for T3 and T4 and 100 µM for Tetrac and stored in aliquots at -20°C. For T3 and T4 stimulation experiments the required cells were incubated 24h prior to the experiment in csFBS medium. Depending on the planned experiment cells were then treated with either csFBS medium and/or cHUH7 medium and 1nM T3 and 100nM T4 with or without 100nM Tetrac. A co-stimulation with 20-400µM $CoCl_2$ was conducted in some experiments.

3.5.13 Stimulation of cells with $CoCl_2$

Cells were stimulated by 50 -200µM of $CoCl_2$ to mimic hypoxic milieu.

3.6 Statistical analysis

Statistical analyses were conducted with Graphpad Prism 6 software or Excel. The statistical significance was marked with asterisk with * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ (plus if needed # = $p<0.05$, ## = $p<0.01$, ### = $p<0.001$) and ns = non-significant. All experiments were performed at least in triplicates. Graphs show mean ± standard deviation. For *Gaussia* Luciferase Assay as well as the Angiogenesis Assay a parametric test, the student's t-test, was used when values were normally distributed.

4 Results

4.1 Implementation and optimization of the Angiogenesis Assay

An important goal of this study was to establish an *in vitro* angiogenesis assay by measuring tube formation.

4.1.1 General conditions

The general conditions required for an *in vitro* angiogenesis assay were evaluated. The assessment was conducted using an inverted microscope and evaluated with ImageJ. Some experiments were performed in collaboration with the work group of Prof. Christine Spitzweg.

In the first sets of experiments, various combinations of factors were tested. Five different materials were evaluated as matrix material. These included: collagen, fibrin, Matrigel®, growth factor reduced Matrigel® and hyaluronic acid as a base matrix for the assay. As a second step, the cells used in the assay were evaluated: endothelial cells (HUVECs), mesenchymal stem cells (hbMSCSap172, in this thesis also called MSCs) and tumor cells (HUH7) were added in two concentrations (10,000 or 30,000) and evaluated as to when tube formation initiated.

Matrix/Gel	Cell amount	Staining	Time	Tube formation
Collagen I	10,000	-	-	Negative (dried up)
Collagen I	10,000	CMFDA	-	Negative (dried up)
Fibrin	10,000	-	-	Negative (dried up)
Fibrin	10,000	CMFDA	-	Negative (dried up)
Matrigel®	10,000	-	1h	**Positive**
Matrigel®	10,000	CMFDA	1h	**Positive**
Matrigel GFR®	10,000	-	1h	**Positive**
Matrigel GFR®	30,000	-	1h	**Positive** (but too many cells)
Hyaluronic acid	10,000	-	-	Not evaluable

Table 5: HUVECs, General conditions used to evaluate the angiogenesis assay, optimization of matrix material used:described in more detail and described in Figure 16.

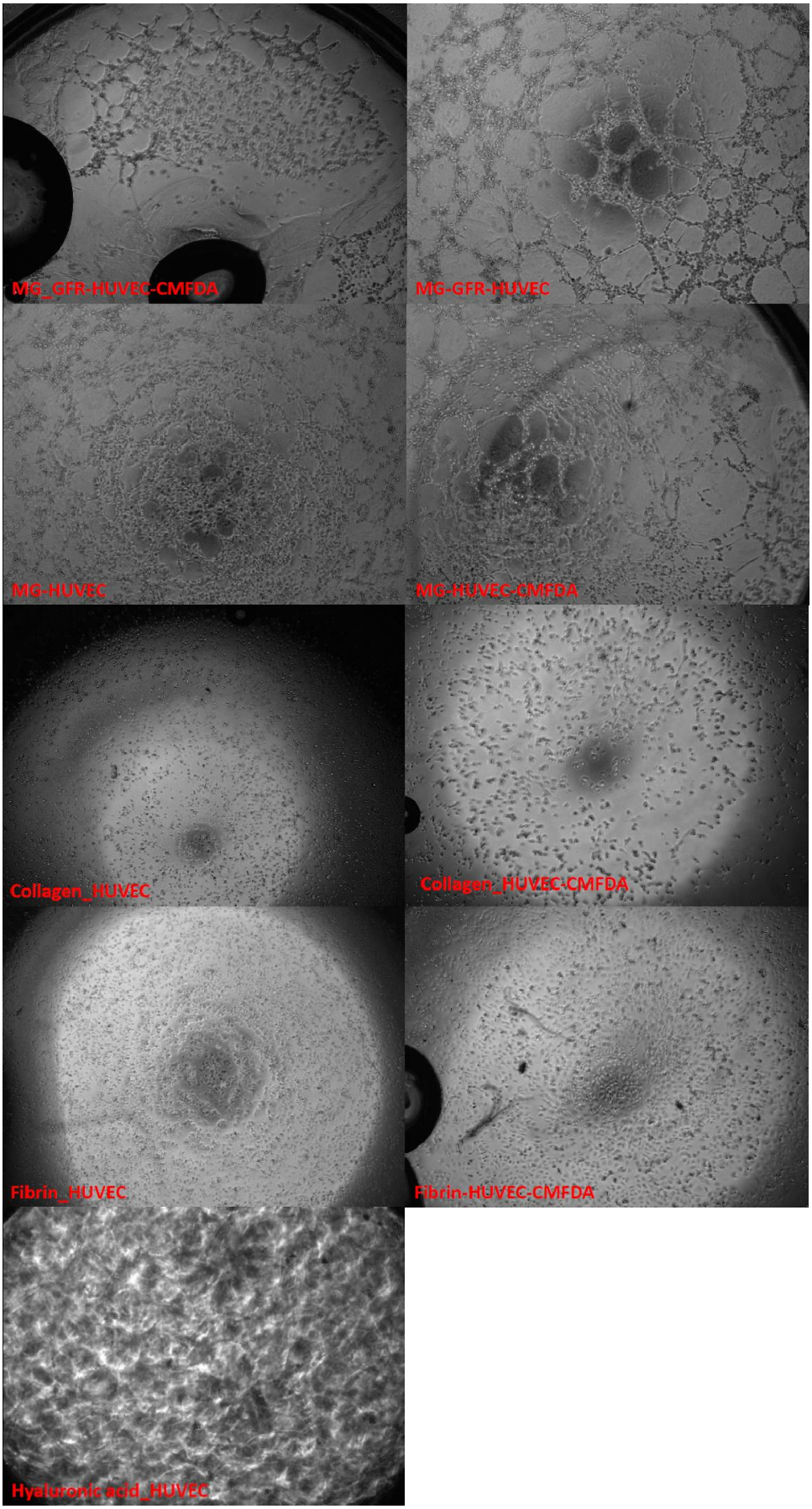


Figure 17: HUVEC General conditions, Tube formation on different matrixes: Tube formation was analyzed with primary human umbilical vein endothelial cells (HUVECs) on five different matrixes (Matrigel®, Matrigel®- Growth Factor reduced,

Collagen, Fibrin, Hyaluronic acid). As described in Table 5, tubes were formed on Matrigel® and Matrigel®- growth factor reduced regardless of staining with CMFDA. 10,000 cells were found to be a good amount. 30,000 cells (tested for Matrigel GFR® only) were considered too many. Pictures were taken after 1h. No tube formation could be seen on Collagen1, Fibrin (both dried out) and Hyaluronic acid (harden up).

Tube formation was first assessed by growing HUVECs on five different matrixes. Tube formation was only seen on Matrigel® and Matrigel® Growth-Factor Reduced after 1h. No difference was seen with staining with CMFDA. No tube formation was seen on Collagen I, Fibrin or Hyaluronic acid. Collagen l and Fibrin quickly dried out while hyaluronic acid hardened not allowing evaluation. As tube formation for Matrigel® GFR worked well, a higher number of cells, 30,000 cells, was tested that did not lead to an evaluable result.

The angiogenesis conditions were then tested using hbMSCs. As hyaluronic acid was not evaluatable in the first experiment, it was not retested. Positive tube formation was again seen on Matrigel® and Matrigel® GFR after 1h, as well as on Collagen l after 3h, but not on Fibrin. Tube formation showed best results for 10,000 cells.

Matrix/Gel	Cell amount	Time	Tubeformation
Collagen l	10,000	3h	**Positive**
Collagen l	30,000	3h	**Positive**
Fibrin	10,000	-	Negative
Fibrin	30,000	-	Negative
Matrigel®	10,000	1h	**Positive**
Matrigel®	30,000	1h	**Positive (too many cells)**
Matrigel GFR®	10,000	1h	**Positive**
Matrigel GFR®	30,000	1h	**Positive (too many cells)**

Table 6: hbMSCSap172, General conditions evaluated for the angiogenesis assay, Tube formation on different matrixes: Analyzed with help of microscopic picture, see in more detail and described in Figure 18.

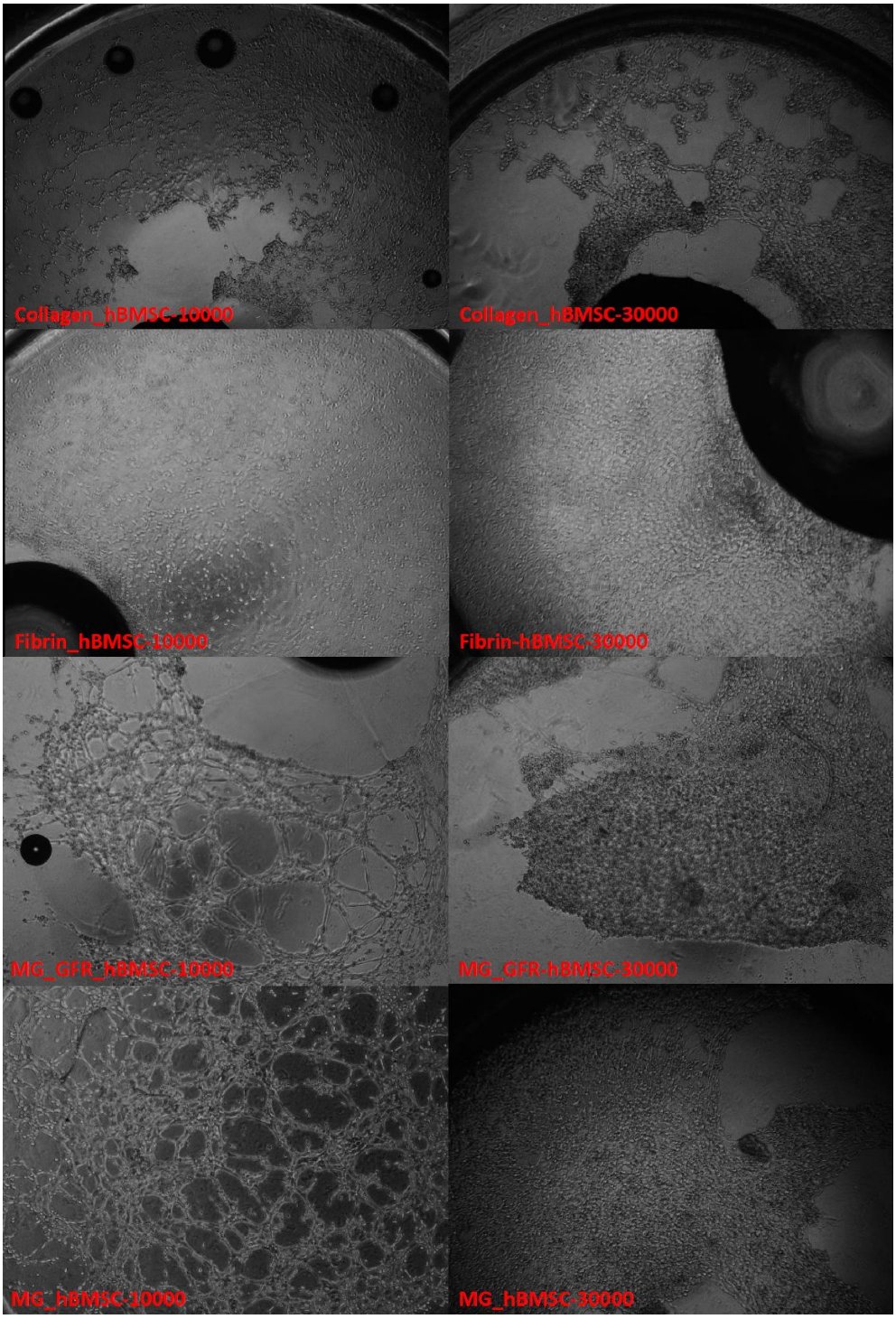


Figure 18: hBMSCSap127 General condition: Tube formation on different matrixes: Conditions for tube formation for hbMSCS were performed as described for HUVECS. Tube formation was seen on Matrigel®, Matrigel®- growth factor reduced after 1h and this time as well for Collagen in a minor extent than for both Matrigels after 3h. 10,000 cells showed good results, 30,000 cells were found to be too high to assess effects.

Pseudoangiogenesis is seen in some tumor settings. To determine if the tumor cells themselves can form tubes, an angiogenesis assay was performed with HUH7 cells. Tube formation was negative for the tumor cells tested (HUH7). No tube formation could be seen on Collagen I and Fibrin.

Matrix/Gel	Cell amount	Time	Tubeformation
Collagen I	10,000	-	Negative
Collagen I	30,000	-	Negative
Fibrin	10,000	-	Negative
Fibrin	30,000	-	Negative
Matrigel®	10,000	2h	**Arrangement**
Matrigel®	30,000	-	**Arrangement (too many cells)**
Matrigel GFR®	10,000	2h	**Arrangement**
Matrigel GFR®	30,000	-	**Arrangement (too many cells)**

Table 7: HUH7 General condition: Tube formation on different matrixes: Analyzed with help of microscopic picture, see in more detail and described in Figure 19.

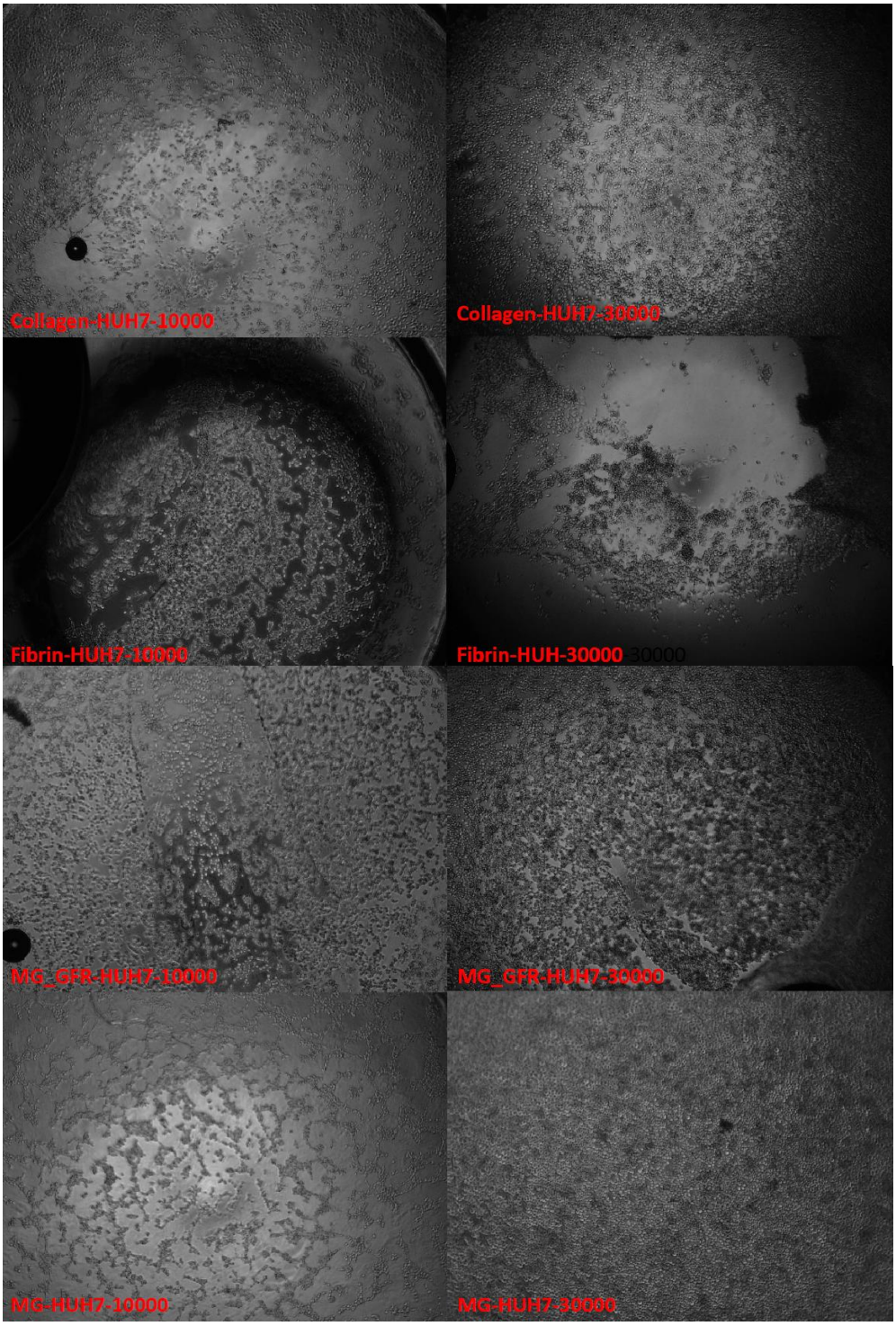


Figure 19: HUH7 General condition: Tube formation on different matrixes: Tube formation was also conducted for HUH7 on Matrigel®, Matrigel®- growth factor reduced, Collagen and Fibrin. An arrangement of cells could be seen on Matrigel® and on Matrigel®- growth factor reduced for 10,000 and 30,000 after 2h. Best results were seen on Matrigel® 10,000 cells with partial forming of tubes. No tube formation on Collagen1 and Fibrin regardless number of cells and time.

In summary, Matrigel® and Matrigel GFR® were found to be the most suitable matrixes for the tube formation assay. Tube formation was seen for endothelial cells and mesenchymal stem cells but not for the tumor cell line tested. The optimal cell number was 10,000 cells and the earliest time of tube formation was seen after 1h in this setting.

To evaluate the optimal time for the tube formation pictures were taken at time points 0h, 4h, 8h, 12h and 24h. The best results were seen after 12h, as shown in Figure 20.

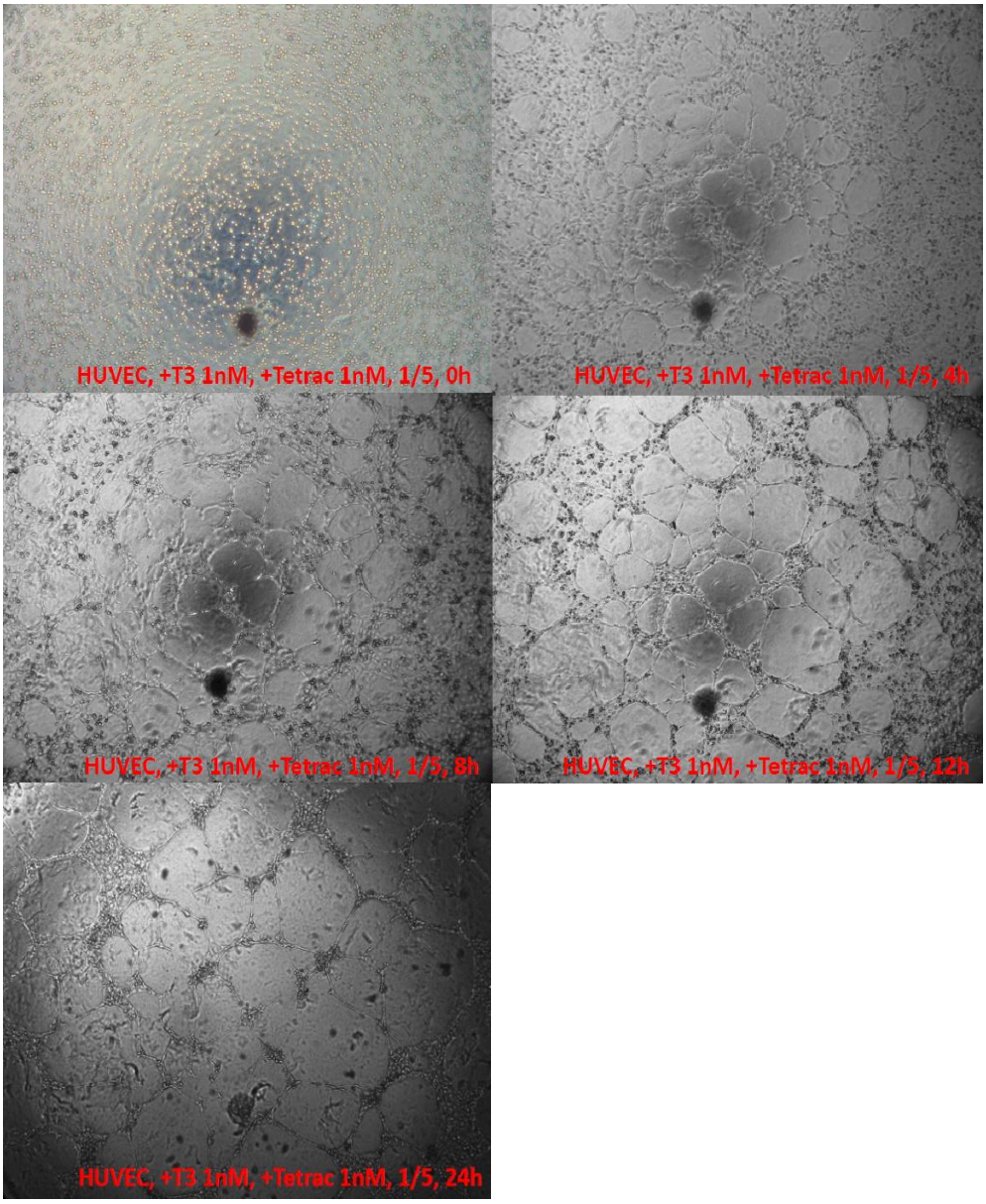


Figure 20: Testing for the best evaluable time point to analyze tube formation in angiogenesis assay: At time point 0h no tube formation was seen. This result parallels the outcome of the first experimental series (see Figure 16- Figure 18) that showed a first measurable time point of tube formation at 1-3h. After 4h tube formation was seen in the middle of each well that spread further to the margins by 8h and reach its maximum at time point 12h. At 24h the well started to dry out leading to loss of formed tubes. (Data taken out of first Angiogenesis Assay of HUVECS treated with thyroid hormones T3/T4 ± Tetrac, see total experiment in results 4.4.2., Figure 37.

4.2 Gateway Cloning to generate expression and reporter vectors to better characterize processes involved in the hypoxia response and angiogenesis network in tumor milieu

The hypoxic response within the tumor milieu represents an important aspect of tumor biology. The transcription factor Hif-1α plays an essential role in this setting. It becomes stabilized in response to a hypoxic milieu and has been linked to the initiation of genes linked to the process of angiogenesis

(see in detail in introduction 1.2.2). It has been suggested that Hif-1α may help drive expression of Tie2 and VEGF and this was investigated in more detail in course of this study.
To better understand the processes involved in tumor stroma formation, and with a special focus on the hypoxia response network and angiogenesis in the context of MSC-based tumor therapy, a series of expression and reporter plasmids were generated. An in-house platform for the efficient generation of gene promoters and reporters was developed that allows monitoring of the activation of the specific pathways thought to be crucial for tumor angiogenesis and formation. Gateway cloning technology as described in 3.3 was used to create the plasmids.

4.2.1. **Generation of Expression Clones/ Reporter Plasmids**

4.2.1.1 Generation of the Hif-1α- constitutive active (Hif-1α-CA) Expression vector

The transcription factor Hif-1α plays a key role in the hypoxic response network linked to stroma formation and angiogenesis. An inducible Hif-1α expression vector was generated using a constitutively active Hif-1α protein to study the effects of Hif-1α overexpression. As a reporter system used in concert with the expression plasmid, a series of synthetic Hif-1α responsive elements (HRE) acting through a minimal CMV promoter were used to drive expression of a secreted form of *Gaussia* luciferase. The final configuration of the plasmid is seen in Figure 21, and a proof of principle was performed (see also results 4.3.3).

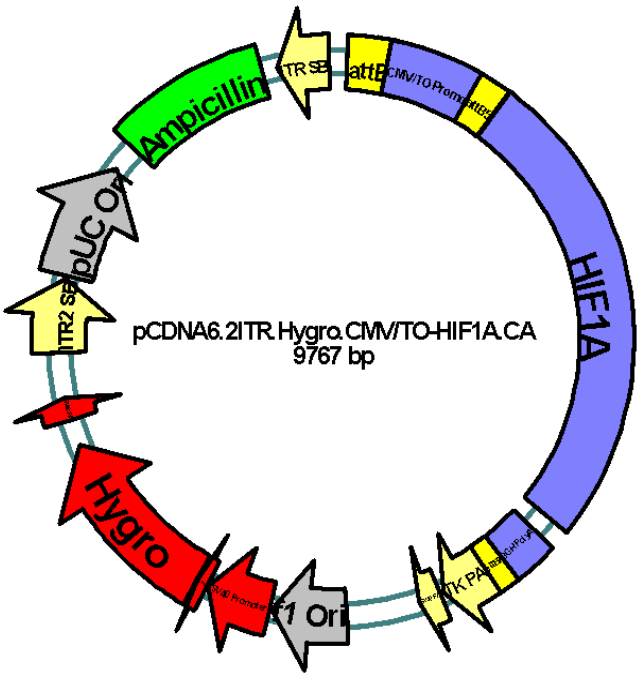


Figure 21: pCDNA6.2ITR_Hygro_CMV/TO-Hif-1α-CA: The Hif-1α- Expression vector (pCDNA6.2ITR_Hygro_CMV/TO-Hif-1α CA) was constructed with a 2-fragment Gateway Reaction out of Gateway Destination Vector pcDNA6.2PLITR.Hygro.CMV/TO-Dest and two Entry Clones pEntr221-*Gaussia* and HA-Hif-1α P402A/P564A-pcDNA3 (described in detail in methods, see 3.3.3). It is an expression vector that transcribes a conditionally active Hif-1α protein

constantly. It was generated to investigate the role of Hif-1α in the hypoxia response during tumor stroma formation and angiogenesis. Proof of principle performed (see results 4.3.3)

The Hif-1α-CA Expression vector (pCDNA6.2ITR_Hygro_CMV/TO-Hif-1αCA) was constructed using a 2-fragment Gateway Reaction into a Gateway Destination Vector pcDNA6.2PLITR.Hygro.CMV/TO-Dest using Entry Clones pEntr221-*Gaussia* and HA-Hif-1α-P402A/P564A-pcDNA3 (see methods 3.3).
The pcDNA-Gluc3-Hif-1α-miniTK reporter plasmid also constitutively expresses the Tet-repressor that blocks transcription of the Hif-1α protein on the second pCDNA6.2ITR_Hygro_CMV/TO-Hif-1α-CA plasmid. The addition of doxycycline de-represses expression of the Hif-1α construct leading to *Gaussia* luciferase production. The pcDNA-Gluc3-Hif-1α-miniTK reporter plasmid was generated (in collaboration with M.Schmitt-Noquiera and C. Jäckel) by ligation of the miniTK-Hif-1α into the pcDNA-Gluc3 plasmid. This plasmid acts as a reporter plasmid to monitor the activity of the Hif-1α regulated signal transduction pathway.

4.2.1.2 Generation of the VEGF reporter plasmids

VEGF is activated in early angiogenesis. To study the effect of thyroid hormones in VEGF activation in the context of the hypoxia response, tumor stroma formation and angiogenesis, a VEGF-promoter reporter plasmid was generated. Four versions of VEGF promoter reporter plasmids were generated using different reporters and antiobiotic resistance genes: pcDNA6.2ITR_BLASTI_Vegf2.1kb.*Gaussia*Luc (see as an example in Figure 21), pcDNA6.2ITR_BLASTI_Vegf2.1kb.*sGFP*, pcDNA6.2ITR_BLASTI_Vegf2.1kb.*Cherry* and pcDNA6.2ITR_HYGRO_Vegf2.1kb.*Cherry*. The plasmids were generated using the 2-fragment Gateway Cloning technology. Either pcDNA6.2PLITR Blasti-Dest or pcDNA6.2.PlITR Hygro-Dest were used as backbone/ Destination vector. For example, the pcDNA6.2ITR_BLASTI_Vegf2.1kb.*Gaussia*Luc, -*sGFP*, -*cherry* Expression vector the Entry Clones pEntr221-VEGF2.1Pro and respective the reporter Entry clones pEntr221*Gaussia*, -*sGFP* or -*Cherry* were incorporated in the backbone using the LR reaction. For creation of the pcDNA6.2ITR_HYGRO_ Vegf2.1kb.*Cherry* Expression vector the Entry Clone pEntr221-VEGF2.1Pro and pEntr221*Cherry* were ligated into the pcDNA6.2.PlITR Hygro-Dest vector.

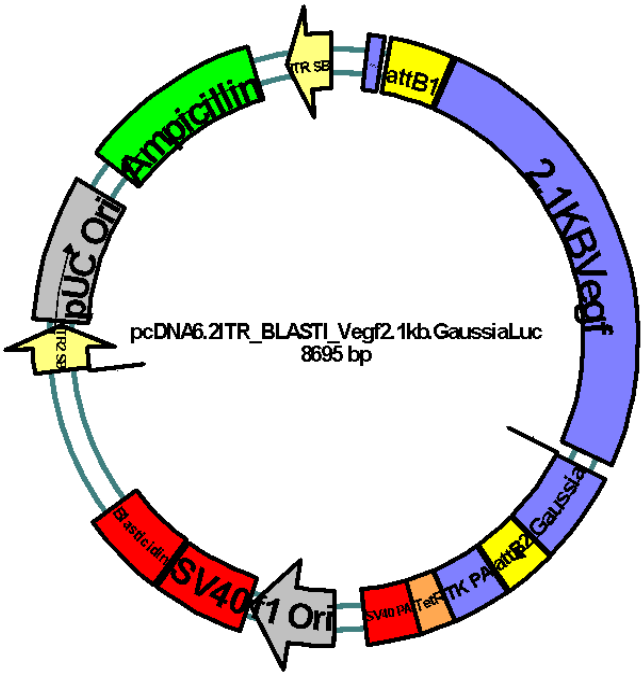


Figure 22: pcDNA6.2ITR_BLASTI_Vegf2.1kb.GaussiaLuc: To Gateway Cloning technology was used to generate the VEGF reporter. Either pcDNA6.2PLITR Blasti-Dest or pcDNA6.2.PlITR Hygro-Dest were used as backbone/Destination vectors. To build the pcDNA6.2ITR_BLASTI_Vegf2.1kb.*Gaussia*Luc, *-sGFP*, *-cherry* Expression vector the Entry Clones pEntr221-VEGF2.1Pro and respective the reporter Entry clones pEntr221*Gaussia*, *-sGFP* or *-Cherry* were incorporated in this backbone by an LR reaction. Here as example seen the pcDNA6.2ITR_BLASTI_Vegf2.1kb.*Gaussia*Luc plasmid.

4.2.1.3 Generation of the Tie2 Destination Vector and Tie2 reporter plasmids

A Tie2 promoter-based reporter plasmid was designed to monitor the underlying signal transduction pathways linked to late angiogenesis events with focus on the interaction of Hif-1α in the hypoxia response network as well as thyroid hormone influences. The activation of Tie2 transcription was measured by quantification of *Gaussia* luciferase or the fluorescent marker *sGFP* (see 4.3.3).

It took several steps to build the pcDNA6TR/ITR-Tie2-Dest. First the Tie2 cassette consisting of a promoter and an enhancer from the pSPTg.T2pAXK plasmid was incorporated in the backbone pcDNA6.2PLITR Blasti-Dest vector. As second step the Hygro-Dest-Gateway-cassette was integrated in order to be able to incorporate a reporter as *Gaussia* luciferase or a fluorescent marker as *sGFP*.

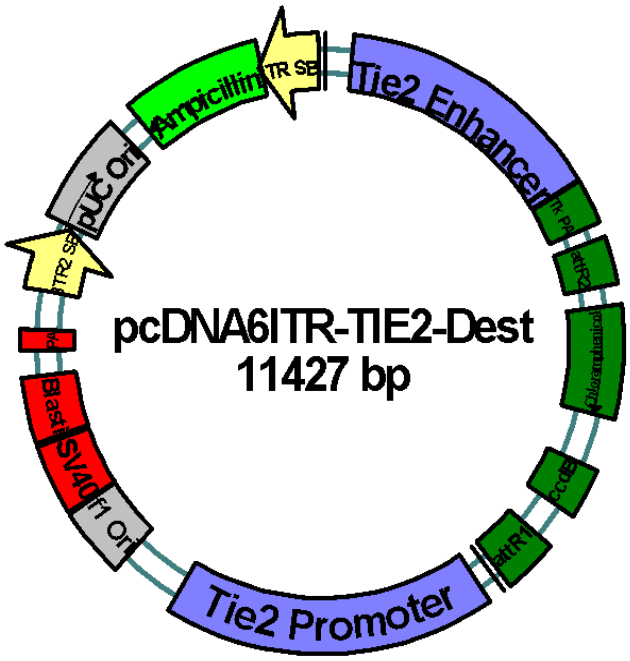


Figure 23: pcDNA6ITR-Tie2-Dest: The Destination vector pcDNA6TR/ITR-Tie2-Dest was designed and built in several steps. First the Tie2 cassette consisting of a promoter and an enhancer were cut out of the pSPTg.T2pAXK plasmid and incorporated into the backbone pcDNA6.2PLITR Blasti-Dest. To be able to get the reporters Entry Clones incorporated into the pcDNA6TR/ITR-Tie2-Dest-vector another vector had to be designed as intermediate step in order to use the Gateway Cloning technology. As final vectors pcDNA6ITR-TIE2-*Gaussia* and pcDNA6ITR-TIE2-*sGFP* were built.

To be able to get the reporters Entry Clones incorporated into the pcDNA6TR/ITR-Tie2-Dest-vector another vector had to be designed as intermediate step in order to use the Gateway Cloning technology. As final vectors pcDNA6ITR-TIE2-*Gaussia* and pcDNA6ITR-TIE2-*sGFP* were built.

4.3 Transfection and establishment of MSC reporter cells

MSCs (hbMSCsap172 and in in short in the experiments called hbMSCs) were used as transfection cells since they play a role in tumor stroma formation in part through their tumor tropism and differentiation into CAFs and pericytes as well as of their well-established role in driving angiogenesis. The hbMSCs were transfected using nucleofection with the SB100 Transposase expression plasmid, and with either the Hif-1α-CA, Hif-1α, or Tie2 and VEGF promoter plasmids - factors thought to be crucial in angiogenesis. The central questions were to better characterize when and how Tie2 and VEGF become activated in the *in-vitro* setting. Another future oriented aspect for this study is the potential use of genetically modified hbMSCs for gene therapy (see 5.3, using hbMSCs as Trojan horse and the tumor specific promoter activation of therapy genes). Transfection of hbMSCS was conducted with the Sleeping Beauty transposon technology as described in detail in 3.4. Quantification was realized with *Gaussia* Luciferase Assay.

4.3.1 Stem cell characterization

To test whether the transfected stem cells maintained their multi-lineage potential we tested for two of the three possible subpopulations adipocytes and osteocytes. Tests were positive for hbMSCs transfected with Tie2, those cells maintained adipogenic and osteogenic potential. Same results for hbMSCs transfected with VEGF cells, those cells as well maintained adipogenic and osteogenic potential. hbMSCs transfected with Hif-1α were positive for osteogenic potential and slightly positive for adipogenic potential.

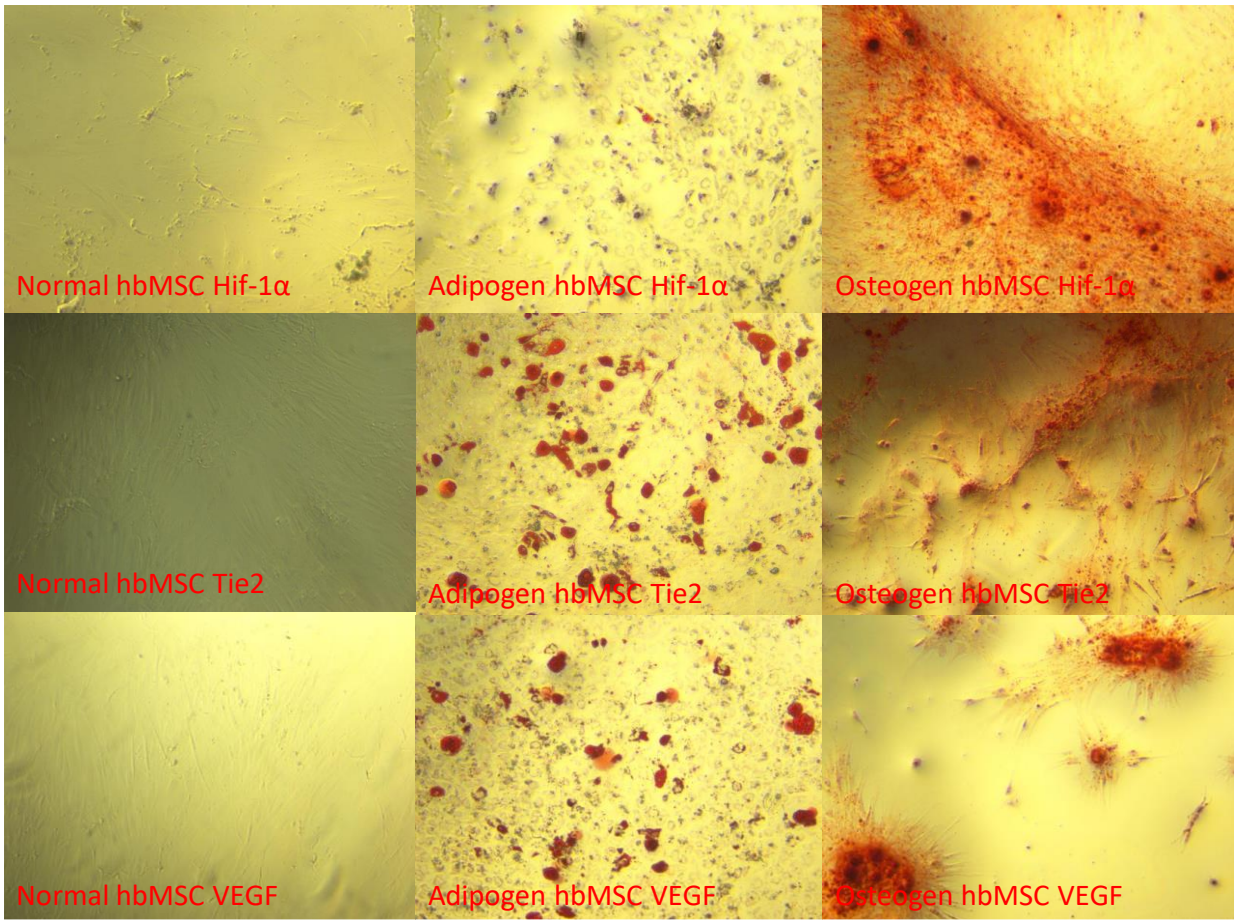


Figure 24: Testing the multi-lineage potential of transfected MSCs: Testing for two of the three possible differentiation pathways: adipocytes and osteocytes. Tests were positive for hbMSC Tie2 cells. The cells maintained adipogenic and osteogenic potential. Similar results were obtained for hbMSC - VEGF cells. hbMSCS - Hif-1α were clearly positive for osteogenic potential and slight positive for adipogenic potential.

4.3.2 Stimulation of transfected hbMSC reporter cells with $CoCl_2$, HUH7 supernatant and VEGF to mimic a hypoxic tumor milieu

After transfection of hbMSCs with the respective plasmids, activation with different stimuli was performed. A hypoxic milieu was mimicked by adding $CoCl_2$ to the cells in culture. $CoCl_2$ leads to stabilization of Hif-1α (Yuan, Hilliard, Ferguson, & Millhorn, 2003) and therefore to activation. To test the effect of tumor-secreted factors on the hypoxic response HUH7-conditioned medium was added. VEGF itself was used to test whether it can activate Tie2 since VEGF gets activated in early and Tie2 in late angiogenesis. The reporter activity was measured using *Gaussia* luciferase.

4.3.2.1 Hif-1α-Gaussia-hbMSCs activation by $CoCl_2$ and HUH7 supernatant

The Hif-1α *Gaussia* reporter plasmid was then tested for its responsiveness to hypoxia by testing with $CoCl_2$ stimulation. A dilution series was performed which showed a concentration dependent response (Figure 23). 50 – 200µM $CoCl_2$ was chosen for subsequent experiments.

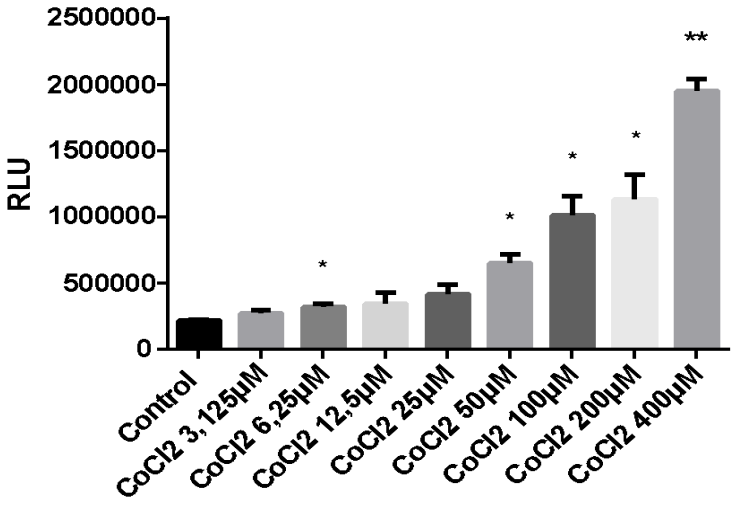


Figure 25: $CoCl_2$ dilution on MSC - Hif-1α- Gaussia reporter cells: To test the Hif-1α-*Gaussia* responsive promoter plasmid a dilution series with $CoCl_2$ was conducted that showed a concentration dependent activation of the construct. 50µM $CoCl_2$ showed a 3-fold, 100µM a 4.6-fold and 200µM a 5.2 fold, 400µM a 8.9-fold increase compared to the control. It was chosen for 50 – 200µM $CoCl_2$ in future experiments to see additional effects induced by other factors. *Gaussia* Luciferase light reaction was measured after 48h. (Experiment as pre-experiment performed once with duplicates, mean+ standard deviation; student's t-test; RLU= relative luciferase unit, significance:* = p<0.05, ** = p<0.01, *** = p<0.001,compared to control)

In a follow-up experiment, hbMSC - Hif-1α- *Gaussia* reporter cells were treated with HUH7-conditioned medium with or without $CoCl_2$. *Gaussia* luciferase measurements were conducted after 48h (Figure 26). Compared to untreated hbMSC - Hif-1α- *Gaussia* reporter cells, stimulation with HUH7 conditioned medium decreased *Gaussia* activation. Interestingly, addition of $CoCl_2$ to the HUH7-conditioned medium led to a 1.5-fold increase of Hif-1α-*Gaussia* activation as compared to exclusive stimulation with HUH7 supernatant.

This results were found to be significant for all three different HUH7 concentrations (100% HUH7 medium, p≈0.003,**; 50% HUH7 medium, p≈0.0001,***; 25% HUH7 medium, p≈0.001,**) tested. The difference between 100, 50 and 25% HUH7 medium stimulation was slight regardless of co-stimulation with $CoCl_2$. Therefore, we concluded that the concentration of HUH7-conditioned medium used does not make a difference. Adding $CoCl_2$ to the control group (hbMSCS in sFCS) showed a slight increase in Hif-1α- *Gaussia* activation but was not significant in this experiment.

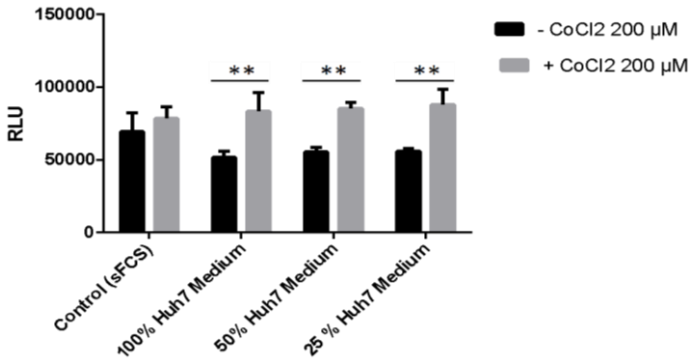


Figure 26: Stimulation of hbMSC – Hif-1α – *Gaussia* reporter cells with Huh Medium + $CoCl_2$: Compared to untreated hbMSCs stimulation with HUH7 medium had an adverse effect and a decrease in Hif-1α- *Gaussia* activation could be measured. Addition of $CoCl_2$ to the HUH7 stimulated hbMSCSs led to respectively circa 1.5-fold increase of Hif-1α- *Gaussia* activation as compared to stimulation with HUH7 supernatant alone. The results were significant for all HUH7 CM concentrations tested (100% HUH7 medium, p≈0.003,**; 50% HUH7 medium, p≈0.0001,***; 25% HUH7 medium, p≈0.001,**). The difference between 100, 50 and 25% HUH7 medium stimulation was minor regardless of co-stimulation with $CoCl_2$. It was concluded that very little HUH7 medium was required to achieve the results seen. (Experiment performed once, in quadruplets, mean+ standard deviation; student's t-test; RLU= relative luciferase unit, significance:* = p<0.05, ** = p<0.01, *** = p<0.001).

4.3.2.2 Tie2- Gaussia-hbMSC activation by VEGF, $CoCl_2$ and/or HUH7-conditioned media

Tie2-*Gaussia*-hbMSCSs were stimulated with $CoCl_2$ and tested in experiments with HUH7 conditioned media and VEGF. A $CoCl_2$ dilution series was performed to determine the effect of Hif-1α activation on the Tie2 promoter. Stimulation was measured after 48 hours. As shown in Figure 27 the Tie2-*Gaussia*-hbMSCSs did not show a strong response.

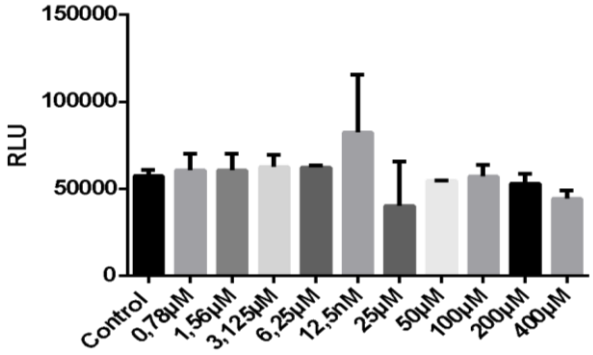


Figure 27: $CoCl_2$ dilution on Tie2-Gaussia-hbMSC reporter cells: Tie2- *Gaussia*- hbMSCs were stimulated with $CoCl_2$. A $CoCl_2$ dilution series did not show a concentration dependent response. No significant activation was seen for any of the $CoCl_2$ concentrations tested. (Experiment performed once, in quadruplets, mean+ standard deviation; student's t-test; RLU= relative luciferase unit, significance:* = p<0.05, ** = p<0.01, *** = p<0.001).

As a next step, stimulation of the Tie2-*Gaussia*-hbMSCS was conducted with HUH7 supernatant with or without $CoCl_2$. (Figure 28) Stimulation with 100%, 50% and 25% HUH7 medium increased Tie2 *Gaussia, but very slightly, it was not significant*. Adding $CoCl_2$ 200mM further reduced the Tie2-*Gaussia* activation.

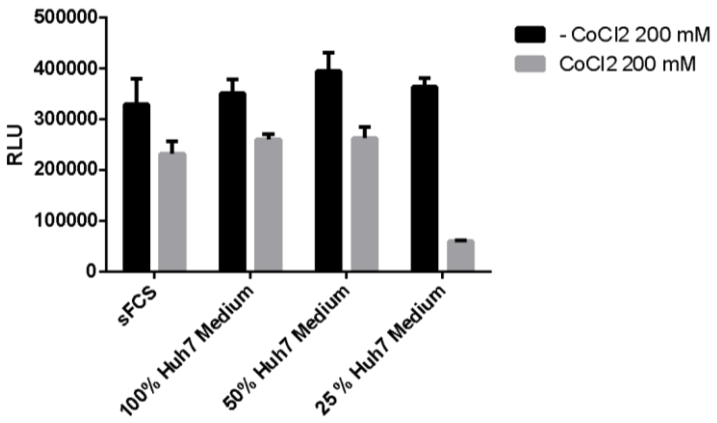


Figure 28: Stimulation of MSC - Tie2 - *Gaussia* reporter cells with $CoCl_2$ and HUH7 medium: Stimulation of hbMSC-Tie2-*Gaussia* with HUH7 medium ± $CoCl_2$ did not show an increase in Tie2-*Gaussia* activation Adding of $CoCl_2$ 200mM to all three HUH7 concentrations even more reduced the Tie2-*Gaussia* activation. (Experiment performed once, in quadruplets, mean+ standard deviation; student's t-test; RLU= relative luciferase unit, significance: * = p<0.05, ** = p<0.01, *** = p<0.001).

Since VEGF is upregulated early in angiogenesis and Tie2 is associated with late stages of angiogenesis, we sought to determine whether recombinant VEGF-A could activate Tie2 transcription in this experimental context. No stimulation of the construct was seen (Figure 29).

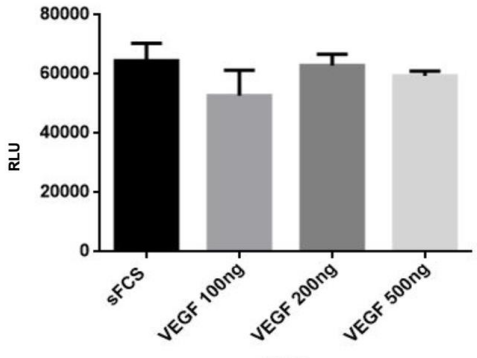


Figure 29: Stimulation of MSCS - Tie2 - Gaussia reporter cells with recombinant VEGF-A: Stimulation of hbMSC-Tie2-*Gaussia* reporter cells with VEGF-A (100ng, 200ng or 500ng) showed no significant activation of the reporter gene. (Experiment performed once, in quadruplets, mean+ standard deviation; student's t-test; RLU= relative luciferase unit, significance:* = p<0.05, ** = p<0.01, *** = p<0.001).

4.3.2.3 VEGF-Gaussia-hbMSC activation by $CoCl_2$ and HUH7 supernatant

The VEGF - *Gaussia* reporter plasmid in hbMSCs was then tested for its response to $CoCl_2$. Activation was not a clear concentration dependent way as seen for the Hif-1α- responsive promoter construct (see results 4.3.2.1). The reporter cells showed a low but significant activation (Figure 30). For example, 200µM $CoCl_2$ (p≈0.0006, *** = p<0.001, highly significant) showed a 2.1-fold increase as compared to untreated VEGF- *Gaussia* hbMSCSs. For future experiments a $CoCl_2$ concentration range of 50 – 200µM was used to assess the additional potential effect of other factors.

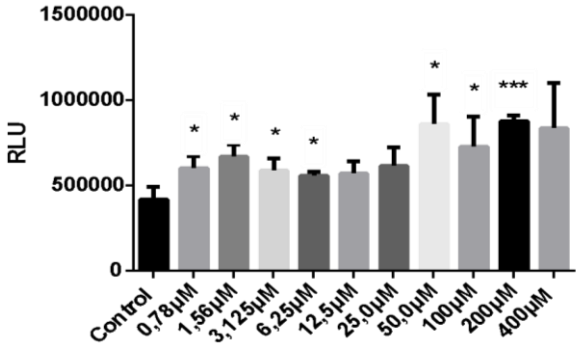


Figure 30: $CoCl_2$ dilution series effect on VEGF-Gaussia-hbMSCSs reporter cells: VEGF- *Gaussia* reporter cells were slightly responsive to $CoCl_2$. Not in an as clear concentration dependent way as seen for the Hif-1α- responsive promoter construct. Two small but significant peaks were found. The best result was seen for 200µM $CoCl_2$ (p≈0.0006, *** = p<0.001, highly significant), with an 2.1-fold increase compared to the untreated VEGF- *Gaussia* hbMSCSs. (Experiment performed once, in quadruplets, mean+ standard deviation; student's t-test; RLU= relative luciferase unit, significance:* = p<0.05, ** = p<0.01, *** = p<0.001)

Additional VEGF-Gaussia-hbMSCs experiments to assess potential effects of additional factors were performed using 50µM and 200µM $CoCl_2$. Activation of VEGF-*Gaussia*-hbMSCSs was conducted with 50µM $CoCl_2$ and 100% HUH7 medium. An increase in *Gaussia* luciferase was again seen with $CoCl_2$ as compared to untreated with 1.7-fold and a high significance (p≈0.001). Co-stimulation with 50µM CoCl2 and 100% HUH7 medium did not show a significant increase over $CoCl_2$ alone.

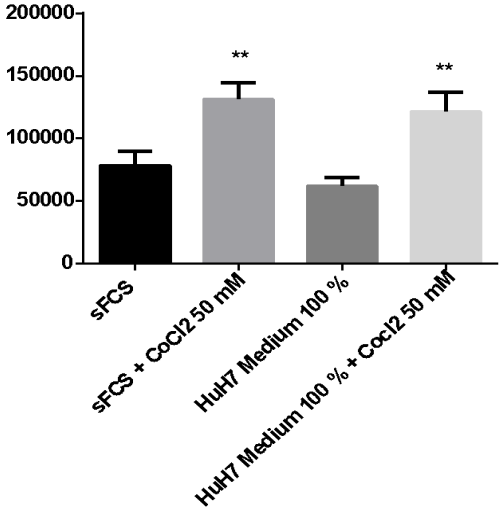


Figure 31: Stimulation of VEGF-Gaussia-hbMSCs with 50µM $CoCl_2$ and 100% HUH7 conditioned medium: As seen in the prior experiment, an increase in *Gaussia* luciferase was seen with $CoCl_2$ compared to untreated with 1.67-fold and a high significance (p≈0.00104, ** = p<0.01). Co-stimulation with 50µM $CoCl_2$ and 100% HUH7 medium showed a level of activation similar to that seen with $CoCl_2$ alone (1.55-fold, p≈0.003, ** = p<0.01). (Experiment performed once, in quadruplets, mean+ standard deviation; student's t-test; RLU= relative luciferase unit, significance: * = p<0.05, ** = p<0.01, *** = p<0.001.).

4.3.3 Co-transfection of hbMSCs with Hif-1α-CA (constitutive active) expression vector + Hif-1α/ Tie2/ VEGF reporter vectors

The effect of Hif-1α was then evaluated using the Hif1α-CA plasmid (constitutively active Hif-1α – induction) and tested with the synthetic Hif-1α reporter plasmid (pcDNA-Gluc3-Hif-1α-miniTK), the Tie2 promoter plasmid (pcDNA6ITR-Tie2-Dest) and the VEGF promoter plasmid (pcDNA6.2ITR_Hygro_Vegf2.1). A Proof of principle was performed to demonstrate functionality of the Hif-1α-CA plasmid (pCDNA6.2ITR_Hygro_CMV.TO-HIF1A CA). We sought to validate that the Hif-1α-CA plasmid could activate the Hif-1α responsive promoter plasmid (pcDNA-Gluc3-Hif-1α-miniTK). In parallel, the effect of Hif-1α-CA over expression on the Tie2 promoter plasmid (pcDNA6ITR-Tie2-

Dest) and the VEGF promoter plasmid (pcDNA6.2ITR_Hygro_Vegf2.1) were evaluated. All three reporter plasmids made use of *Firefly* Luciferase as the reporter gene and the experiments were performed using transient transfection of primary MSCs using the Neon electroporation. As described in 3.5.6, the resultant *Firefly* activity was normalized using transfection with a constitutively expressed *Renilla* Luciferase control plasmid. To control the active transcription of Hif-1α-CA a second plasmid was used where a CMV/TO promoter controlled by the Tet-repressor (pcDNA6TR/ITR_TK) was used. The Tet-repressor protein blocks the CMV/TO promoter from driving the Hif-1α-CA transgene (in detail described in 3.3.2.). The addition of doxycycline caused a de-repression of the CMV/TO promoter leading to transcription of the Hif-1α-CA construct. MSCs were co-transfected with the tet-repressor (pcDNA6TR/ITR_TK) as negative control.

The Hif-1α-CA plasmid was shown to activate the Hif-1α responsive promoter plasmid (pcDNA-Gluc3-Hif-1α-miniTK) containing a synthetic Hif-1α responsive promoter plasmid consisting of a series Hif-1α responsive elements (HRE) driving a minimal promoter. A significant higher activation was seen compared to the control with the Tet-repressor ($p<0.014$, * = $p<0.05$, 1st experiment). For the MSCs transfected with Hif-1α-CA and VEGF-Firefly, and Hif-1α-CA and Tie2-Firefly, no significant difference Hif-1α-driven activation was seen (data not shown).

4.3.3.1 Co-transfection of hbMSCs with Hif-1α-CA expression vector and Hif-1α responsive promoter plus stimulation with T3/T4 and $CoCl_2$

After validating that the Hif-1α-CA plasmid could activate the Hif-1α responsive promoter, another proof of principle was performed to investigate whether an inducible version of the construct could be activated via doxycycline (same system as described in 4.3.3, see methods described in 3.3.2, Tetracycline-controlled transcriptional activation.). Furthermore, it was tested for an additional effect of T3 /T4, in their physiological doses T3 1nM and T4 100nM as well as $CoCl_2$ in 50µM and 100µM in this setting. We then determined if diverse concentrations of T3/T4 ± $CoCl_2$ could modulate the reporter activity, but the results did not show further significant effects on Hif-1α reporter activity as (Figure 32) compared to the control group.

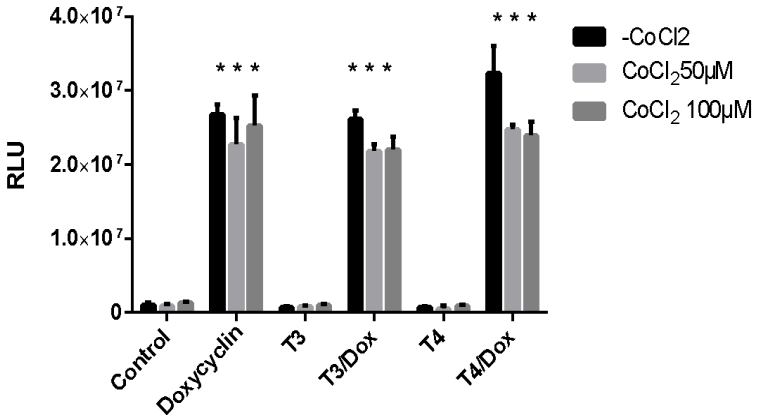


Figure 32: Proof of principle Co-transfection of hbMSCSs with Hif-1α expression vector and Hif-1α reporter plus stimulation with T3/T4 and $CoCl_2$: Proof of principle to prove the correct construction of the Hif-1α-CA plasmid (pCDNA6.2ITR_Hygro_CMV.TO-HIF1A CA). It was tested if the Hif-1α-CA plasmid activates the Hif-1α responsive promoter plasmid (pcDNA-Gluc3-Hif-1α-miniTK). *Gaussia* Luciferase was used as reporter and was measured. Adding doxycycline led to transcription of Hif-1α-CA and Hif-1α reporter activity which was highly significant for all tested variations with T3/T4/$CoCl_2$ in their physiological levels T3 1nM and T4 100nM as well as $CoCl_2$ in 50µM and 100µM. In the dox+ group additional adding of T4 was almost significant with $p\approx0.053$ (Experiment performed once with triplets, Mean+ standard deviation; student's t-test; RLU= relative luciferase unit, significance: * = *** = $p<0.001$).

4.3.4 Stimulation with T3/T4 to assess thyroid hormone effect on transfected MSCs

To investigate whether the thyroid hormones T3/T4 can influence the hypoxia response during angiogenesis and tumor stroma formation, cells engineered with the three reporter constructs Hif-1α, Tie2 and VEGF were stimulated with thyroid hormones. To simulate a hypoxic milieu, co-stimulation with $CoCl_2$ was performed. These experimental series were performed without tetrac. See results 4.4, for effects of tetrac as inhibitor of the non-genomic receptor pathway of thyroid hormones.

4.3.4.1 Hif-1α-Gaussia-MSC activation by T3/T4 ± $CoCl_2$

To investigate if T3/T4 could have an influence on the hypoxia responsive network during angiogenesis and tumor stroma formation the effects of T3 and T4 on Hif-1α reporter plasmid ± $CoCl_2$ was performed. Stimulation of hbMSC -Hif-1α-*Gaussia* with T3/T4 ± $CoCl_2$ was performed. Co-stimulation with T3 1nM, 10nM and 50µM $CoCl_2$ increased the Hif-1α *Gaussia* light reaction significantly for 1nM T3. It was significant as compared to T3 1nM alone (*≈ p<0.0104) and as compared to $CoCl_2$ alone (*≈p≈0.01 with 1,3-fold increase)). For T4 10nM and T4 100nM there was an increase seen after co-stimulation with $CoCl_2$, but compared to the control it overall showed a lower activation.

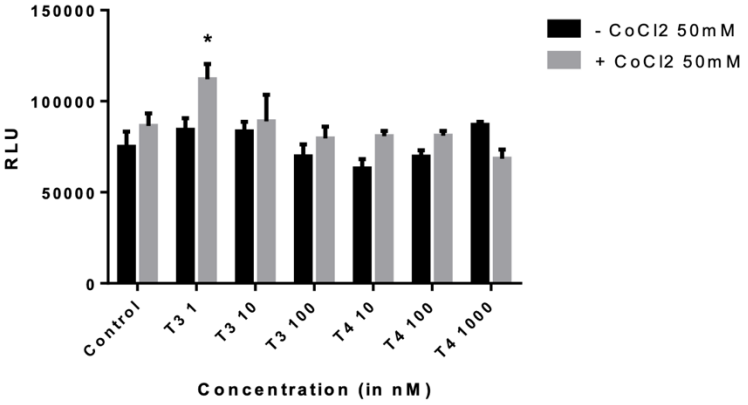


Figure 33: Stimulation of hbMSCs - Hif-1α - Gaussia with T3/ T4 ± $CoCl_2$. To investigate if T3/T4 could have an influence on the hypoxia responsive network during angiogenesis and tumor stroma formation the effects of T3 and T4 on Hif-1α reporter plasmid ± $CoCl_2$ was performed. Stimulation of hbMSC -Hif-1α-*Gaussia* with T3/T4 ± $CoCl_2$ was performed. Co-stimulation with T3 1nM, 10nM and 50µM $CoCl_2$ increased the Hif-1α *Gaussia* light reaction significantly for 1nM T3. It was significant as compared to T3 1nM alone (*≈ p<0.0104) and as compared to $CoCl_2$ alone (*≈p≈0.01 with 1,3-fold increase)). For T4 10nM and T4 100nM there was an increase seen after co-stimulation with $CoCl_2$, but compared to the control it overall showed a lower activation. (Experiment performed once with triplets, mean+ standard deviation; student's t-test; RLU= relative luciferase unit, significance:* = p<0.05, ** = p<0.01, *** = p<0.001, compared to control (± CoCl2); # = p<0.05, ## = p<0.01, ### = p<0.001, compared to - $CoCl_2$).

4.3.4.2 Tie2-Gaussia-MSCs activation by T3/T4 ± $CoCl_2$

We then set up the same experiment to investigate the effects of T3/T4 on the Tie2 receptor. Stimulation of hbMSCS-Tie2-*Gaussia* with T3/T4 ± $CoCl_2$ were performed. Adding T3 led to an increase in Tie2 *Gaussia* luciferase activity for T3 1nM, T3 10nM and T3 100nM, the increase was significant for T3 10nM and T3 100nM (T3 10nM,** = p≈0.0038, 1,2-fold increase; T3 100nM, * = p≈0.037, 1.12-fold increase (no $CoCl_2$ added). Single stimulation with $CoCl_2$ did not show an increase (similar to results performed in 4.3.2.2) instead it showed a significant decrease in light activation. But co-stimulation of $CoCl_2$ with T3 1nM till T4 1000nM showed an increase in the Tie2 *Gaussia* light reaction, for all doses of T3 and T4 it was significant compared to the control with just $CoCl_2$ (''= for T3 1nM p≈0.0018, 1.41-fold; for T3 10nM, p≈0.0078, 1.66-fold; for T3 100nM p≈0.0082, 1,71-fold; for T4 10nM p≈0.0062, 1,68-fold; for T4 100nM p≈0.019, 1.25-fold and T4 1000nM, p≈0.037, 1.28-fold

increase. For T4 100nM, the increase after adding $CoCl_2$ compared to - $CoCl_2$ was significant (# = p≈0.03, 1.2-fold increase compared to - $CoCl_2$).

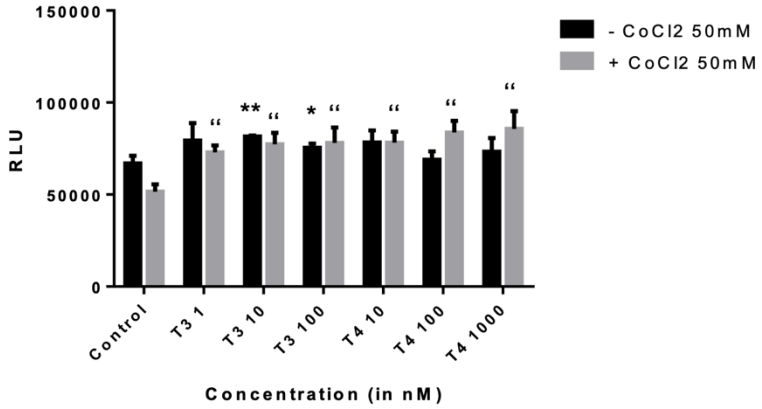


Figure 34: Stimulation of MSCS-Tie2-Gaussia with T3/ T4/ +- $CoCl_2$: Stimulation of MSCS-Tie2-*Gaussia* with T3/ T4/ +- $CoCl_2$: ($CoCl_2$ in 50µM, T3 1-100nM, T4 10-100nM). Adding T3 led to an increase in Tie2 *Gaussia* luciferase activation. The increase was significant for T3 10nM and T3 100nM (T3 10nM,** = p≈0.0038, 1.2-fold increase; T3 100nM, * = p≈0.037, 1.12-fold increase (no $CoCl_2$ added). Single stimulation with $CoCl_2$ did not show an increase (similar to results performed in 4.3.2.2) instead it showed a significant decrease in light activation. But co-stimulation of $CoCl_2$ with T3 1nM till T4 1000nM showed an increase in the Tie2 *Gaussia* light reaction, for all doses of T3 and T4 it was significant compared to the control with just $CoCl_2$ (''=for T3 1nM p≈0.0018, 1.41-fold, for T3 10nM, p≈0.0078, 1.66-fold, for T3 100nM p≈0.0082, 1,71-fold, for T4 10nM p≈0.0062, 1,68-fold, for T4 100nM p≈0.019, 1.25-fold and T4 1000nM, p≈0.037, 1.28-fold increase). For T4 100nM, the increase after adding $CoCl_2$ compared to - $CoCl_2$ was significant (# = p≈0.03, 1.2-fold increase compared to - $CoCl_2$). (Experiment performed once with triplets, mean+ standard deviation; student's t-test; RLU= relative luciferase units, significance:* = p<0.05, ** = p<0.01, *** = p<0.001, compared to control (- $CoCl_2$); # = p<0.05, ## = p<0.01, ### = p<0.001, compared to - CoCl2; ‘= p<0.05, ''= p<0.01, ''' = p<0.001, compared to control (+ $CoCl_2$)).

4.3.4.3 VEGF-Gaussia-MSCs activation by T3/T4 ± $CoCl_2$

To test the effects of thyroid hormones on the VEGF plasmid we treated the VEGF *Gaussia* MSCs with T3 and T4 ± $CoCl_2$. T3 led to a significant increase in VEGF *Gaussia* luciferase activation for T3 10nM (T3 10nM,* = p≈0.031, 1.31-fold increase). Co-Stimulation of $CoCl_2$ with T3 1nM, 10nM and 100nM elevated the *Gaussia* light reaction compared to single stimulation with T3. If compared to the untreated + $CoCl_2$ control there was for all three T3 doses a significant increase (T3 T1nM, ''= p≈0.0096, 1.33-fold increase; T3 10nM,‘= p≈0.010, 1.61-fold increase; T3 100nM, ‘= p≈0.025, 1.43-fold increase), compared to the untreated control the T3 10nM was significant (:* = p≈0.035, 1.44-fold increase). T4 10nM with $CoCl_2$ was still significantly higher than stimulation with just $CoCl_2$ (T4 10nM = p≈0.047, 1.14-fold increase).

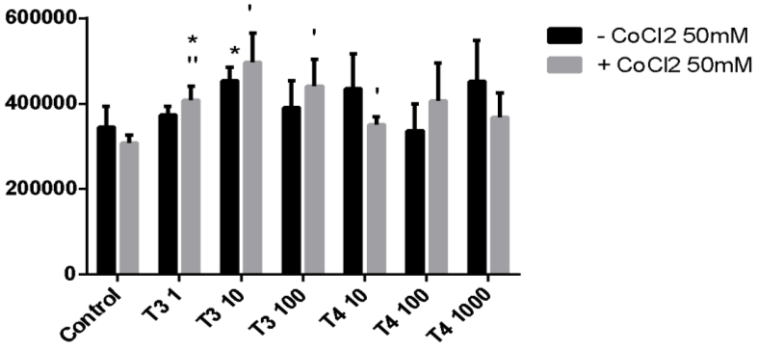


Figure 35. Stimulation of MSCS-VEGF-Gaussia with T3/ T4 and $CoCl_2$: To test the effects of thyroid hormones on the VEGF plasmid we treated the VEGF *Gaussia* MSCs with T3 and T4 ± $CoCl_2$. T3 led to a significant increase in VEGF *Gaussia* luciferase activation for T3 10nM (T3 10nM,* = p≈0.031, 1.31-fold increase). Co-Stimulation of $CoCl_2$ with T3 1nM, 10nM and 100nM elevated the *Gaussia* light reaction compared to single stimulation with T3. If compared to the untreated + $CoCl_2$ control there was for all three T3 doses a significant increase (T3 T1nM, ''= p≈0.0096, 1.33-fold increase; T3 10nM,‘= p≈0.010, 1.61-fold increase; T3 100nM, ‘= p≈0.025, 1.43-fold increase), compared to the untreated control the T3 10nM

was significant (:* = p≈0.035, 1.44-fold increase). T4 10nM with $CoCl_2$ was still significantly higher than stimulation with just $CoCl_2$ (T4 10nM = p≈0.047, 1.14-fold increase). (Experiment performed once with triplets, mean+ standard deviation; student's t-test; RLU= relative luciferase unit, significance:* = p<0.05, ** = p<0.01, *** = p<0.001, compared to control (- $CoCl_2$); # = p<0.05, ## = p<0.01, ### = p<0.001, compared to - $CoCl_2$; '= p<0.05, ''= p<0.01, ''' = p<0.001, compared to control (+ $CoCl_2$)).

4.4 Thyroid hormone treatment in the Angiogenesis Assay

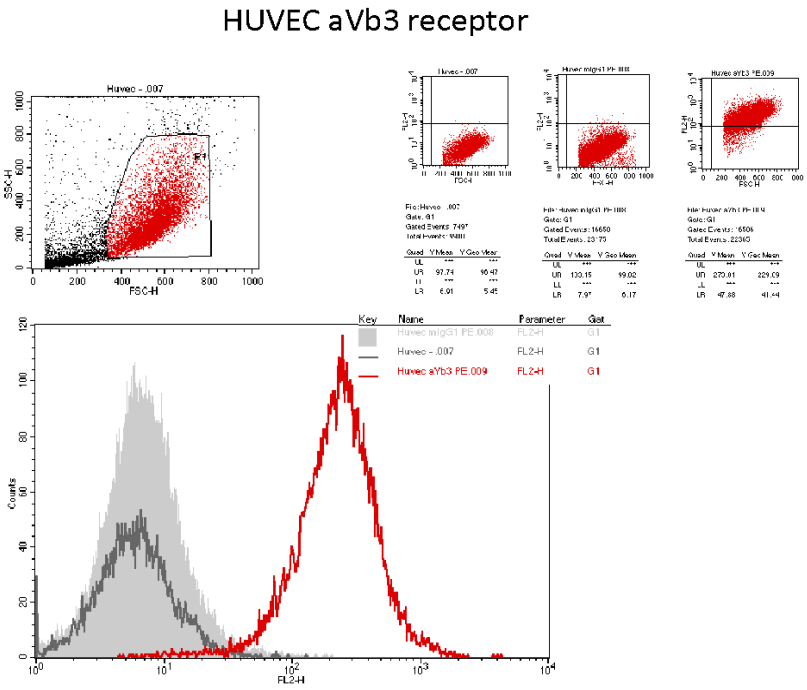
Thyroid hormones play a role in cancer metabolism by stimulating tumor growth, and angiogenesis by driving endothelial cell proliferation, migration and tube formation. (P. J. Davis et al., 2018; Schmohl, Mueller, et al., 2019) As stated in chapter 1.5, two signaling pathways are linked to the actions of the thyroid hormones T3 and T4, referred to as the genomic and non-genomic pathways. Thyroid hormone action through the non-genomic pathway is mediated by the αvβ3 integrin. This integrin is expressed on many tumors, and on endothelial and mesenchymal stem cells. (Bergh et al., 2005; Schmohl et al., 2015; Schmohl, Nelson, & Spitzweg, 2019)

T3/T4 has been previously shown to have an influence on the tumor tropism of MSCs as well as their differentiation into CAFs. In addition, previous experiments have shown an effect of the thyroid hormones on Hif-1α activity (Muller et al., 2016; Schmohl, Muller, Nelson, & Spitzweg, 2019).

The effects of thyroid hormones were investigated on endothelial cell tube formation (see results 4.4.2). As a second step, the effects of thyroid hormones on the various reporter engineered MSCs was investigated in experimental angiogenesis (see results 4.5.).

4.4.1 FACS analysis of cells used in angiogenesis assay

FACS analysis was performed to test for avβ3 integrin receptor expression on the cells used in the angiogenesis assay (HUVECS, hbMSCS and HUH7 cells). The results showed surface expression for HUVECs and hbMSCSs but were negative for the HUH7 cell line.



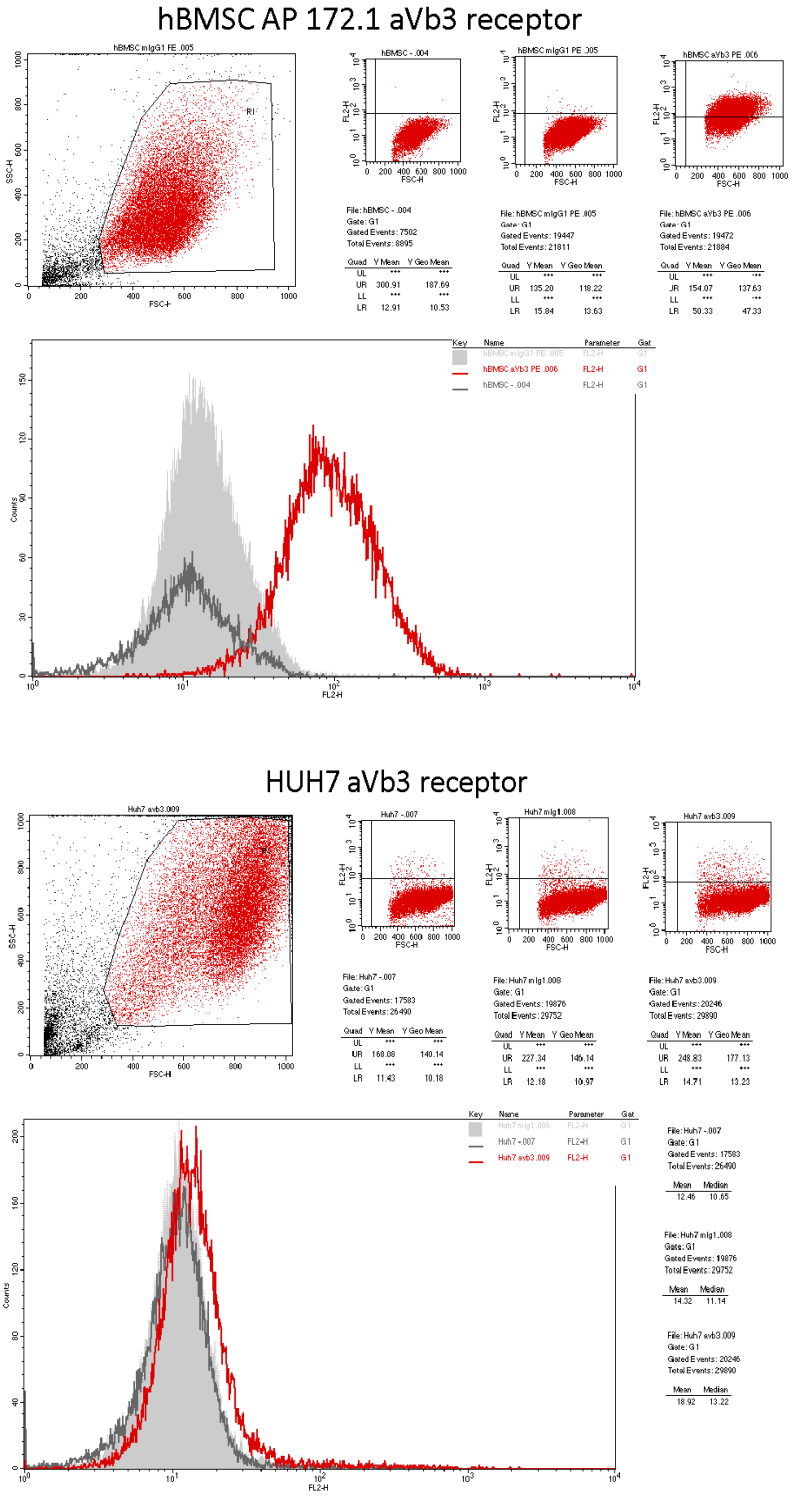


Figure 36: FACS analysis of cells used in angiogenesis assay: A FACS analysis was performed to test for the avβ3 integrin / thyroid hormone receptor on the cells used in the angiogenesis assay. It was tested for HUVECS, hbMSCSs and HUH7. Results were positive for HUVECs and hbMSCSs and negative for HUH7.

4.4.2 Thyroid hormone treatment in the context of the experimental angiogenesis assay

After establishing the general conditions for the angiogenesis assay (see 4.1.1.) we tested for effects of thyroid hormones on endothelial cell tube formation. In earlier studies our group investigated the pro-angiogenic effects of thyroid hormones and could show that these effects are most likely

mediated via the non-genomic thyroid hormone receptor αvβ3 (Schmohl et al., 2015) This could be examined through use of Tetrac, a deaminated T4 derivate that blocks the integrin-based receptor.

MSCs play a crucial role in angiogenesis (see introduction 1.3.2). We then investigated the impact of thyroid hormones on endothelial tube formation (Figure 37) and MSCs (Figure 38-Figure 40). These experiments were performed in co-culture (HUVECs + MSCS) or using MSC-conditioned medium (MSC-CM). HUH7 were again used as tumor model (Figure 39- Figure 40) to evaluate the effects of tumor cells on experimental angiogenesis.

Primary human umbilical vein endothelial cells (HUVECs) were stimulated with different concentrations of T3 (1-100nM), T4 (1-1000nM) with or without the integrin αvβ3-specific inhibitor tetrac and with MSC-CM, HUH7-CM or co-culture-CM. Pictures were taken under a microscope after 12h and analyzed with Image J plug-in Angiogenesis Analyzer regarding total tube length, junction and meshes. In the first experiment (Fig.37), HUVECs were stimulated with T3, T4 ± tetrac. A dose dependent trend can be seen for T3 stimulation of HUVECs compared to untreated HUVECS and this in a tetrac-dependent manner for stimulation with T3 10nM and 100nM, even though it was not significant. Treatment with T4 shows also a dose dependent increase of all three evaluated doses, even though it is not as clearly as the data with T3 and does not happen in a tetrac dependent manner. Data as well not significant.

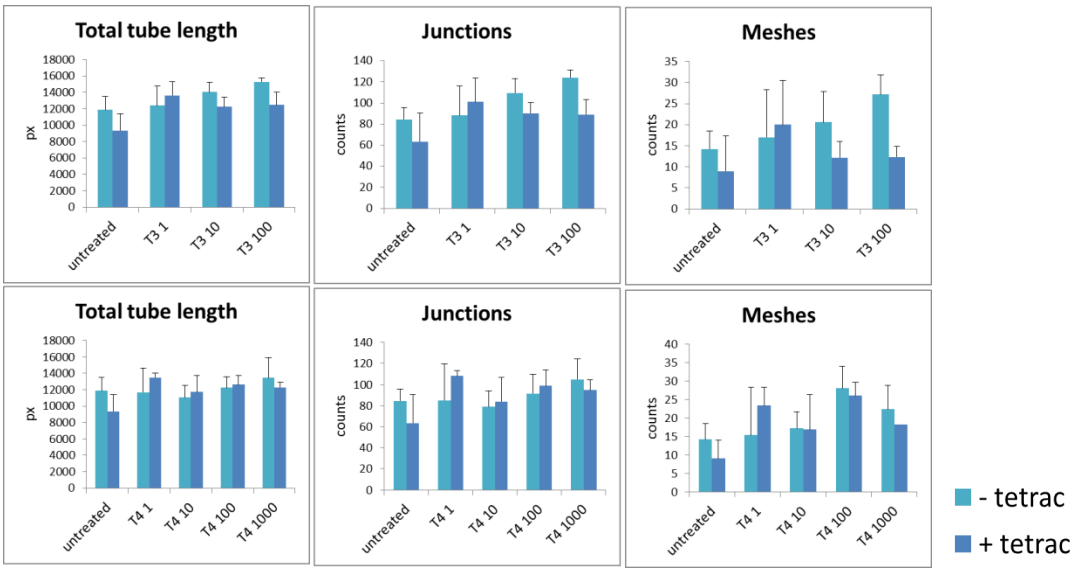


Figure 37: Angiogenesis Assay HUVEC + T3/T4/Tetrac 12h: Primary human umbilical vein endothelial cells (HUVEC) were seeded onto growth factor-reduced Matrigel®, treated with different concentration of T3 (1-100nM), T4 (1-1000nM) ± integrin αvβ3-specific inhibitor tetrac and incubated at 37°C for 12h (5 replicates per respective stimulation). Microscopic pictures were taken at 12h and analyzed with Image J plug-in Angiogenesis Analyzer regarding total tube length, junction and meshes. In contrast to untreated HUVECS a dose dependent trend can be seen for T3 stimulation of HUVECs and this in a tetrac-dependent manner (T3 10nM and 100nM), except of T3 1nM whereas after co-stimulation with tetrac an increase in total tube length, junction and meshes can be seen. Treatment with T4 shows also a dose dependent increase of all three evaluated data, even though it is not as clearly as the data with T3. In contrast to stimulation with T4 it does not do so in a tetrac dependent manner. (Experiment performed once, respectively in quintuplets, Mean+ standard deviation; student's t-test; RLU= relative luciferase unit, significance:* = p<0.05, ** = p<0.01, *** = p<0.001).

Additional treatment of HUVECs with MSC-CM shows less tube formation compared to control HUVECs (Fig.38) but co-stimulation of HUVECs with MSCS-CM plus T3 and T4 led to an increase in tube length, junction and meshes (Figure 38) as compared to HUVECs just stimulated with MSC-CM. It does not appear to be dose or tetrac dependent.

Tube formation was then tested on HUH7-CM treated HUVECs with thyroid hormone stimulation. In comparison with tube formation without treatment of conditioned medium, MSC-CM or coculture conditioned medium (CoC-CM) showed the highest total tube length, junctions and meshes (Figure 39). This suggests that factors related to the tumor milieu helps drive angiogenesis (see discussion 5.2). In contrast to tube formation evaluated in control medium and MSC-CM, no enhancement was seen after stimulation with T3 and T4 (Figure 39). Tetrac had no effect (Figure 39).

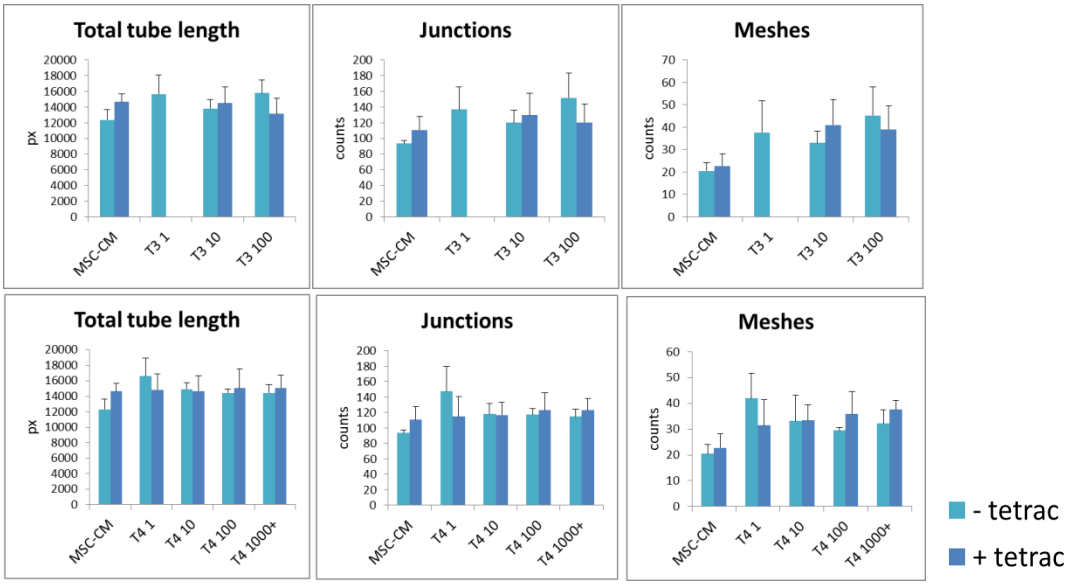


Figure 38: Angiogenesis Assay HUVEC + T3/T4/Tetrac +MSC-CM 12h: Primary human umbilical vein Endothelial cells (HUVEC) were seeded onto growth factor-reduced Matrigel®, treated with different concentration of T3 (1-100nM), T4 (1-1000nM) ± integrin αvβ3-specific inhibitor tetrac and incubated in MSC-conditioned medium were at 37°C for 12h (5 replicates per respective stimulation). Microscopic pictures were taken at 12h and analyzed with Image J plug-in Angiogenesis Analyzer regarding total tube length, junction and meshes. An increase in total tube length, junction and meshes can be seen for T3 as well as for T4. It is not dose-dependent and not Tetrac- dependent. (Experiment performed once, respectively in quintuplets, Mean+ standard deviation; student's t-test; RLU= relative luciferase unit, significance: * = p<0.05, ** = p<0.01, *** = p<0.001).

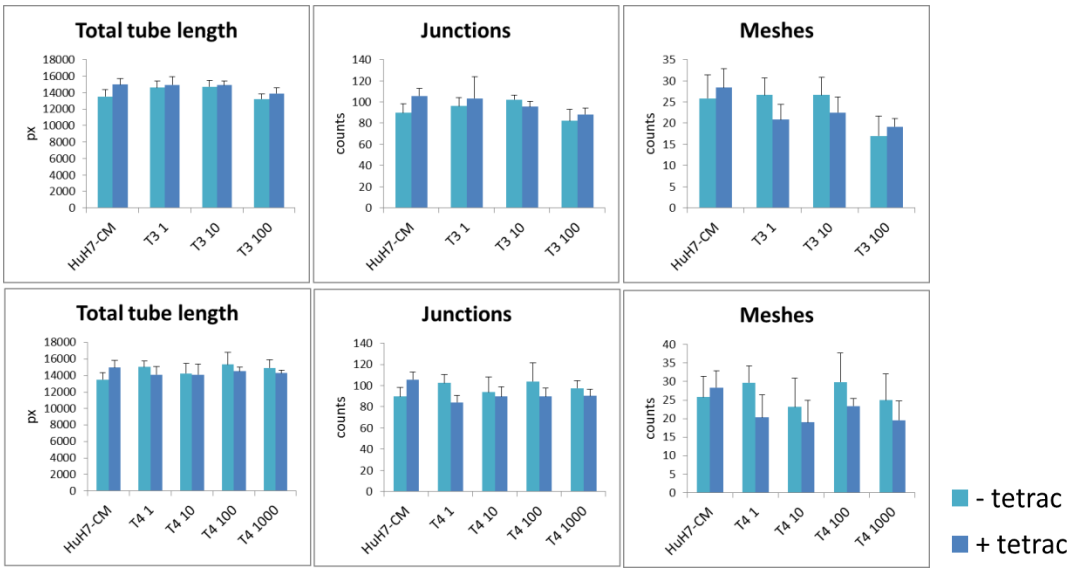


Figure 39: Angiogenesis Assay HUVEC + T3/T4/Tetrac +HUH7-CM 12h: Primary human umbilical vein Endothelial cells (HUVEC) were seeded onto growth factor-reduced Matrigel®, treated with different concentration of T3 (1-100nM), T4 (1-1000nM) ± integrin αvβ3-specific inhibitor tetrac and incubated in HUH7-conditioned medium at 37°C for 12h (5 replicates per respective stimulation). Microscopic pictures were taken at 12h and analyzed with Image J plug-in Angiogenesis

Analyzer regarding total tube length, junction and meshes. There is no clear difference in tube formation between stimulation with or without T3 and T4 after stimulation with HUH7-CM. Tetrac had no significant effect. (Experiment performed once, respectively in quintuplets, Mean+ standard deviation; student's t-test; RLU= relative luciferase unit, significance: * = p<0.05, ** = p<0.01, *** = p<0.001).

To investigate the potential interplay between MSCs and tumor milieu, the tube formation assay was conducted using co-culture-conditioned medium (CoC- CM/ (HUH7/MSCs)) and thyroid hormones as well as the integrin inhibitor tetrac. CoC-CM showed a similar activation as seen with HUH7 medium with regards to tube length and junction formation. No enhancement of tube formation was seen by stimulation with T3/T4 treatment. Adding Tetrac on CoC- CM HUVECs treated with T4 decreased tube formation.

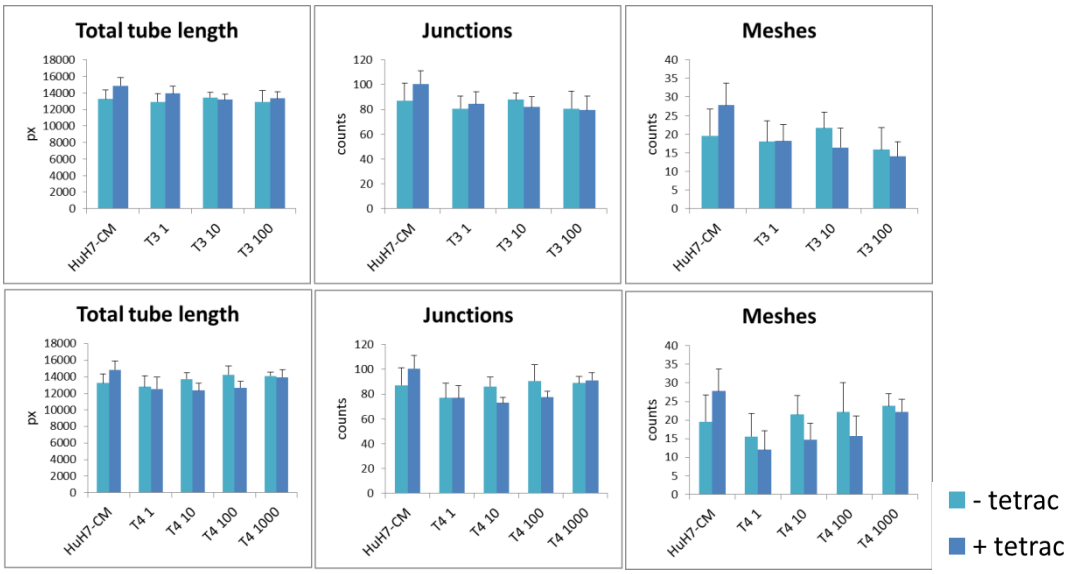


Figure 40: Angiogenesis Assay HUVEC + T3/T4/Tetrac +co-culture-CM (HUH7/MSCS) 12h: Primary human umbilical vein Endothelial cells (HUVEC) were seeded onto growth factor-reduced Matrigel®, treated with different concentration of T3 (1-100nM), T4 (1-1000nM) ± integrin αvβ3-specific inhibitor tetrac and incubated in co-culture-CM (HUH7/MSCS) at 37°C for 12h (5 replicates per respective stimulation). Images were taken at 12h and analyzed with Image J plug-in Angiogenesis Analyzer to evaluate total tube length, junction and meshes. No clear difference between stimulation with or without T3 and T4 was seen in total tube length, junction or meshes. Tetrac modulates effects of T3 treatment. (in quintuplets, Mean+ standard deviation; student's t-test; RLU= relative luciferase unit, significance: * = p<0.05, ** = p<0.01, *** = p<0.001).

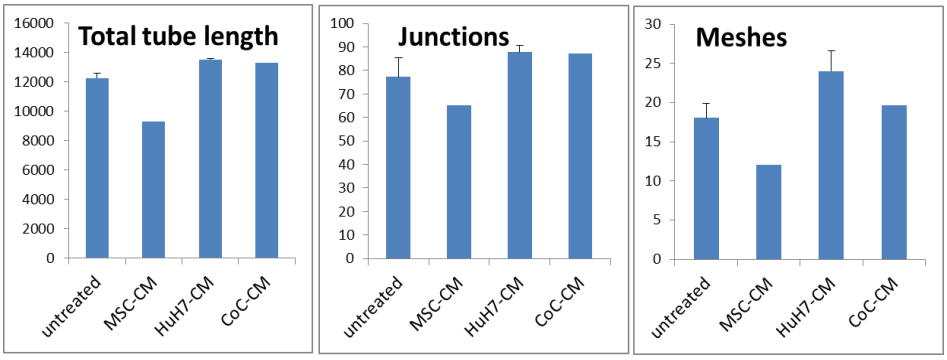
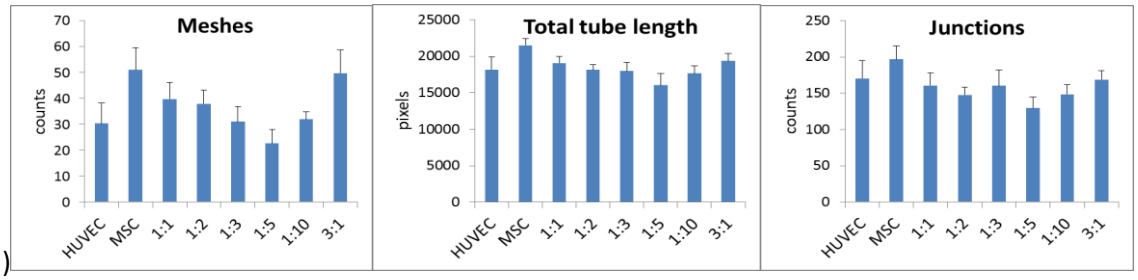


Figure 41: Angiogenesis Assay, untreated versus divers CM 12h: Tube formation was analyzed regarding total tube length, junction and meshes for untreated HUVECS in comparison to MSC-, HUH7-, CoC-CM. Highest outcome can be seen for HUVECs in HUH7-CM medium, immediately followed by CoC-CM. Compared to the other stimulations MSC-CM did not stimulate the HUVECs as much as untreated HUVECS and additional stimulation with HUH7 and CoC -CM. In contrast to HUH7-CM and CoC-CM, MSC-CM was sensitive to T3 and T4 treatment. (See figure 37-40.) (Experiment performed once, respectively in quintuplets, Mean+ standard deviation; student's t-test; RLU= relative luciferase unit, significance:* = p<0.05, ** = p<0.01, *** = p<0.001).

To investigate the effects secreted factors by MSCs, co-culture experiments of MSCs and HUVECs were conducted. The ratio of endothelial cells (HUVECs) and MSCs was investigated. For further experiments a ratio of 1:1 seemed to be good since the maximum of tube formation was not fully reached (Figure 42) but it showed to best outcome compared to 1:2 to 1:10 (MSCS : HUVEC) ratios.

a)



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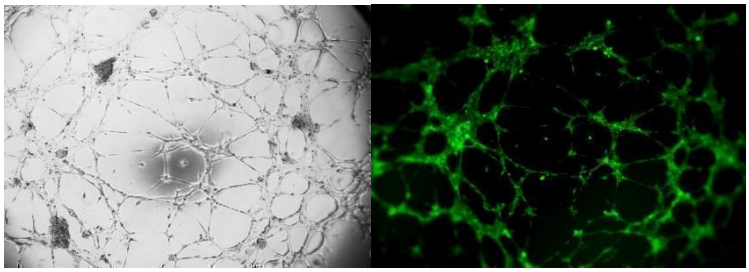

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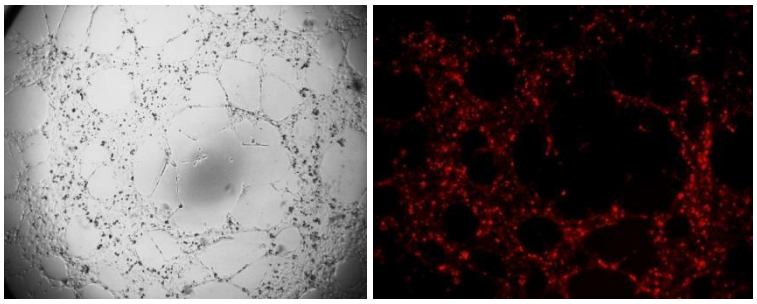

d)

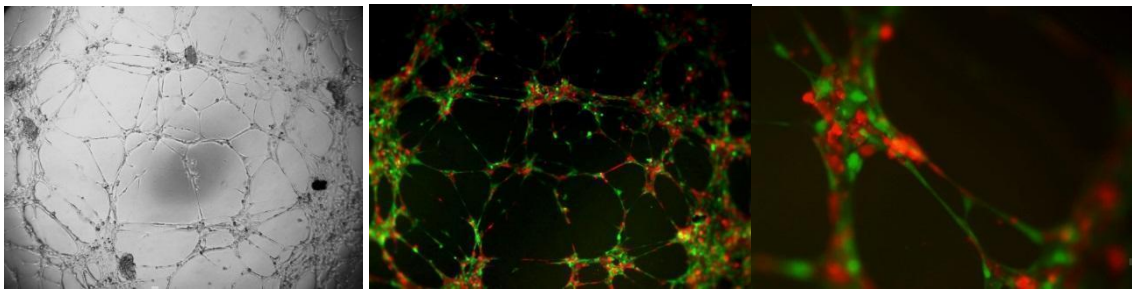

Figure 42: Angiogesis Assay co-culture HUVEC and MSCs: a) Comparison all ratios co-culture HUVEC + MSCS; b) MSC, phase contrast 5x and fluorescence 5x c) HUVEC, phase contrast 5x and fluorescence 5x d) Co-culture HUVEC + MSC, 1:1 ratio, phase contrast 5x, fluorescence 5x and fluorescence 20x (Experiment performed once, respectively in quintuplets, Mean+ standard deviation; student's t-test; RLU= relative luciferase unit, significance:* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$).

4.5 Testing thyroid hormone treatment in angiogenesis assay with genetically modified reporter MSCs

As a last step, the reporter MSCs were tested in the angiogenesis assay. First aim was to investigate if the specific pathways become activated during tube formation. The constructs investigated were as above Hif-1α, Tie2 and VEGF. And again, additional effects of thyroid hormones were tested. In the first experiment, endothelial cells, HUVECs, un-transfected MSCs as well as genetically modified MSCs, MSC - Tie2 and MSC – VEGF, were tested in the Angiogenesis assay, alone or as co-culture ± $CoCl_2$ as extra hypoxic activation. In general, MSCs have a higher baseline activation rate than

HUVECs. For MSCs transfected with Tie2, hbMSCS-Tie2-*Gaussia*, there was a higher *Gaussia* activation compared to Huvecs and MSCs itself, but not significant. Co-transfection with $CoCl_2$ even increased this effect, even though not significant as well. Co-culture of hbMSCS-Tie2-Gaussia and Huvecs showed a lower activation of *Gaussia*, but $CoCl_2$ again increased it, but overall lower than hbMSCS-Tie2-*Gaussia*. For MSCs transfected with VEGF the baseline activation was 3-4-fold higher as compared to HUVECs. hbMSCS- VEGF-*Gaussia* treated with $CoCl_2$ showed no further light reaction, but co-stimulation of hbMSCS- VEGF-Gaussia and HUVECs showed a significant increase as compared to hbMSCS- VEGF-*Gaussia* (p=*≈0.045, 1.19-fold increase). Additional adding of $CoCl_2$ to co-cultured hbMSCS- VEGF-*Gaussia* and HUVECS showed higher results for the co-culture alone, as compared to hbMSCS- VEGF-*Gaussia* (p=*≈0.021, 1.54-fold increase).

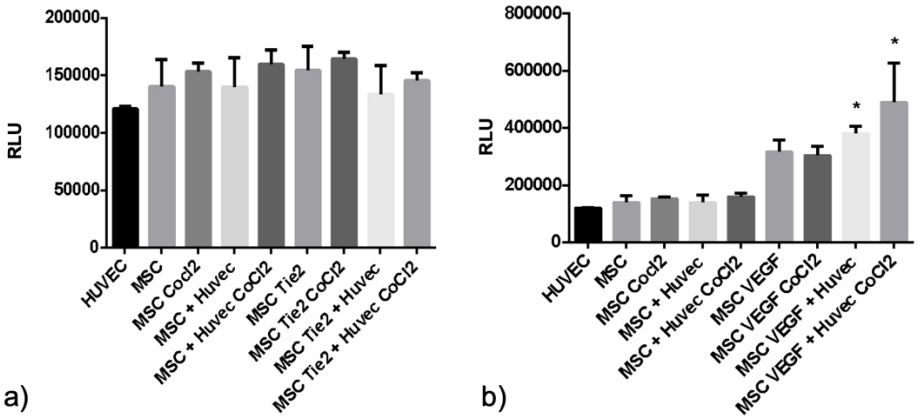

Figure 43: Angiogenesis Assay co-culture HUVEC + MSCS, Tie2- MSCs, VEGF-MSCs 48h: a) In general, MSCs have a higher baseline activation rate than HUVECs. For MSCs transfected with Tie2, hbMSCS-Tie2-*Gaussia, there was a* higher *Gaussia* activation compared to Huvecs and MSCs itself, but not significant. Co-transfection with $CoCl_2$ even increased this effect, even though not significant as well. Co-culture of hbMSCS-Tie2-*Gaussia and Huvecs showed a lower activation of Gaussia, but* $CoCl_2$ *again increased it, but overall lower than* hbMSCS-Tie2-*Gaussia.* b) For MSCs transfected with VEGF the baseline activation was 3-4-fold higher compared to HUVECS. hbMSCS- VEGF-*Gaussia* treated with $CoCl_2$ showed no further light reaction, but co-stimulation of hbMSCS- VEGF-*Gaussia* and HUVECS showed a significant increase compared to hbMSCS-VEGF-*Gaussia* (p=*≈0,045, 1,19-fold increase). Additional $CoCl_2$ on co-culture hbMSCS- VEGF-*Gaussia* and HUVECS was higher than results for the co-culture alone, compared to hbMSCS- VEGF-*Gaussia* and was significant (p=*≈0.021, 1.54-fold increase). Fig.43d shows the whole experiment with Tie2 MSCs, Fig.43e with VEGF MSCs.(Experiment performed once, in triplets to sextuplets, Mean+ standard deviation; student's t-test; RLU= relative luciferase unit, significance:* = p<0.05, ** = p<0.01, *** = p<0.001).

The genetically modified MSCs were then treated with physiologic concentrations of thyroid hormones (T3 1nM and T4 100nM) with or without tetrac to evaluate the effects of thyroid hormones on MSCs in the context of experimental angiogenesis. Genetically modified MSCs (hbMSC-Tie2, hbMSC-VEGF and hbMSC-Hif-1α) were tested alone or as co-culture with HUVECs.
For HUVECs an enhancement after treatment with T3 and T4 was seen and after co-treatment with tetrac. For non-engineered MSCs a similar picture was seen, co-stimulation of T3 and T4 with tetrac led to an increase. Stimulation of co-cultured HUVECs and MSCs showed an increase after T3 plus tetrac stimulation, T4 itself and co-stimulation of T4 and tetrac. All results not significant. See results in Fig. 44. For hbMSCs - Tie2, stimulation with either T3 and T4 with or without tetrac showed no significant changes. Co-culture of hbMSC-Tie2 and HUVECs stimulated with T3 and T4 with and without tetrac did as well not show a difference in light reaction. All results were not significant. For hbMSC-VEGF as well as co-culture hbMSC-VEGF and HUVECs there was no increase seen after stimulation with T3 and T4. Adding tetrac led to a decrease as compared to the respective stimulations without tetrac.
For hbMSC-Hif-1α, there was a significant increase seen after stimulation with T3. Additional stimulation with tetrac showed no effect (hbMSC- Hif-1α plus T3, p=***>0.0001, 1.7-fold increase,

compared to untreated, hbMSC- Hif-1α plus T3 and tetrac, p=***>0.0001 compared to untreated, compared to T3 only p=***≈0.049). Stimulation with T4 as well as co-stimulation with T4 with and without tetrac did not show an increase. Co-culture of hbMSC-Hif-1α and HUVECs stimulated with T3 slightly showed an increase in light reaction, but not significant. Additional treatment with tetrac did not show a difference. Similar to stimulation of hbMSC -Hif-1α alone, the co-culture hbMSC-Hif-1α and HUVECs stimulated with T4 with or without tetrac showed no increase in light reaction, all of those results were not significant.

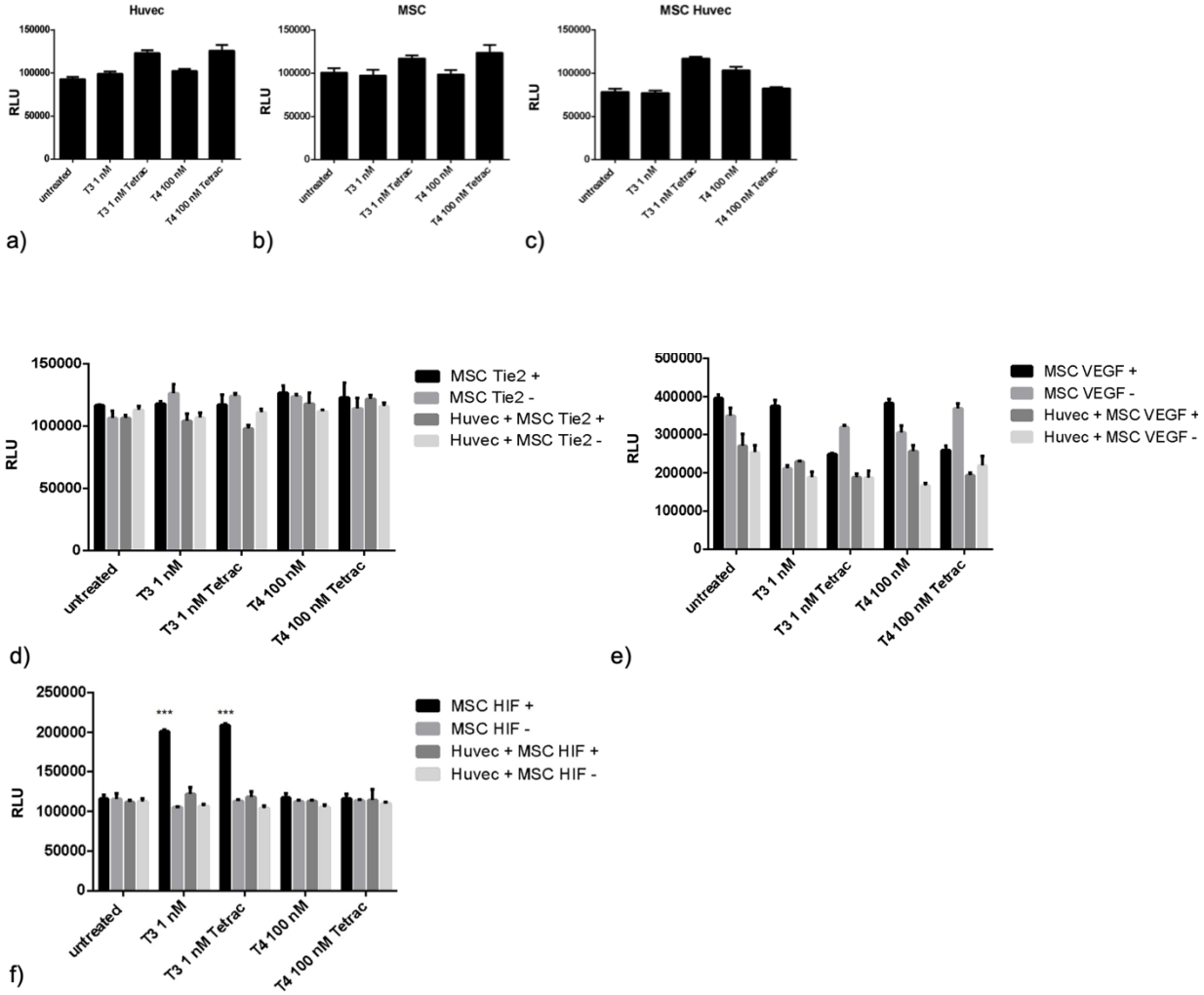

Figure 44. Effects of Thyroid hormone (T3 and T4) and Matrigel on genetically modified MSCs: a) For HUVECs no significant effect was detected. b) For non- engineered MSCs a similar pictures was seen. c) For co-culture of MSCs and Huvecs treatment with T4 100nM did not show a significant effect. d) For MSC-Tie2, there was no significant change. e) For MSC-VEGF as well as co-culture MSC-VEGF and MSC-VEGF plus HUVEC there was no increase to be seen after stimulation with T3 and T4. Interestingly adding tetrac a significant decrease compared to the respective stimulations without tetrac. (MSCS VEGF T3+ tetrac, p=***≈0.00015 compared to T3 only, T4+ tetrac, p=***≈0.000138 compared to T4 only; MSCS VEGF HUVEC T3+ tetrac, p=***≈0.00093 compared to T3 only, T4+ tetrac, p=**≈0.0025 compared to T4 only.) f) For Hif-1α, there was a significant increase after stimulation with T3 seen. Additional stimulation with tetrac did not make a difference (MSCs Hif T3, p=****>0.0001, 1.7-fold increase, compared to untreated, T3+ tetrac, p=****>0.0001 compared to untreated, compared to T3 only p=***≈0.049). Stimulation with T4 as well as co-stimulation with T4 and tetrac did not show an increase. Similar to stimulation of Hif-1α alone, the co-culture MSC-Hif-1α plus HUVEC stimulated with T4 showed no increase in light reaction. Co-stimulation with tetrac did not make a difference. All of those results were not significant. (Experiment performed once, respectively in quintuplets, Mean+ standard deviation; student's t-test; RLU= relative luciferase unit, significance:* = p<0.05, ** = p<0.01, *** = p<0.001).

5 Discussion

5.1 Genetically engineered MSCs in targeted cancer gene therapy

MSCs can be used as a 'trojan horse'- like vehicle to transport diagnostic or therapeutic genes into the tumor stroma. (Hanahan & Weinberg, 2011; Ozawa et al., 2008) An advantage of MSCs is their fast and easy isolation, relatively uncomplicated culture and flexibility regarding genetic modulation. (Bao et al., 2012) We and others investigated the use of herpes simplex virus-thymidine kinase (TK) gene as a suicide transgene in MSCs. Adding the prodrug ganciclovir (GCV) leads to a potent toxin and cell death in the tumor. (Conrad et al., 2011; Knoop et al., 2011; Müller et al., 2016; Rainov, 2000) Other groups have investigated the effects of interferon beta (IFN-beta) eMSCs which inhibits malignant tumor growth. (Studeny et al., 2004). We and others have also evaluated the use of the sodium iodide symporter (NIS) as a theranostic gene as it can be used for diagnostic effects by using I^{123} - scintigraphy and positron emission tomography I^{24}-/F^{18}-TFB as well as a therapeutic transgene through application of I^{131}. (see 5.1.1).

5.1.1 Hypoxia and angiogenesis related transgenes (Hif-1α, Tie2, VEGF) in genetically engineered MSCs

In this thesis we studied the hypoxia and angiogenesis response network in the context of MSC biology as a means of enhancing specific targeted cancer therapy. Hypoxia plays an important role in the tumor tropism of MSCs since it leads to the remodeling process as it takes place in tumor or wound healing. (Horwitz et al., 1999) Hypoxia can initiate angiogenesis and neovascularization. We made use of engineered MSCs with transgenes linked to the hypoxia responsive network - Hif-1α, Tie2 and VEGF to better understand the underlying processes. A synthetic Hif-1α (HRE containing) promoter activated in response to hypoxia, VEGF activated early in angiogenesis and Tie2 thought to be activated at later (Jeltsch et al., 2013) stages of angiogenesis were evaluated.

5.1.2 Establishment of a vector platform for the rapid and efficient engineering of MSCs

One aim of this study was the development of a vector platform for the generation of the expression and reporter plasmids described in 3.3, results in 4.2. The platform makes use of Gateway cloning technology as described in 3.3. that allows different combinations of promoter and reporter genes as well as the ability to monitor the activation of specific pathways crucial for tumor angiogenesis and formation. (Jäckel et al., 2016)

Transfection of plasmids into hbMSCs was conducted using the Sleeping Beauty transposase technology as described in 3.4. In comparison with other transfection technologies such as retro- or adenoviruses, this non-viral technology is less effective, but allows more complex transgenes to be introduced into the target cells (Bao et al., 2012; Young, Searle, Onion, & Mautner, 2006)

In earlier studies, Conrad et al. generated a Tie2 promoter TK transgene and established stable transfected MSCs. This construct was re-cloned using the gateway cloning technology and in a Sleeping Beauty compatible vector. Building the Tie2 vector via Gateway cloning technology was technically complicated and required much time to establish the vector. (see results 4.2.1.3).

The VEGF promoter generated in this study via the gateway cloning platform was combined with *Gaussia*, GFP and Cherry (see results 4.2.1.2).

A co-transfection with the Hif-1α reporter plasmid containing a series of Hif-1α responsive elements (HRE) driving a *Gaussia* reporter gene was performed as proof of principle to validate the correct construction of the plasmids. A second proof of principle was performed by co-transfecting the constitutive active Hif-1α expression vector with the Hif-1α reporter plasmid and doxycycline.

This system was then used to determine if the Hif-1α transcriptional factor could activate the Tie2 and VEGF gene promoters in the stem cells (see results 4.3.3.). In this study, an inducible version of the transcription factor – Hif-1α-CA (constitutively active) plasmid vector was validated to activate the Hif-1α responsive promoter plasmid consists of a Hif-1α responsive element (HRE) demonstrating a positive control for the Hif-1α-CA construct. However, the co-transfection of MSCs with Hif-1α-CA and the VEGF-Firefly or Tie2-Firefly showed no significant activation of the reporter genes. Importantly, other groups have suggested that Tie2 can be increased through enhanced Hif-1α levels. In their study Hif-1α deficiency in Tie2-Cre knockout mice showed reduced wound healing as compared to the control group expressing Hif-1α. The evidence that Hif-1α activates VEGF has been replicated by different studies but could not be demonstrated in the context of the present study. (Ahluwalia & S Tarnawski, 2012; Jo A Forsythe et al., 1996)

To mimic factors present in a tumor milieu and to then test the possible activation of the genetically engineered MSCs in this context, the cells were stimulated with $CoCl_2$, VEGF and HUH7 supernatant. $CoCl_2$ functioned as *in vitro* means of Hif-1α induction. $CoCl_2$ treatment can lead to blockage of the enzyme Hippel- Lindau (VHL) tumor suppressor that normally hydroxylates and degrades Hif-1α, and thus stabilizes the transcription factor (see Introduction 1.2.2.) (Yuan, Hilliard, Ferguson, & Millhorn, 2003). HUH7-derived conditioned media was chosen to mimic the presence of soluble factors in the tumor milieu.

Compared with the interaction between Hif-1α and Tie2 which is not generally well understood, there are many studies which show clear interaction between Hif-1α and VEGF. (Ahluwalia & S Tarnawski, 2012; Chen et al., 2015) Activation of Tie2 by $CoCl_2$ could not be demonstrated in the present study (see results 4.3.2.2.). Several reasons may explain this. Possible activation of Tie2 by Hif-1α may take place indirectly. It is also possible that this activation may be context dependent – such that it does not occur in MSCs but it may occur in other tissue settings. Kakali Sarkar et al. concluded that Hif-1α is needed in Tie2 + cells for homing of bone marrow derived stem cells towards wounds potentially by driving expression of factors needed for facilitated migration. (Sarkar et al., 2012) Licht et al. showed that mouse embryos deficient on Hif-1α had significantly lower vessel development and lower levels of Tie2 based on a dominant-negative Hif-1α mutant which blocked the transcriptional function of Hif-1α. (Licht et al., 2006) Eckardt et al. was able to show the effect of hypoxia (and inflammatory cytokines) on Tie2 in Huvecs. (Willam et al., 2000)

Co-stimulation of the three constructs with $CoCl_2$ and HUH7-conditioned medium were performed to mimic signals from the tumor milieu. The addition of $CoCl_2$ to the HUH7 medium did not significantly increase Hif-1α- *Gaussia* activation compared to stimulation with HUH7 supernatant alone.
Stimulation of Tie2 MSCs with the HUH7 medium with or without $CoCl_2$ showed no significant activation. It could be due to the same reasons as stated above in the discussion of activation of Tie2 just with $CoCl_2$. And on top of that it could also be due to toxic effects of the HUH7 medium with and without $CoCl_2$. Stimulation of hbMSC-Tie2-Gaussia with VEGF did not activate the reporter gene. The question we tried to solve was if VEGF since it gets transcribed early in angiogenesis can activate Tie2 in late angiogenesis. The reason for not getting activated by VEGF could be that VEGF indeed initiates angiogenesis but may not necessarily directly activate Tie2. There are studies that support that VEGF can stimulate shedding of Tie2 via the PI3K/Akt and p38 MAPK pathway. (Findley et al., 2007) Stimulation of hbMSCS-VEGF-Gaussia cells with HUH7 conditioned medium showed no significant effect.

5.2 Effects of thyroid hormones in angiogenesis and the hypoxia response network in MSCs

5.2.1 Implementation of a protocol of an angiogenesis assay

As the first step, an angiogenesis assay was established. In an overview by Auerbach et. al, of different angiogenesis assays *in-vitro* and *in-vivo* were compared. One of the conclusions drawn was if you want to perform an angiogenesis with more than one cell line and evaluated component, than *in-vitro* experiments are the better choice as compared to *in-vivo* angiogenesis assays. (Auerbach et al., 2003; Terman & Stoletov, 2001)

Another overview regarding *in-vitro* and *in-vivo* angiogenesis pointed out the difficulties between possible interesting findings in in-vitro and *in-vivo* studies but problems of proceeding in phase III/IV clinical trials. (Staton et al., 2009) In this thesis, analysis of tube formation for the endothelial cells were conducted with the angiogenesis analyzer by ImageJ. Carpentier et al. has recently made a study/comparison about the use of 'Endothelial tube formation assay', which we used in this study as a 2D model, and the 'Fibrin bead assay', as an 3D assay suggesting that these methods represent an intermediate step relative to an in-vivo angiogenesis assay. (Carpentier et al., 2020)

5.2.2 Effects of Thyroid hormones in angiogenesis

After implementing the basic settings for the angiogenesis assay, we then tested the cells in the angiogenesis assay in response to thyroid hormones.

We had previously shown that thyroid hormones can enhance MSC tumor tropism and are necessary when the NIS gene is used for the MSC-based therapy approach. As stated above, thyroid hormones reduce the expression of the physiological NIS in the thyroid gland. (Schmohl et al., 2019) In addition there is growing evidence that thyroid hormones have an influence on angiogenesis by inducing the differentiation of endothelial cells and by initiating angiogenesis by supporting vessel formation by activating crucial angiogenic factors such as Hif-1α and VEGF. (Mousa et al., 2006)

As described in 1.5., two different pathways are mediated via the non-genomic thyroid hormone receptor $\alpha v\beta 3$. The PI3K/Akt, activating Hif-1α, and the ERK1/2 (MAPK) pathway activating VEGF and endothelial proliferation. (Chen et al., 2015; Davis et al., 2009; Mousa et al., 2006) We investigated the impact of thyroid hormones T3 and T4 on endothelial tube formation on its own, and in combination with the evaluation of potential pro-angiogenic effects of non-genetically modified MSCs (see 5.2.1 for genetically modified MSCs), and tumor cells. A dose-dependent activation was seen for T3 stimulation of endothelial cells. Treatment with T4 also showed a dose dependent increase of all three evaluated doses, even though it is not as clear as the data with T3 and did not happen in a tetrac dependent manner. (Schmohl et al., 2019)

Additional treatment of HUVECs with MSC-CM led to less tube formation but co-stimulation of HUVECs with MSC-CM plus T3 and T4 lead to an increase in tube length, junction formation and meshes as compared to HUVECs stimulated with MSC-CM. These observations suggest that thyroid hormones have a direct effect on angiogenesis and that they may unfold their effects on angiogenesis by effecting the production of pro-angiogentic factors in MSCs.

Testing endothelial tube formation with HUH7-CM led to the highest rate of total tube length, junction formation and meshes seen. This supports the suggestion that factors secreted within the tumor milieu helps drive angiogenesis. Further stimulation with thyroid hormones had no additional effect. Further experiments and studies are clearly required to get a full picture of the biology at work.

Stimulation of endothelial cells with co-culture conditioned medium CoC-CM (HUH7/MSCS) activated tube formation as high as treatment with HUH medium alone, again with no enhancement by co-stimulation with T3/T4 treatment. (Schmohl et al., 2019)

5.2.3 Effects of thyroid hormones on the hypoxia and angiogenesis response network in genetically engineered MSCs in and without angiogenesis assay

In earlier studies by our group, we showed effects of thyroid hormones and tumor supernatant on MSC biology. In these experiments, MSCs stimulated with HUH7 medium and thyroid hormones showed influenced ANG, ANGPT2 and Hif-1α expression. Interestingly Tie2 and its ligand ANGPT1 were not found to be activated in this context. (Schmohl et al., 2019)

Thyroid hormones can have an influence on the hypoxia response network in the tumor milieu. T3 and T4 have been shown to influence Hif-1α and hence activation of hypoxia responsive genes. (Müller et al., 2016; Salb, 2018; Schmohl et al., 2019). In the present study, we used various Hif-1α transgenes to study potential effects on Tie2 and VEGF to hypoxia-related effects.

Stimulation of hbMSCS-Hif-1α-*Gaussia* with T3/T4 ± CoCl2 led to an increase in Hif-1α Gaussia luciferase activation for T3 1nM and 10nM. Co-stimulation with T3 1nM and 10nM with $CoCl_2$ increased the Hif-1α-*Gaussia* light reaction significantly for T3 1nM. For T3 10 nM an additional effect could also be seen, even though it was not significant. Those results support the thesis that T3 in co-stimulation with Huh7 conditioned medium- functioning as in-vitro tumor milieu - possibly have an effect via Hif-1α on the hypoxia response system. Similar results were described in studies by our working group and others. (Knoop et al., 2011) The effects of T4 were not significant.

Stimulation of hbMSCS-Tie2-*Gaussia* was performed with T3/T4 ± $CoCl_2$. The addition of T3 led to a significant increase in Tie2 -*Gaussia* luciferase activation for T3 at 10, 100nM. Co-stimulation of $CoCl_2$ showed a significant increase for all doses of T3 and T4 tested. The successful use of the same Tie2 gene promoter to drive a therapy gene (thymide kinase) expression in engineered MSC was previously demonstrated by our group where the approach was used to treat experimental breast cancer. (Niess et al., 2011)

Stimulation of hbMSC-VEGF-*Gaussia* with T3/T4 ± CoCl2 was also performed. The addition of T3 led to a significant increase in VEGF *Gaussia* luciferase activation for T3 10nM. Single addition of T4 did not lead to a significant increase in VEGF *Gaussia* luciferase activity. Co-stimulation of $CoCl_2$ with T3 1nM, 10nM and 100nM significantly elevated the *Gaussia* light reaction as compared stimulation with T3 alone. Co-stimulation of hbMSC-VEGF-*Gaussia* with T4 and $CoCl_2$ showed for T4 10 and 1000nM a decrease in light reaction compared to single treatment of T4. Overall, these results suggest that stimulation with thyroid hormones can enhance activation of Hif-1α, Tie2 and VEGF slightly, and in combination with a hypoxic stimulus the results were partially significant. The results support the hypothesis that thyroid hormones have an influence on the hypoxia and angiogenesis network in tumor growth. (Davis et al., 2009; Mousa et al., 2006)

The modified transgene reporter cells (MSCs Hif-1α, Tie2 and VEGF) were then tested in the *in vitro* angiogenesis assay, and potential effects of thyroid hormones tested. HbMSCS- VEGF-*Gaussia* treated with $CoCl_2$ showed no increased light reaction, but co-stimulation of hbMSCS- VEGF-*Gaussia* and HUVECS showed a significant increase as compared to hbMSCS- VEGF-*Gaussia* cells. The addition of $CoCl_2$ to the co-cultured hbMSCS- VEGF-*Gaussia* and HUVECS showed more pronounced results relative to the co-culture alone. The results for the hbMSCS-Tie2-Gaussia in the angiogenesis assay were unclear, even though additional treatment with $CoCl_2$ showed a positive tendency.

We then treated the genetically modified MSCs with thyroid hormones at physiologic concentrations (T3 1nM and T4 100nM) with or without tetrac to investigate the effects of thyroid hormones on the Hif-1α, Tie2 and VEGF gene reporters engineered into MSCs in the experimental angiogenesis assay, alone and in co-culture with HUVECs.

For Hif-1α there was a significant increase after stimulation with T3 seen. Additional stimulation with tetrac did not make a difference. Stimulation with T4 as well as co-stimulation with T4 and tetrac on Hif-1α as well as Hif-1α and Huvec did not show an increase. Co-culture of Hif-1α and Huvec stimulated with T3 slightly showed an increase in light reaction, tetrac did not show a difference.

For hbMSCSs Tie2 Gaussia as well as co-culture Tie2 and Huvec no significant effect was seen. Stimulation with T3 or co-stimulation with T3 and tetrac did not show an increase. All results were not significant. Stimulation with T3 in co-culture of Tie2 – MSC reporter cells and Huvec did not show a difference in the reporter levels, tetrac decreased it but not significantly. For VEGF as well as co-culture VEGF and Huvec there was no increase to be seen after stimulation with T3 and T4.
Our data suggest that thyroid hormones T3 and T4 can influence tumor stroma formation, in particular in the context of the response to hypoxia.

5.2.4 The use of tumor stroma - active gene promoters in the context of MSC-driven gene therapy of cancer

Using a gene promoter that is activated by signals found in tumor stroma are thought to help reduce potential off-target side effects and thus enhance the efficacy of gene therapy. (Müller et al., 2016; Schug et al., 2019) In earlier studies of our working group, we made use of angiogenesis and hypoxia related promoters (Conrad et al., 2011; Müller, 2017) as well as inflammatory related transgenes such as the RANTES/CCL5 promoter. (Hagenhoff, 2018; Zischek, 2011) (Knoop et al., 2011)
Conrad et al. showed that the Tie2 promoter driving the red fluorescent protein (RFP) in eMSCs became activated in tumor stroma. When the RFP gene was replaced by the suicide gene herpes simplex virus-thymidine kinase (TK) gene and the animals were treated with the prodrug ganciclovir resulted in a dramatic reduction in tumor growth. (Conrad et al., 2011)
Müller et al. investigated the efficacy of a synthetic Hif-1α responsive promoter driving the theranostic NIS symporter gene that can be used a diagnostic as well as therapeutic transgene. The study showed that the transgene became specifically activated in hypoxic areas of the tumor as determined by imaging using ^{123}I - scintigraphy and positron emission tomography ^{24}I-/^{18}F-TFB. The application of ^{131}I as a therapeutic agent showed a robust therapeutic effect (Müller, 2017) Importantly, in these studies, the animals were pretreated with T3/T4 to reduce thyroid expression of NIS. This is protective for the thyroid and also enhances the circulating levels of ^{131}I. The work shown here suggests that the thyroid hormones may also directly impact the MSCs in the context of tumor angiogenesis.

5.3 Outlook

Stem cell gene therapy provides an individualized cancer gene therapy. Using genetically engineered MSCs with specific promoters activated in the tumor milieu may help us better understand the underlying pathways and potentially enhance treatment of cancer. This thesis was performed to provide insight into relevant processes behind individualized stem cell - gene therapy and to better understand aspects of tumor biology specifically in the context of MSC differentiation during angiogenesis with a special emphasis on the interplay of thyroid hormones with this biology.

6 Addendum

6.1. Tables of tables

6.2. Tables of figures

6.3. Abbreviations

Akt ..... Protein Kinase B
Ang-1 ..... Angiopoeitin 1
Ang-2 ..... Angiopoeitin 2
Ang-4 ..... Angiopoeitin 4
ATP ..... Adenosine Triphosphate
BSA ..... Bovine serum album
CAF ..... Cancer Associated Fibroblasts
CCL2 ..... Monocyte Chemotactic Protein 1
CCL5 ..... CC-chemocine ligande 5
CMFDA ..... 5-chloromethylfluorescein diacetate, *Siehe* 5-chloromethylfluorescein diacetate
CMTMR ..... 5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine
CMV ..... Cytomegalie Virus
$CoCl_2$ ..... Cobalt (ll) Chlorid Hexahydrate
DMEM ..... Dulbecco's Modified Eagle Medium, Dulbecco's Modified Eagle Medium
DMSO ..... Dimethyl Sulfoxide
DPBS ..... Dulbeccos phosphate buffered saline
E.coli ..... Escherichia coli
EDTA ..... Ethylenediaminetetraacetic acid
ERK2 ..... Extracellular-signal Regulated Kinase
AK ..... Focal adhesion kinase
FCS ..... Fetal calf serum
Flt-1 ..... Vascular Endothelial Growth Factor recpetor 1
GFP ..... Green Fluorescent Protein
GLOBOCAN ..... Global Cancer Observatory
hbMSCs ..... Human Bone Marrow Stromal Cells
HCC ..... Hepatocellular Carcinoma
HDBEC ..... Human dermal blood endothelial cells
HGF ..... Hepatocyte Growth Factor
Hif-1α ..... Hypoxia inducible factor- 1α
HLA ..... Human Leucocyte Antigene
HMECS ..... Human Mammary Epithelial Cells
HRE ..... Hormone Responsive Element

HT1080 ..... Fibrosarcoma Cell Line
HUH7 ..... Hepatocellular carcinoma cell line
HUVECs ..... Human Umbilical Vein Endothelial Cells
IL-6 ..... Interleukin (IL)-6
iMSCs immortalized MSCS
IU International unit
L87 ..... immortalized mesenchymal stemcells
MAPK1 ..... Mitogen-activating Proteinkinase 1
MCP ..... Monocyte Chemoattractant Protein
MSCs ..... Mesenchymal st em cells
ml *milliliter*
NEB New England Biolabs
NIS ..... Nitric Oxide Synthase
ORF Open Reading Frame
PBS ..... Phosphate-buffered saline
PCR ..... Polymerase Chain Reaction
PDGF ..... Platelet-Derived Growth Factor
PGF ..... Prostaglandin F
PI3K Phosphatidylinositol-3-KinaseKDR/Flk-1
PS Penicillin/Streptomycin
PTK2 ..... Protein-Tyrosinkinase 2
rpm Revolution per minute
RPMI ..... Roswell Park Memorial Institute, Roswell Park Memorial Institute
T3 ..... 3,3', 5,5'-Triiodo-L-Thyronin-sodium acid
T4 ..... Thyroxine
TEK ..... Tyrosin Endothelial Cell Kinase
Tetrac 3,3', 5,5'-Tetraiodothyroacetic acid
TGF-ß ..... Tumor Growth Factor ß
Tie-1 ..... Tyrosinkinase 1
Tie2 ..... Angiopoietin-1 receptor
TNF-α ..... Tumor Necrosis Factor
TRH ..... Thyrotropin-Releasing Hormone
TSH ..... Thyroid Stimulating Hormone
V54 ..... immortalized mesenchymal stemcells
VEGF ..... Vasular endothelial growth factor
WHO ..... World Health Organisation

7 References

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9 Eidesstattliche Versicherung

Ich, **Maike Dohmann**, erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Titel „**Engineered mesenchymal stem cells as therapeutic vehicles for tumor therapy: Angiogenesis-based targeting and the influence of thyroid hormones“** selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

Maike Dohmann

Hamburg, den 22.05.2025

10 Übereinstimmungserklärung der gebundenen Ausgabe der Dissertation mit der elektronischen Fassung

Ich, Maike Dohmann, erkläre hiermit, dass die elektronische Version der eingereichten Dissertation mit dem Titel: **Engineered mesenchymal stem cells as therapeutic vehicles for tumor therapy: Angiogenesis- based targeting and the influence of thyroid hormones** in Inhalt und Formatierung mit den gedruckten und gebundenen Exemplaren übereinstimmt.

Maike Dohmann

Hamburg, den 22.05.2025