universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

„Über die Rolle von Desmin bei der Entwicklung von Herzzellen“

„On the intracellular role of desmin in cardiomyogenesis“

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1.Introduction

1.1. Intermediate filament proteins

The cytoskeleton of all metazoan cells contains three major types of filament systems: the actin microfilaments (MFs), the microtubules (MTs), and the intermediate filaments (IFs). The name “intermediate filaments” comes from their diameter (10-12nm) being intermediate of that from MTs (25nm) and MFs (7-10nm). The network of the three filament systems is responsible for the mechanical integrity of the cell and is critically involved in processes such as cell division, plasticity and motility. *In vivo* IFs function in close association with cytoskeletal components such as motor proteins and plakin-type cross bridging proteins. IFs harbour unique structural features, which make them different from other filament systems: First, the elementary unit of an IF is an elongated (45nm) and thin (2-3nm) rod-like dimer. In contrast to that, elementary units of MF and MT are globular proteins consisting of actin monomers and αβ-tubulin heterodimers, respectively. Second, assembled IFs have no polarity: they are oriented in “up” and “down” direction along the same filament. In contrast to that, actin MFs and MTs are polar. This polarity allows active transport of motor proteins, such as myosin and kinesin, along these filaments. Third, *in vivo* IFs appear to be the most dynamic filament type: assembly and disassembly can occur along the whole filament not only at the ends, as it is the case in MFs and MTs *(S.V.Strelkov, 2003)*.

The intermediate filament protein family was classified into five different major subtypes as listed in the table below. The classification is based on comparison of amino acid sequences.

Table 1.1: Intermediate filament proteins*: groups I-IV are considered (predominantly) cytoplasmic, while the lamins (group V) are found in the nuclei of all cell types.*.

Type	Protein	Size (kDa)	Site of expression
I	Acidic keratins (~15 proteins)	40–60	Epithelial cells
II	Neutral or basic keratins (~15 proteins)	50–70	Epithelial cells
III	Vimentin	54	Fibroblasts, white blood cells, and other cell types
	Desmin	**53**	***Muscle cells***
	Glial fibrillary acidic protein	51	Glial cells
	Peripherin	57	Peripheral neurons

IV	Neurofilament proteins		
	NF-L	67	Neurons
	NF-M	150	Neurons
	NF-H	200	Neurons
	A-Internexin	66	Neurons
V	Nuclear lamins	60–75	Nuclear lamina of all cell types
VI	Nestin	200	Stem cells of central nervous system

The classification of the intermediate filament protein nestin is quite controversial. Some authors count it to class III, because of its amino acid sequence, but according to its gene organisation it also may fit into class IV. However, it is, like synemin, unable to form filaments on its own, and in the table above it is listed in class VI.

Despite the diversity of intermediate filament proteins in size and amino acid sequence, the various intermediate filament proteins share a common structural organization, which is shown in figure 1.1.1.

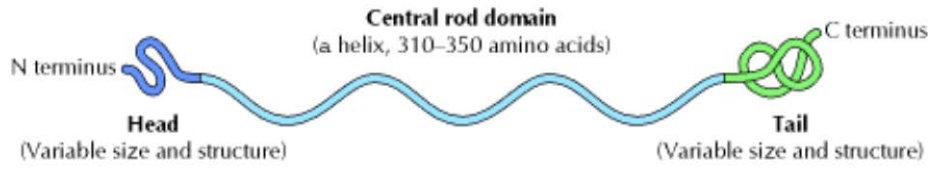


Fig.1.1.1: The structural organization of an intermediate filament protein *(from "The Cell a molecular approach"2nd edition;Geoffrey M.Cooper, 2000)*

Figure 1.1.1 shows a typical monomer of a type III intermediate filament protein. The central alpha(α)-helical rod domain is needed for filament assembly. The amino(N)-terminal and carboxy(C)-terminal portions are highly variable among the different subtypes and determine the specific functions of the intermediate filament proteins. In case of desmin the N-terminus can interact with a variety of other proteins and DNA *(Tolstonog et al. 2005; Costa et al.2004)*. Desmin was found to interact with other IF-proteins, with nebulin, plectin, dynein, the myogenic regulator protein myoD and DNA. In addition to that, the N-terminus contains specific serine residues (phosphorylation sites), which might be of importance in the control of the cell cycle and probably even in development. (*Höllrigl et al. 2007)* Together, these findings and the variety of its *in-vitro* interaction partners suggest that desmin is more than just a mechanically important structural protein.

Another classification of intermediate filament proteins is made with respect to their assembly properties. Here three different classes were described: Group I consisting of acidic and basic keratins, group II with desmin and other mesenchymal, muscle, or neuronal IF proteins, and group III, built by the nuclear lamins. Only the members of the same assembly

group are able to co-assemble in a productive manner. Most of the assembly studies have been done on vimentin *(Herrmann and Aebi, 1998b)*. Although vimentin and desmin exhibit high amino acid sequence identity and may co-assemble with each other, they differ in some aspects: Desmin filaments are thicker and assemble much faster than vimentin *(Herrmann et al., 1999)*. *In vitro* studies of IF assembly showed, as first step, the formation of a parallel coiled-coil dimer, then two dimers associate in a half-staggered, anti-parallel manner to form a tetramer. These tetrameric units are the smallest stable soluble complexes found *in vitro*. Within seconds, they associate laterally to form a so-called "unit-length filament" (ULF). ULFs measure about 58 nm in length and 16 nm in diameter. In the next step ULFs anneal longitudinally to form loosely packed filaments. Within minutes after initiation of assembly, these loosely packed filaments undergo a process of "radial compaction". The radial compaction reduces filament diameter to about 14 nm (in desmin). Finally, a mature apolar filament is obtained. Apolarity results, according to data obtained from chemical cross-linking, from the fact that individual dimers alternate their orientation along the filament and associate in a heads-to-tails manner *(Bär H. et al., 2004)*.

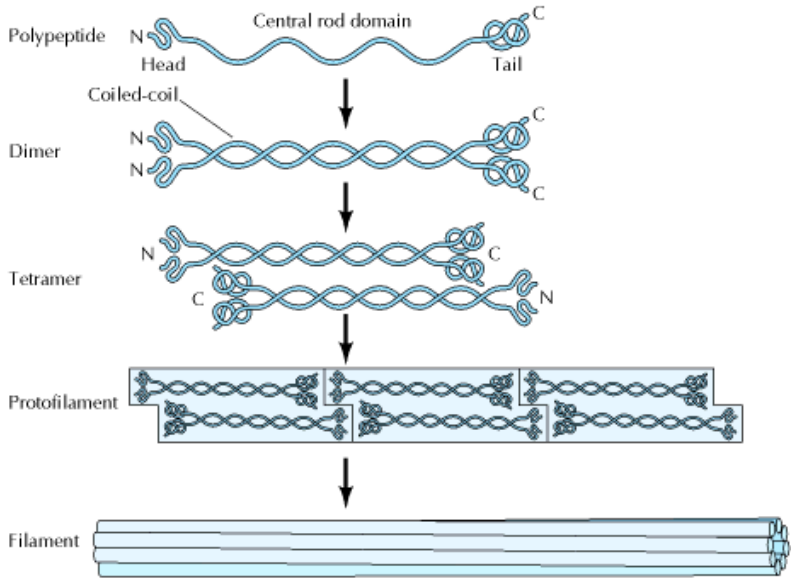


Fig.1.1.2: The Modular arrangement of IF protein monomers in filament assembly: *Two IF protein monomers wind around each other in a head to head orientation. The resulting dimers associate with another dimer in a heads to tails orientation and form a tetramer. The tetramers further assemble into proto-filaments, where N-terminal heads always face C-terminal tails. ("The Cell a molecular approach"2nd edition; Cooper, 2000)*

1.2. About "Desmin"

Desmin protein was purified first almost at the same time by J.V.Small and A.Sobieszek and by E.Lazarides and B.D.Hubbard in 1976 *(Lazarides & Hubbard 1976; Small & Sobieszek 1977)*. Lazarides and Hubbard purified a 50 kilo Dalton (kDa) protein from chicken gizzard

(smooth muscle). They also succeeded to produce antibodies against it and used them for indirect immunofluorescence staining. The immunofluorescence staining located the protein in muscle. However, to indicate the proteins most obvious function, the discoverers termed it after the greek word "δεσμός", which means "bond" or "link". The *desmin* gene was characterized in 1984 *(Capetanaki et al.1984),* its transcriptional regulation by the myogenic transcription factors MyoD, myogenin, MRF4, and Myf5 was elucidated in 1993 *(Li et al. 1993)* and knockout animals were generated in 1996 *(Milner et al. 1996).* Nowadays, research on desmin is more focussed on desmin-associated disease such as desminopathy or desmin-related myopathy.

Desmin is a muscle specific type III intermediate filament (IF) protein. It is one of the earliest known myogenic markers and it is occurring exclusively in muscle and endothelial cells. As a cytoplasmic protein it plays a crucial role in the maintenance of cellular architecture. In muscle cells there are other IF proteins such as nestin, peripherin, and vimentin, besides the ubiquitous lamins, but in contrast to desmin, they are all not unique to muscle *(Costa et al. 2004).* The desmin protein connects the sarcomeres, which are the contractile elements of a muscle cell, along so-called Z-lines (or Z-discs). In addition to that, desmin interconnects myofibrils to form muscle fibers and anchors myofibrils to the inner portion of the cell membrane (sarcolemma) and to the outer portion of the nuclear membrane. It also serves to connect neighbouring cells via desmosomes (in cardiac muscle) or costameres (in skeletal muscle) and therefore contributes to inter-cellular mechanical integrity.

Figure1.2.1:

A) The organization of a skeletal muscle. *(from "The Cell a molecular approach"2nd edition; Cooper, 2000)*

B) The intracellular location of desmin intermediate filaments *(from Bär et al.2004)*

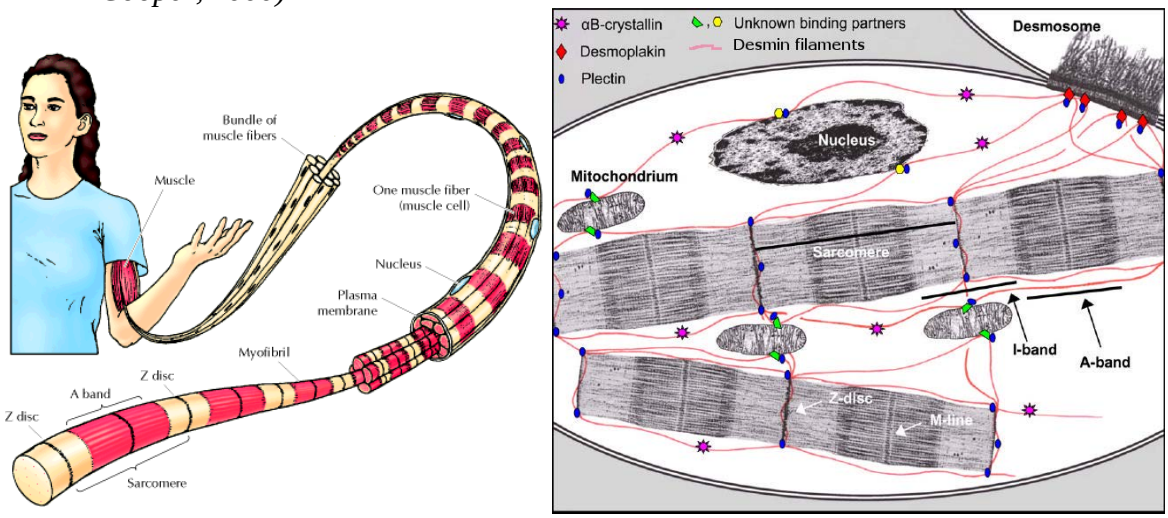


Figure 1.2.1A: *The organization of a skeletal muscle as a bundle of muscle fibers. Each of these muscle fibers consists of interconnected myofibrils. The myofibrils are formed by syncytic cells, which contain sarcomeres. The sarcomeres are the contractile units in a muscle*

cell. ***B)*** *A schematic drawing of the localization of Intermediate filaments in muscle cells. It depicts the IF proteins cellular localization and its interaction partners (from Review in Journal of Structural Biology Vol 148, Bär et al. 2004); Desmin filaments are shown as pink lines.*

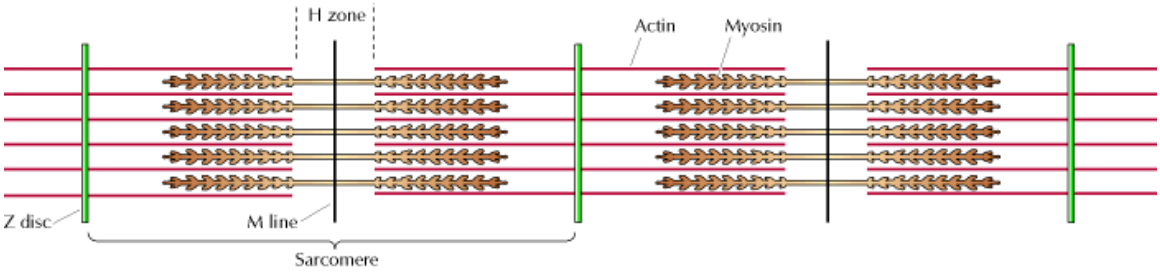


Fig.1.2.1C: The structure of a sarcomere: *(from "The Cell a molecular approach"2nd edition; Cooper, 2000). The sarcomeres contain actin and myosin filaments in a staggered array. Z-lines mark the regions where the sarcomeres are in contact. Desmin interconnects the sarcomeres and the myofilaments at the Z-lines.*

The sarcomeres, which are the contractile subunits in a muscle cell, consist of actin and myosin filaments. During muscle contraction the actin and myosin filaments slide along each other. This leads to a reversible shortening of each sarcomere along a muscle. One sarcomere measures about 2.3µm in length. The ends of the sarcomeres are defined by the so-called Z-discs. Here desmin filaments interconnect the sarcomeres as it is mentioned above. The sarcomeres contain several distinc regions: The "I-band", which consists of actin filaments only whereas the dark "A-band"(A stands for anisotropic) contains both, the actin and the myosin filaments. At the "H zone"the myosin filaments are anchored at the midline (M line).

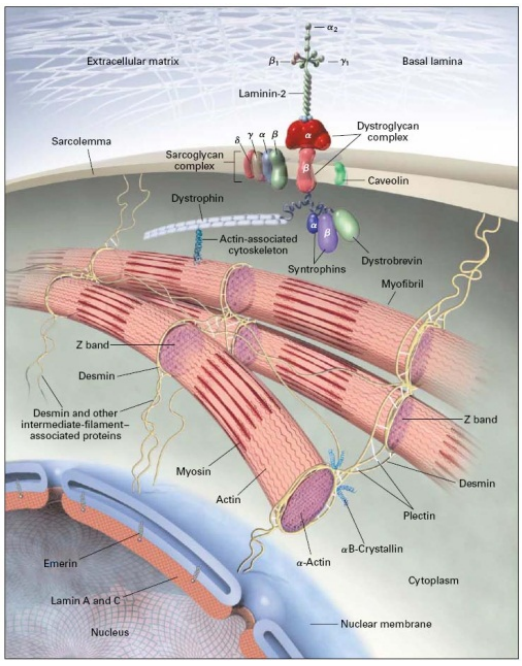


Fig.1.2.2: Desmin in maintenance of structural integrity of myocytes: *Desmin as a component of a muscle cell serves for stability structural integrity and interconnection of cellular compartements and cells in a tissue. (Goldfarb et al. 2008)*

1.3. The biochemical aspects of desmin

The human desmin protein is encoded by a single copy gene (*des)*, located on chromosome band 2q35. It encompasses nine exons within an 8.4 kilobases (kb) region and codes for 476 amino acids. The gene is highly conserved among the vertebrate species. The human desmin protein weights 52 kDa and has an isoelectric point (pI) at 5.4 *(Costa et al.2004).*

Desmin, like all IF proteins, is very resistant to chemical denaturation. After homogenization of myofibrils, desmin filaments are highly enriched in the insoluble high-salt-resistant residue, which is a characteristic property of all IFs. To solubilize this fraction of cytoplasmic proteins, high concentration of denaturing agents, like 8 M urea or 6 M guanidine hydrochloride is needed. Upon removal of denaturing agents, IFs can completely be re-natured. In short, IFs are very stable proteins, which are able to self re-assemble in the appropriate ionic strenght after chemical denaturation. This fact unfortunately impairs extensive study of desmin's structural traits. So, only little information from crystallographic studies is available, and most knowledge comes from studies of vimentin, which is very similar to desmin in its amino acid sequence, structural traits and folding behaviour. *(Bär et al.2004)*

In accordance with its function, the desmin molecule is organized into three domains: a highly conserved alpha(α)-helical core of 308 amino acid residues flanked by globular amino(N)- and carboxy(C)-terminal ("head" and "tail") structures as shown in figure 1.3. The α-helical core maintains a seven residue (heptad) repeat pattern with a typical sequence of hydrophobic and hydrophilic amino acids. This heptad repeat structure guides two polypeptides into formation of a homo-polymeric coiled-coil dimer, the elementary unit of an intermediate filament. The heptad periodicity within the helical rod is interrupted in several places. This led to determination of the four consecutive helical segments 1A, 1B, 2A, and 2B, which are connected by short non-helical linker regions (L1, L12, and L2). The segments 1A and 2B contain highly conserved regions that are very important in association of two dimers to form tetramers. The 2B segment located at the C-terminal part of the desmin core domain contains a discontinuity in the heptad repeat pattern, a so called "stutter". The "stutter" is an obligate feature of all IF proteins and its position is absolutely conserved. Experimental "straightening out" of the stutter by addition of three amino acids would restore the heptad pattern but leads to inability of this "stutterless" molecule to anneal into longer filaments.

Another thoroughly examined structure is the 405-YRKLLEGEESRI-416 motif at the C-terminal end of the 2B helix. Here the coiled-coil structure loosens and the α-helices gradually separate, eventually bending away from each other. *In vitro* data demonstrated that this motif directs the proper formation of tetramers and controls the number of segments per filament cross section.

The desmin C-terminal "tail" is involved in longitudinal "heads-to-tails" tetramer assembly and control of lateral packing, stabilization and elongation of the filament structure. However, the tails major function seems to be interacting with other cytoskeletal proteins to establish a cytoplasmic intermediate filament network. *(Bär et al.2004)*

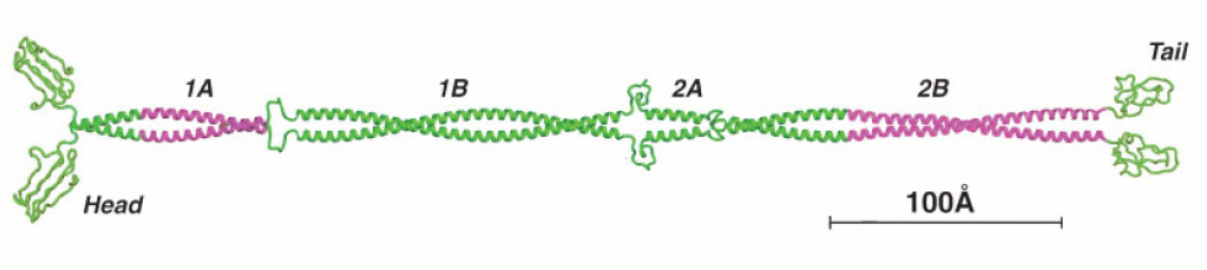


Fig1.3.: A model of an intermediate filament dimer *(Strelkov et al. 2003).*
Originally this picture was shown as a model for vimentin, but the two type III IFs exhibit high amino acid sequence identity and the modular arrangement of α-helical structure is shared by desmin. It is mainly built by a series of coiled-coils. Periodicity is interrupted at several sites, which led to determination of four consecutive α-helical segments (1A, 1B, 2A, and 2B).The segments 1A and 1B together form coil 1, 2A and 2B form coil 2. The α-helical segments are interconnected by relatively short linkers (L1, L12, and L2). The regions shown in purple are highly conserved and are critically involved in dimer-dimer interaction.

Desmin- like all type III intermediate filaments- can form homopolymers and heteropolymers. It normally interacts with many other structural proteins, including intermediate filament associated proteins (IFAPs), which cross-link desmin filaments to a dense network that anchors the cytoskeleton. Mutated desmin is thought to be impaired in its interaction with these proteins, and in consequence to trigger disease.

Desmin usually is phosphorylated at several distinct serine residues. The phosphorylation is thought to regulate polymerisation of desmin. Signaling molecules such as cAMP-dependent kinase and protein-kinase C were shown to increase the state of phosphorylation of desmin and to interfere with the filament assembly *(Kitamura et al., 1989).* In consequence of desmin over-phosphorylation, changes in the structure of the filament network were observed *(Huang X. et al., 2002)*. Moreover, Aurora-B kinase, which phosphorylates serine and threonine residues, was shown to be engaged in phosphorylation of desmin at the cleavage furrow during cytokinesis, probably to serve for proper segregation of desmin filaments to daughter cells.

1.4. Development of the heart

The heart is part of the circulatory system, together with the blood cells, and a system of blood vessels. It serves to nourish the developing vertebrate embryo. The heart is the first functional organ in the developing organism. The circulatory system is built from the lateral plate mesoderm. The lateral plate mesoderm itself divides into two separate regions: the first is the dorsal **somatic (parietal) mesoderm**, which underlies the ectoderm. The second is the ventral **splanchnic (visceral) mesoderm**, which overlies the endoderm. The heart itself is built from two regions of splanchnic mesoderm, one at each side of the body. The splanchnic mesodermal regions, which will form the heart, are influenced by their surrounding tissue. The presumptive heart cells originate from a region, which is close to the primitive streak and lateral to the node. The cells of the **cardiogenic mesoderm** migrate anteriorly between the ectoderm and the endoderm towards the midline of the embryo. The direction of migration seems to be implied by the foregut endoderm. This is assumed, because of the finding, that when the cardiac region endoderm is rotated with respect to the rest of the embryo, the direction of migration of the cardiogenic mesodermal cells is reversed. It is thought that **fibronectin** is the factor responsible for the direction of the migration. The endodermal factor fibronectin most probably provides the cardiogenic mesoderm cells with a concentration gradient to move along. In 1988, Linask and Lash showed that antibodies against fibronectin stop this migratory process, while antibodies against other extra-cellular matrix proteins had no influence on the migration of cardiogenic mesoderm cells. The presumptive heart cells receive inductive signals from the endoderm and the primitive streak. One of these inductive signals is the TF **Cerberus**, another might be **BMP2**. Together, these TFs induce the synthesis of the **Nkx 2.5 TF** in the migrating mesodermal cells. Nkx 2.5 is a critical protein in the specification of the presumptive heart field. It instructs the mesoderm to become cardiac tissue and induces the activation of other heart-relevant TFs, like the **GATA** family and the **MEF2** family members. Working together, these TFs activate the gene expression of cardiac muscle-specific proteins, like **atrial natriuretic factor (ANF)**, **α myosin heavy chain (αMHC)**, and **cardiac actin**. *(Gilberts Developmental Biology 6th edition)*

As the presumptive heart cells of the cardiogenic mesoderm are still migrating towards midline, they begin to express N-cadherin, which makes them to join in an epithelium. This epithelium is the **myocardium**.

N-cadherin is a calcium-dependent binding factor, which serves a role in various developmental processes, like in migration. In addition to that, it serves a role in cell-cell contacts, at the desmosomes. The cadherins, in general, often function in cell-cell contacts at

adherens junctions. In development during embryonic morphogenesis they provide cells, which carry cadherin, with polarity. It may also function in signal transduction. Some of the migrating cardiogenic cells downregulate the expression of N-cadeherin and delaminate from the myocardial epithelium, to form the **endocardium**. The myocardium will form the heart muscle itself, while the endocardium will produce the heart valves. In addition to that, the endocardium will secrete proteins, that stimulate the growth of the myocardium and it will regulate the innervation of the cardiac tissue. *(Gilberts Developmental Biology 6th edition)*

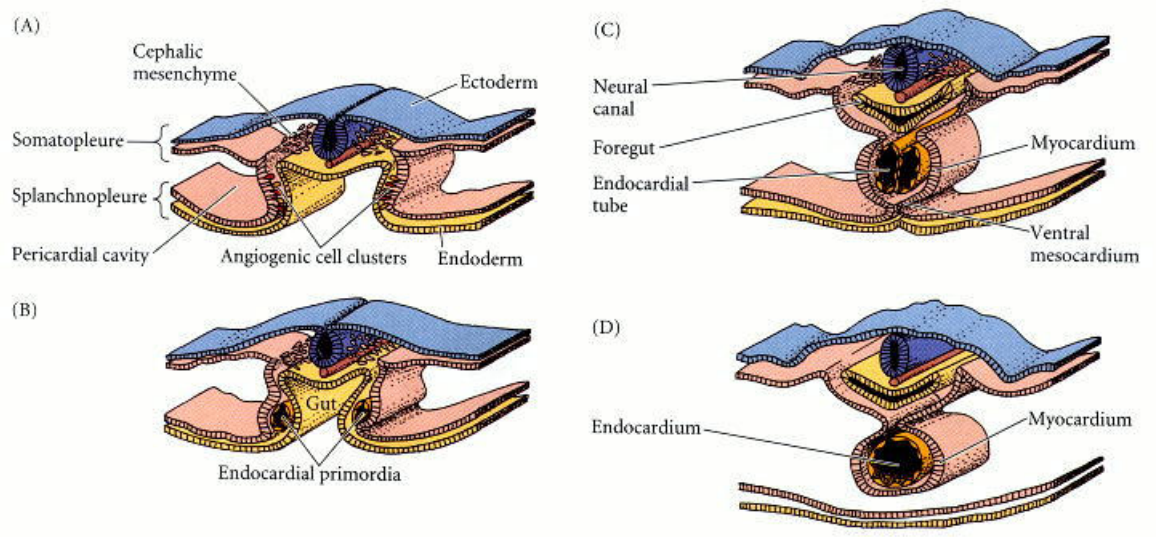


Fig.1.4.1: Shows the formation of the chick heart from the lateral plate mesoderm.
(Gilberts "Developmental Biology" 6th edition)
Schematic drawing of the formation of the chick heart from the splanchnic lateral plate mesoderm. The endocardium forms the inner lining of the heart, the myocardium forms the heart muscles. Transverse sections through the heart-forming region of the chick embryo are shown at (A) 25 hours, (B) 26 hours, (C) 28 hours, and (D) 29 hours.

The inward folding of the splanchnic mesoderm during neurulation, leads to the convergence of the endocardial primordia. Finally, these primordia further develop to heart tubes, which fuse to one endocardium. By week three of human gestation, the fusion of the two heart tube primordia occurs. While the paired primordia still are fusing, the pulsation of the heart begins. The pacemaker of the initial contractions is the sinus venosus. Here the contractions begin, and are continued in a wave-like manner along the heart tube. The heart is able to pump blood even before the system of valves has established. The heart muscle cells have an own inherent ability to continue beating, even if they are isolated from the body. In the context of an embryo the contractions of the heart become regulated by electrical stimuli from the medulla oblongata via the vagus nerve. *(Gilberts Developmental Biology 6th edition)*

In a five week human embryo the heart is a two chambered tube, with one atrium and one ventricle. Thereafter, the heart undergoes a looping, which converts the original anterior-

posterior polarity to a right-left polarity. Thus, the portion of the heart, which was designated to become the right ventricle, lies anterior to the portion, which was designated to become the left ventricle. The looping of the heart tube is dependent on the left-right patterning proteins **Nodal** and **Lefty-2**. Nodal and Lefty-2 are two paracrine factors in the lateral plate mesoderm of the left side of the embryo. The expression of these two factors is ascribed to the left-ward motion of the cilia on the nodal cells. Nodal itself activates the *pitx*-2 gene, which codes for a product, which activates left sided properties in tissue. *(Gilberts Developmental Biology 6th edition)*

In the heart primordium, Nkx 2.5 regulates the expression of **Hand1** and **Hand2** transcription factors. Initially, the expression of the Hand proteins seems to be uniformly distributed in the heart tube. Later when the looping begins, the Hand1 protein becomes restricted to the left ventricle and the Hand2 protein becomes restricted to the right ventricle. Another TF, critical for heart looping, is **Pitx-2**. It is activated at the left side of the lateral plate mesoderm only, and it is assumed that it also regulates the expression of proteins, such as extra cellular matrix protein flectin, which in turn is thought to regulate the physical tension of the heart tissue on the different sides. The TFs Nkx 2-5 and MEF2C activate the *Xin* gene. The **Xin** protein is thought to mediate cytoskeletal changes in the heart tissue, which are essential for the looping of the heart tube. *(Gilberts Developmental Biology 6th edition)*

The separation of the atrium from the ventricle is achieved when the cells express several TFs, which are restricted to either the anterior side or the posterior side of the embryo. The partition of the heart tube is accomplished, when the cells of the myocardium express a factor, which drives the cells of the endocardium to detach and to migrate between these two layers to build the **cardiac jelly**. In humans this leads to the formation of the **endocardial cushion**, which divides the heart tube into the right and the left atrioventricular channels. The formation of the septa usually occurs in the seventh week of human development. When the septa are formed, the heart is a four chambered structure, as shown in figure 1.4.2. In the four chambered heart, the pulmonary artery is connected to the right ventricle, and the aorta is connected to the left ventricle. *(Gilberts Developmental Biology 6th edition)*

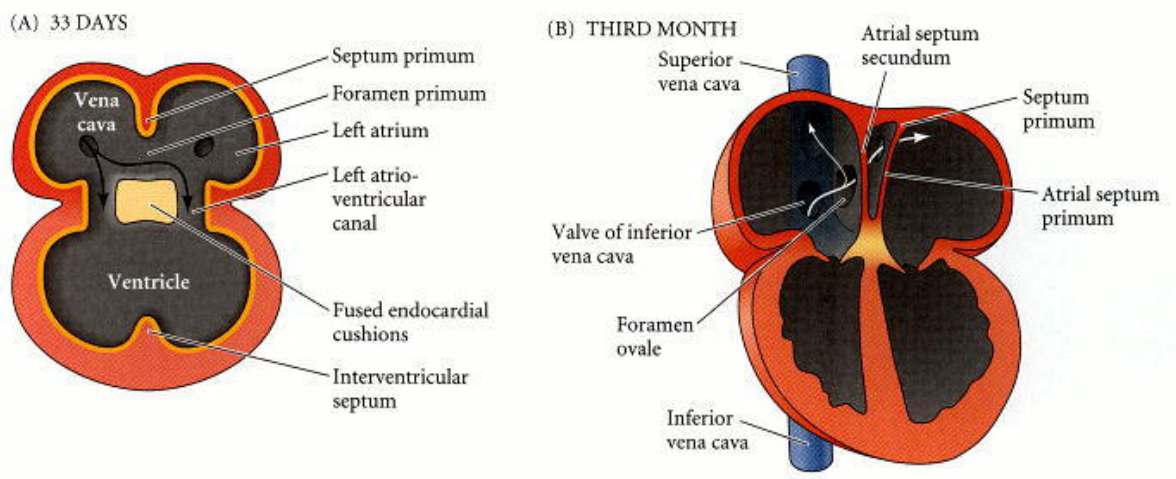


Fig.1.4.2: The formation of the heart chambers. *(Gilberts Developmental Biology 6th edition)*

(A) Diagrammatic cross section of the chambers of the human heart at 4.5 weeks. The septa of the atrium and the ventricle are growing towards the endocardial cushion.
(B) Diagrammatic cross section of the human heart during the third month of gestation. Before the first breath, the blood can cross from the right side of the heart to the left side through openings in the atrial septa. The first breath causes these openings to close, and the newborn's circulation and oxygen supply now depends on the lung.

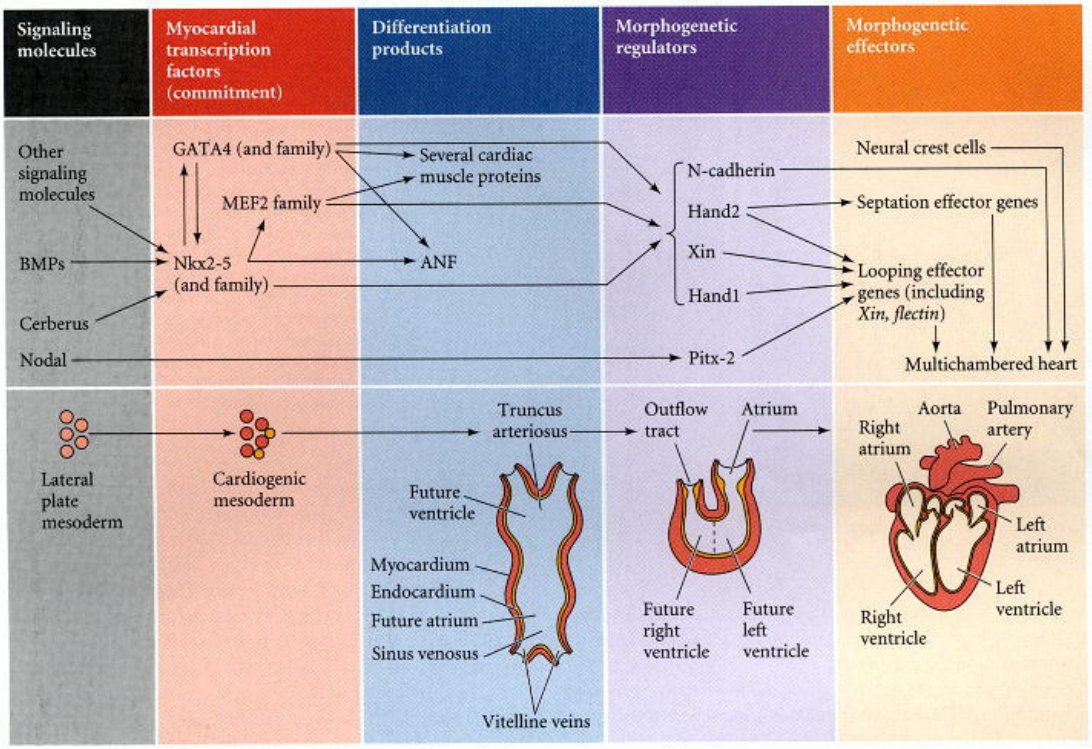


Fig.1.5.3: Summary of the developmental events in the formation of the heart.
The correlation between the morphological stage and the transcription factors present in the nucleus of the heart precursor cells. Cardioblasts are the committed heart precursor cells when they contain Nkx2-5 and GATA family proteins. These proteins convert the cardioblasts into cardiomyocytes (heart muscle cells), which express the cardiac muscle-specific proteins. The cardiomyocytes join together to form the cardiac tube. Under the influence of the Hand proteins, Xin, and Pitx-2, the heart loops and the chambers are formed. (Gilberts Developmental Biology 6th edition)

Molecular signals in heart development

Very few of the genes involved in cardiac morphogenesis are restricted to the cardiac mesoderm. Many signalling molecules, that control cardiac development, are found in numerous other cell types than mesodermal precursors of muscle. To achieve transcriptional specificity in a certain cell type at a given time, it is necessary to interpret the intercellular and intracellular signals combined with local cellular identity. Therefore, to understand how gradients and thresholds of signals are coupled with combinatorial interactions among transcriptional regulators on individual transcriptional modules remains a major challenge for the future. Equally important are the additional layers of regulation that are imposed on the cis-regulatory information and its associated TFs. For example, alterations in chromatin conformation, and epigenetic mechanisms, such as DNA-methylation, play key roles in the interpretation of DNA sequence information. In addition to that, there are protein-protein interactions among transcriptional activators and repressors that do not bind DNA directly. These are also involved in modulating the activities of TFs, which are directly bound to DNA. The convergence of these different regulatory strategies provides the rich diversity of gene expression patterns that serve as the basis for development.
There are four major signalling pathways involved in the induction of the cardiogenic program. *(Gilberts Developmental Biology 6th edition)*

The first signalling pathway acts via **BMPs-** the bone morphogenetic proteins. BMPs are expressed in the endoderm of the cardiogenic region as well as in ectoderm and cardiac mesoderm itself. The inductive signal for cardiac development is **Sonic hedgehog** (**Shh**) from the node. Shh positively influences BMP signalling and the activation of cardiogenic TFs. A role of BMPs in cardiomyogenesis was implied, because the application of BMPs on explants of cardiac mesoderm and non-cardiogenic tissue activates the expression of the early cardiac markers, such as Nkx2.5, GATA4, Tbx2 and MHC. In addition to that, the inhibition of BMP signalling blocks *nkx2.5* gene expression and cardiac differentiation. If BMP-soaked beads are placed to head mesoderm Nkx 2.5 is induced ectopically in this tissue. In *bmp2* knock out mice the development of the heart completely fails and a dominant inhibition of BMP signalling inhibits cardiogenesis. *(Gilberts Developmental Biology 6th edition)*

Downstream of BMP and TGFβ signalling Smad proteins act as effectors. The Smad proteins are transcription factors (TFs), which transduce extracellular signals from TGFβ-receptor family members. Smad 1, 3 and 5 are regulated by BMPs during cardiac induction. The BMP-Smads appear to directly activate early cardiac TF genes, including *nkx2.5.*

The second major signalling pathway, which is involved in the induction of the cardiogenic program, acts via **WNTs** and **β-catenin**. Wnts are secreted glycoproteins, which are expressed in the dorsal neural plate and act repressive on cardiogenesis. The ectopic expression of Wnt antagonists can overcome the repressive effect of the dorsal neural plate on cardiogenesis. For example, the Wnt antagonists of the *Dickkopf* and *Frizzled-like* families are expressed in the node and in the endoderm, which surrounds the cardiac mesoderm. These antagonists can activate cardiogenesis in non-cardiogenic mesoderm in the frog and chick embryo. The ectopic application of Wnt-antagonists leads to the formation of ectopic beating heart tubes in the frog. Thus the elimination of Wnt/β catenin signalling is essential for cardiogenesis. The balance of Wnts and their antagonists seems to shape the cardiac field. The TF, β-catenin, is one of the best characterized effectors of the Wnt signaling pathway. The stabilization of cytoplasmic β-catenin within the canonical Wnt pathway, allows for its translocation to the nucleus. In the nucleus it associates with TFs of the TCF/LEF family and activates the expression of target genes. Normally, in the absence of Wnt signals, the GSK3β, a serine/threonine kinase, serves to destabilize β-catenin. When GSK3β is overexpressed, it can also initiate cardiogenesis in non-cardigenic mesodermal explants of *Xenopus laevis*. The targeted inactivation of β-catenin in the node, the notochord and the endoderm of developing mice, was found to lead to ectopic formation of hearts along the anterior-posterior axis. *(Gilberts Developmental Biology 6th edition)*

The third major signalling pathway, which is involved in the induction of the cardiogenic program, acts via **WNTs** and the c-Jun-N-terminal Kinase (**JNK**). In contrast to the Wnt/β-catenin pathway, which is inhibitory for cardiogenesis, there is a non-canonical Wnt/JNK pathway, which is essential for cardiac induction in the frog and chick embryo. Upon the activation of JNK and the small GTPases RhoA and cdc42, Wnt 11 is expressed in the mesoderm. Wnt 11 and has been implicated in regulation of cell polarity and movement during gastrulation. In frogs, the ectopic expression of Wnt 11 or activation of protein kinase C (PKC) and JNK induces a cardiogenic phenotype. In contrast to that, the inhibition of Wnt 11, PKC or JNK inhibits endogenous or induced cardiogenesis. Wnt 11 also was shown to inhibit the canonical Wnt/β-catenin pathway, and therefore to silent the repressive signals from Wnt/β-catenin pathway. The Wnt-inhibitors of the Dickkopf and Frizzled family stimulate the Wnt/JNK pathway at the same time as they block Wnt/β-catenin signalling. Thus, the activation of Wnt/JNK signalling by factors expressed in endoderm and mesoderm promotes cardiogenesis. In contrast to that, the inhibitory Wnts from the neural plate help to limit the extension of the cardiac fields. *(Gilberts Developmental Biology 6th edition)*

The fourth major signalling pathway, which modules cardiogenesis, acts via the fibroblast growth factor (**FGF**) family of signalling molecules. Explant experiments revealed that FGF-4 in combination with BMP-2 can induce cardiac differentiation in non-cardiac mesodermal explants. The FGF signal is required transiently to induce the cardiac fate in non-cardiac cells, but it is also required to sustain the expression of *Nkx2.5* and Serum Response Factor (SRF) genes. In another study in mouse and chick embryos, the inducing mesoderm was excised and FGF-8 was applied ectopically. The ectopic application of FGF-8 prevented the loss of cardiac gene expression. Furthermore, the expression of cardiac markers is expanded when FGF-8 soaked beads are placed lateral to heart fields. FGF-8 only has a cardiogenic effect in presence of BMP signalling and the *fgf8* gene appears to lie downstream of BMPs. *(Gilberts Developmental Biology 6th edition)*

The signalling pathways described above are highly interconnected and cross-talk to each other. There are positive regulatory circuits as well as negative regulatory circuits.

The gene of our interest, *nkx 2.5*, also lies downstream of BMP signalling.

The *nkx 2.5* gene is a vertebrate orthologue of the Drosophila *tinman (tin)* gene. It is one of the first genes expressed in the developing heart of early stage vertebrate embryos. In the mouse, *nkx-2.5* is one of the initial markers of the cardiogenic region and is expressed in the heart throughout development and in the adult. In addition to that, *nkx 2.5* is expressed in the developing pharyngeal arches, the spleen, the thymus, the thyroid, the stomach and the tongue. Likewise to *tin*, *nkx 2.5* gene expression is regulated by a complex series of positive and negative regulatory circuits. The *nkx 2.5* gene is known to be regulated by TFs, which bind to a 505 bp regulatory element, that contains multiple GATA, NKE, bHLH, HMG and HOX consensus binding sites. This 505 bp regulatory element was shown to be sufficient for gene activation in the cardiac crescent and in the heart outflow tract, pharynx and spleen by reporter gene assays with the *lacZ* gene. If the paired GATA sites within this element are mutated, the activation of *nkx 2.5* is eliminated in transgenic embryos. The dependence of this *nkx-2.5* regulatory element on GATA sites for gene activity is evidence for a GATA-dependent regulatory mechanism controlling *nkx-2.5* gene expression. As there are not only GATA binding sites on the distal regulatory element, it is most likely a combinatorial interaction of multiple factors, which is responsible for the initial activation of *nkx 2.5* in the cardiac, thyroid and spleen primordial (*Searcy et al.1998*).

1.5. Desmin in cardiomyogenesis

In all types of mature striated muscle, desmin links individual myofibrils laterally at the level of Z-discs. In addition to that, it connects the contractile apparatus with mitochondria *(Capetanaki et al.2002; Milner et al.2000)*, the nucleus, the sarcolemma, the T-tubules, and the post-synaptic areas of motor end plates. Last but not least, desmin is also found to be concentrated at myotendinous junctions of skeletal muscle and at the desmosomes of cardiac muscle *(Bär et al. 2004)*. In mammalian skeletal muscle, desmin is one of the first marker proteins for muscle, where it is detected in the somites and myoblasts *(Herrmann et al. 1989; Kaufman and Foster 1988)*.

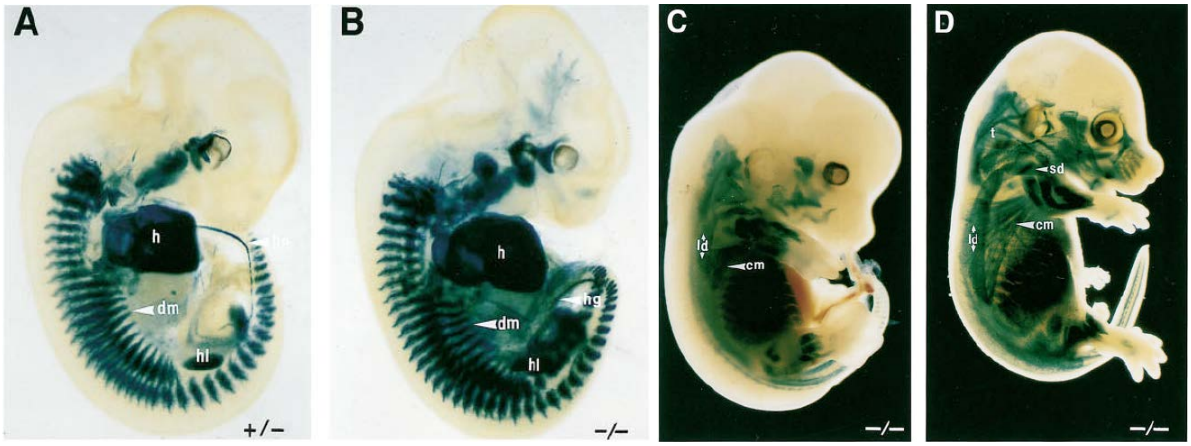


Fig.1.5.1: Transgenic mice deficient in desmin.
Representative developmental stages of transgenic mice deficient in desmin (des::lac Z) are shown in figure 1.5.1: The desmin gene was heterozygously or homozygously disrupted by an E.coli lacZ gene (Li et al. 1997). Therefore the sites where desmin would be expressed can be easily located in β-galactosidase staining. ***(A)*** *Shows the formation of the myotome in heterozygous $des^{+}/lac\ Z^{+}$ mouse at day 11 p.c.* ***(B)*** *Shows the formation of the myotome in homozygous $des^{-/-}$ ($lacZ^{+/+}$) mouse at day 11 p.c.: Tissue areas, which are stained in blue, correspond to sites, where the expression of the desmin gene normally takes place (h=heart, dm=dermomyotome, hl=hind limb, hg=hind gut). More intensive blue staining in (B) is due to homozygosity in the lacZ gene.* ***(C)*** *Shows the growth of muscle in mice, aged 13.5 days p.c. and* ***(D)*** *day 15 p.c. Both pictures show normal formation of muscle, although desmin gene expression is homozygously replaced by lac Z. (ld= latissimus dorsi, cm=cutaneous maximus)*

In mouse the desmin protein first appears at day 8.25 post coitum (pc) in the neurectoderm, where it is transiently co-expressed with keratin, vimentin, and nestin. At day 8.5 pc the protein localizes to the heart rudiment and by day 9 to 9.5 it forms longitudinal filaments associated with the Z-disc within the somites. During early differentiation of mammalian skeletal and cardiac muscle, desmin is transiently co-expressed with keratin, vimentin, and nestin. Later in development the protein has been described to co-localize with synemin and paramin *(Capentanki et al.1984)*. During skeletal muscle differentiation, the expression of the *desmin* gene precedes the expression of all other muscle specific genes. It even precedes the

expression of transcriptional regulators for muscle differentiation, the myoD family members, with the exeption of the myogenic factor 5 (myf5). Transcripts of *myf5* could be detected as early as day 8 pc in the myotome, which is in contrast to *desmin* transcripts appearing at day 9 pc. Due to the fact that the expression of the *myf5* gene is preceding the expression of *desmin* and because the expression of the *desmin* gene is preceding the expression of myoD family members, it has been proposed that myf5 may be involved in the initial activation of the *desmin* gene and on the other hand, that desmin might play a regulatory role in myogenic development as well. However, the next myogenic marker to appear in developing mouse embryos is myogenin at day 8.5 pc (or 9pc), followed by myoD at day 10 pc and finally MRF4. The *desmin* gene contains various cis-acting elements in its 5´- flanking region, such as several GC-boxes, three myoD- binding sites (E-boxes), one MEF2 binding site, and a region with homology to an M-CAT motiv. Co-transfection studies of myoD, myogenin, MRF4, and myf5 with a desmin-CAT construct revealed that the *desmin* gene is transactivated in 10T ½ fibroblast *(Li et al.1993).* Therefore, it is assumed that these factors transactivate desmin at different stages during development, alone or in combination. Studies with *myoD*$^{-/-}$ myoblasts showed that the *desmin* gene expression is 55-fold higher in wild type myoblasts than in the *myoD*$^{-/-}$ myoblasts. This supports the idea that the myogenic transcription factor, myoD, is one of the major regulators of *desmin* gene expression. The *desmin* gene is most probably regulated by myoD via the E-boxes (myoD-binding sites) in the 5'-flanking region of *desmin* gene *(Asakura et al., 2007).* To further investigate the role of desmin in myogenic development and differentiation, several *in vitro* studies were done in the past decades. For instance, antisense RNA-technology was used in C2C12 myoblasts. The *desmin* antisense RNA interferes with *desmin* gene expression, which in the C2C12 myoblasts blocked the fusion of myoblasts and the formation of myotubes. Furthermore, the antisense-RNA negatively interfered with the normal induction of the muscle specific gene expression. In addition to that, the *desmin* antisense-RNA downregulated the expression of the myogenic helix-loop-helix (HLH) regulators of transcription myoD and myogenin *(Capetanaki et al.1997).* In another *in vitro* experiment embryonic stem cells (ESC) were used to investigate the consequences of a lack of functional desmin. In this study ESC with wildtype alleles of *desmin* and ESC with homozygously or heterozygously mutated alleles of *desmin* were used. The ESC were allowed to differentiate *in vitro* in an embryoid body (EBs) model, which normally recapitulates the *in vivo* cardiac, skeletal and smooth muscle myogenesis of mouse embryos. The ESC/EB study revealed that skeletal myogenesis in *desmin* null mutant EBs was totally inhibited. The inhibition of skeletal myogenesis was manifested by the absence of

myotube formation, the absence of any contracting movement, and the absence of myogenic marker expression, such as myoD, myogenin, myf5, and myosin heavy chain. Formation of smooth muscle tissue also was completely missing in the absence of desmin. On the other hand, no obvious effect on cardiac differentiation could be observed, as *desmin* null EBs were still able to develop fields of beating cardiomyocytes. The data of this *in vitro* study suggests that desmin is indispensable for skeletal and smooth muscle development, but in contrast seems to be negligible for cardiac differentiation.

However, the *in vitro* data is not supported by *in vivo* experiments. Desmin knockout mice were generated in 1996 by D.J.Milner and colleagues. The *desmin* null mice were viable, which implicates that prenatal muscle development can take place in the absence of *desmin*. These mice display no obvious anatomical defects, when compared to wild type or heterozygous littermates. Capetanaki and colleagues *(1997)* ascribed the absence of any obvious anatomical defects in *desmin* null mice to compensatory mechanisms. To prove this hypothesis, the expression levels of other IF genes were analysed, but there was no unusual maintenance or up-regulation of gene expression detected, that could serve for compensation. As myofibrils are properly assembled in *desmin* null mice, it is obvious that desmin is not necessary for assembly and initial lateral alignment of myofibrils, as it was originally suggested by Hill and colleagues *(1986).* An analysis of muscle from *desmin* null mice has shown that the absence of desmin does not influence the localization or expression levels of some individual myofibrillar proteins. A comparison of the localization and expression levels of α-actinin, cardiac troponin, nebulin and myosin heavy chain proteins in *desmin* null and *desmin* wildtype mice showed no significant difference *(Capetanaki et al.1997).*

In mice the expression of the *desmin* gene becomes visible at day 7.25 p.c. *(Kuisk et al.1996)* in murine pre-cardiac mesoderm, emanating from *brachyury* and *goosecoid* expressing mesodermal precursors. In embryoid bodies (EBs), *desmin* mRNA was detected at day 5 *(Weitzer et al.1995),* before cardiomyogenesis takes place. Later in development, *desmin* is also expressed in skeletal and smooth muscle cells and it is known to influence myogenesis at the transcriptional level by modulating the basic helix-loop-helix (bHLH) TF genes *myogenin, myoD,* and *myf5 (Li et al.1994; Weitzer et al.1995). In-vivo* and *in-vitro* studies revealed that desmin is dispensable for cardiomyogenesis *(Weitzer et al.1995; Li et al.1996; Milner et al.1996).* Initially, transgenic mice which lack a functional *desmin* gene develop normal. Later, these mice develop lesions in cardiac muscle *(Li et al.1996; Milner et al.1996)* which are accompanied by the disorganization of myofibrils, intercalated discs, and mitochondria *(Capetanaki et al. 2002).*

However, the early expression of desmin in pre-cardiac mesoderm and its influence on transcription of early myogenic transcription factor genes in skeletal muscle *(Li et al.1994; Weitzer et al.1995)*, suggests a role for desmin in very early developmental processes like cardiomyogenic commitment and differentiation.

To test the hypothesis that desmin affects very early developmental processes, *in-vitro* cardiomyogenesis was observed in EBs. The EBs had been generated from four different murine ESC lines, which divert in their alleles of the *desmin* gene. The first ESC line, which was used to generate EBs, carries the normal wild-type alleles of the *desmin* gene ($des^{+/+}$). The second ESC line, which was used to generate EBs, holds an additional wild-type allele of the *desmin* gene and therefore is expressing desmin ectopically ($des^{+/+}$ $des^{\mathbf{ect}}$). The third ESC line, carries disrupted alleles of the *desmin* gene ($des^{-/-}$) and therefore does not produce any functional desmin *(Weitzer et al. 1995)*. The fourth ESC line, carries an amino-terminally truncated version of *desmin* ($des^{\Delta 1\text{-}48/\Delta 1\text{-}48}$), which leads to the production of N-terminally truncated desmin Δ1-48 protein *(Höllrigl et al. 2002)*. These ESC were subjected to *in-vitro* differentiation in the embryoid body (EB) hanging drop model. Usually, in wild-type (WT) EBs clusters of beating cardiomyocytes appear at day 7. Interestingly, in the $des^{+/+}$ $des^{\mathbf{ect}}$ EBs the onset of cardiomyogenesis took place one day prior compared to the WT EBs, as shown in figure 1.5.2.A. The development of beating cardiomyocytes from the $des^{-/-}$ ESC, which were differentiated in the EB hanging drop model, was delayed by about one day compared to WT ESC. In addition to the delay of cardiomyogenesis in the $des^{-/-}$ EBs, the clusters of beating cardiomyocytes were smaller, as shown in figure 1.5.2.C.

The EBs, which were generated from $des^{\Delta 1\text{-}48/\Delta 1\text{-}48}$ ESC, poorly developed small clusters of beating cardiomyocytes with a delay of two days, compared to WT EBs *(Hofner et al.; 2007)*. In addition to that, the data demonstrates that the expression of the N-terminally truncated desmin (desmin Δ1-48) is more disadvantageous to cardiomyogenesis than the absence of desmin (desmin-/-). From these results it is reasonable to propose a regulatory role for desmin in commitment and early differentiation of cardiomyocytes.

In the same piece of work, Hofner and colleagues investigated the influence of desmin on the morphology of cardiomyocytes. Therefore, EBs which were derived from *in-vitro* differentiation of ESC of the respective genotypes, were subjected to immunofluorescence imaging (data not shown). The EBs had been stained with specific antibodies for cardiac troponin T (cTnT) and desmin. The immunofluorescence staining revealed that, compared to $des^{+/+}$ EBs, the size of clusters of cardiomyocytes in EBs from $des^{-/-}$ ESC was decreased

significantly. In contrast to that, the EBs derived from *des*$^{+/+}$ *des*^{ect} ESC showed very large areas of cardiomyocytes, which completely covered the central area of the EBs. The *des*$^{\Delta 1\text{-}48/\Delta 1\text{-}48}$ EBs showed similar reduced areas of cTnT-staining as the *des*$^{-/-}$ EBs. This demonstrates a dominant-negative effect of desminΔ1-48 on cardiomyogenesis. Further, this suggests that differentiation and proliferation of ESC towards a cardiac fate is severely disturbed by the presence of an amino-terminally truncated version of the desmin protein. The constitutive desmin expression in the *des*$^{+/+}$ *des*^{ect} ESC, in EB differentiation model led to the observation that these EBs develop larger and more interconnected clusters of beating cardiomyocytes. In addition to that, these clusters show increased rates of synchronous contraction compared to the wild-type EB-clusters, as it is shown in figure 1.5.2.B.

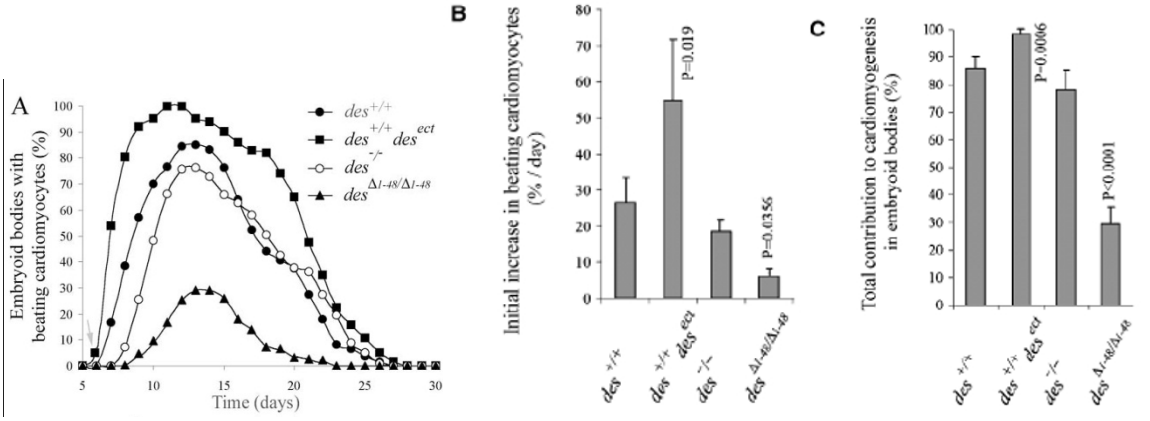


Fig.1.5.2ABC: *EB in vitro differentiation study (Hofner et al.; 2007)*
A) *Compared developmental time course of beating cardiomyocytes in EBs. Note that the development of beating cardiomyocytes in EBs from des*$^{+/+}$ *des*^{ect} *ESC is one day ahead (day 6), compared to the wild-type (day 7). The expression of* des$^{\Delta 1\text{-}48/\Delta 1\text{-}48}$ *is more disadvantageous for cardiomyogenesis, than the complete absence of desmin (des*$^{-/-}$*).*
B) *The initial increase in clusters of beating cardiomyocytes per day. (Hofner et al.;2007). The acceleration of development to beating cardiomyocytes by desmin is demonstrated.*
C) *The extent of cardiomyogenesis from ESC of the different genotypes. (Hofner et al. 2007) The size of the bars refer to the ability of ESC of the four different genotypes to develop into cardiomyocytes. More than 85% of the des*$^{+/+}$ *ESC contrasted by less than 30% of des*$^{\Delta 1\text{-}48/\Delta 1\text{-}48}$ *ESC contribution to cardiomyogenesis.*

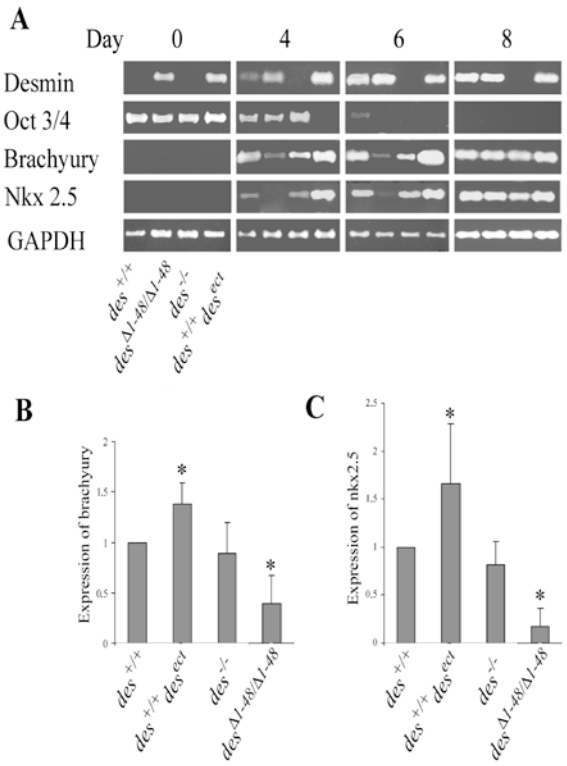
After the observation of the reported morphological and physiological changes, the effect of differential desmin expression was further investigated on a molecular level. Therefore, total mRNA was purified from EBs of the four different ESC at day 0 to day8 of *in-vitro* differentiation. The mRNA was reverse transcribed and used to assess the levels of mesodermal- and cardiac- marker genes in a semi-quantitative PCR. In wild-type EBs, *desmin* transcripts were detected at the beginning of mesoderm development around day 4 of *in-vitro* differentiation, as shown in figure 1.5.3A. Interestingly, the absence of *desmin* in EBs from

des$^{-/-}$ ESC had a weak negative effect on the expression of the mesodermal TF gene *brachyury* and a significant delay in the expression of the *mlc1v* gene. The constitutive expression of the truncated desminΔ1-48 protein caused a downregulation of the mesodermal TF genes *brachyury* and *goosecoid*. In addition to that, the expression of the cardiac TF genes *nkx 2.5* and *mef2C* is completely abolished between day 4 and day 6. The expression of the cardiac specific marker gene *mlc1v* was delayed by two days. The marker gene of skeletal muscle development, *mhcα*, was unaffected.

Furthermore, the constitutive overexpression of desmin in *des*$^{+/+}$ *des*$^{\mathbf{ect}}$ ESC seemed to promote differentiation of ESC. This promotion of ESC differentiation became evident, because the stemness factor gene *oct 3/ 4* was downregulated and the expression of brachyury and goosecoid, two primitive mesodermal markers, and the expression of the early cardiac TF nkx2.5 was reported to be significantly increased *(Hofner et al.; 2007)*. *Nkx 2.5* together with T-box transcription factors are known to synergistically promote cardiogenesis *(Bruneau et al. 2002)*. The ability of desmin to directly bind to DNA via its N-terminus was shown in 2003 by Li and colleagues and in 2005 by Tolstonog and colleagues. The fact that desmin is able to directly bind to DNA, allows for the suggestion of a regulatory role for desmin in cardiomyogenesis.

The findings from Hofner and colleagues, as shown in figure 1.5.3ABC, strengthened the hypothesis that desmin influences cardiomyogenesis by direct or indirect interaction with regulatory elements within heart relevant transcriptional circuits.

Fig.1.5.3.: *The influence of desmin on the expression of mesodermal and cardiac markers (Hofner et al.; 2007*



A) *Shows the mRNA levels of the genes as indicated.The levels of desmin expression are shown. Oct 3/4 is a stemness factor, which is expressed at high levels in undifferentiated embryonic stem cells. Oct 3/4 expression ceases in course of differentiation. Brachyury is known as a mesodermal TF. Nkx 2.5 is a cardiac TF. GAPDH expression levels were used for internal normalization of the template cDNA.*

B) *Demonstrates the up-regulation and down-regulation of brachyury expression in* $des^{+/+}$ des^{ect} *EBs and* $desmin^{\Delta 1\text{-}48/\ \Delta 1\text{-}48}$ *EBs respectively relative to* $des^{+/+}$ *EBs.*

C) *Demonstrates the up-regulation and the downregulation of the cardiac TF nkx 2.5 expression in* $des^{+/+}$ des^{ect} *EBs and* $desmin^{\Delta 1\text{-}48/\ \Delta 1\text{-}48}$ *EBs respectively relative to* $des^{+/+}$ *EBs.*

The hypothesis that desmin influences cardiomyogenesis is further strengthened by the data, which is presented in the work of Höllrigl and colleagues from 2007 and shown in figure 1.5.4. From previous experiments, the scientists had learned that the expression of desminΔ1-48 in ESC elicits a severe phenotype in *in-vitro* differentiated EBs. It was already known before, that the amino-terminus of intermediate filament proteins influences their assembly and can interact with a variety of other proteins and with DNA, and that it therefore might be essential, but still the severity of the phenotype was surprising.

At the N-terminus of native intermediate filament proteins of type III, there are specific serine residues, which might be reversibly phosphorylated during the cell cycle or during developmental processes. Usually, the phosophorylation of serine or threonine residues is accomplished by kinases, like the protein kinases A and C, the small kinase cdc42, the Rho-dependent kinase, and many others. To elucidate the significance of desmin and especially the N-terminal serine residues in more than just structural means, Höllrigl and colleagues performed a series of mutations in the N-terminal region of the desmin protein. The serine residues 6,7, and 8 of the desmin protein were heterozygously mutated to alanine in ESC ($des^{+/S6,7,8A}$).

In parallel, the serine residues 31 and 32 were mutated heterozygously to alanine in ESC ($des^{+/S31,32A}$). Both mutant variants of the *desmin* gene were expressed under the control of the native desmin promoter. Upon the exchange of the indicated serine residues by alanine, these affected residues are no more phosphorylated by kinases. The hetero-zygously mutated ESCs were allowed to differentiate *in-vitro* in the EB hanging drop model. The heterozygous expression of the mutant desminS6,7,8A variant resulted in delayed cardiomyogenesis and reduced proliferation, as reported by Höllrigl and colleagues in 2007 *(Höllrigl et al.; 2007).* In addition to that, the expression of this mutant desmin variant affects myofibrillogenesis and results in altered morphology of cardiomyocytes (data not shown). Furthermore, the expression of desminS6,7,8A led to the downregulation of the TF genes *brachyury, goosecoid, nkx2.5,* and *mef2C* , as it is shown in figure 1.5.4. Höllrigl and colleagues demonstrated, that with the onset of desmin expression in the pre-cardiac mesoderm of embryoid bodies, the mutant desminS6,7,8A variant caused a delay in the onset of the expression of the mesodermal transcription factor genes *brachyury* and *goosecoid*. The expression of *nodal*, a gene of the TGF-β family, which is specifically expressed in mesoderm, was partially and temporally down-regulated. In contrast to that, the expression of the stemness factor *oct3/4* in the primitive ectoderm was not affected. In parallel to the observed delay of cardiomyocyte development in EBs, which were generated from $des^{+/S6,7,8A}$ ESC, the expression of the early myocardial TF *nkx2.5* was delayed significantly. The expression of *mef2C* was found to be completely blocked at day 6 and partially blocked at day 8. The expression of the *myosin light chain* gene (*mlc1v*) was partially delayed. In contrast, the expression of the genes coding for *mhcα* and *vimentin* was not affected significantly. The mhcα and vimentin proteins are not restricted to cell types, which express the mutant desminS6,7,8A. In summary, the timing and the degree of the expression of the myocardial TFs *nkx2.5* and *mef2C* was much more affected by the expression of the mutant desminS6,7,8A variant, than the expression of the mesodermal TFs *brachyury* and *goosecoid*. The degree of the delay in the expression of the TF genes *brachyury, goosecoid, nkx2.5* and *mef2C* in $des^{+/S6,7,8A}$ EBs relative to $des^{+/+}$ EBs (1.0) is shown in figure 1.5.4B.
These results suggest that the expression of the mutant desminS6,7,8A variant specifically affects mesodermal cells with a cardiac fate.

In contrast, the expression of the mutant desminS31,32A variant did not show the same impact on the expression of myocardial TFs, as it was observed for the expression of the mutant desminS6,7,8A variant. Consequently, desminS6,7,8A but not desminS31,32A severely interferes with the molecular mechanisms, which are involved in the transcriptional

control of the TF genes, which guide the commitment of the mesoderm to the cardiomyogenic lineage.

Further, the expression of the mutant desminS6,7,8A variant seemed to cause increased apoptosis of presumptive mesoderm and differentiating cardiomyocytes. The cardiomyocytes, which remained viable, were lacking myofibrils. These results suggest an important role for temporarily phosphorylated serine residues 6,7, and 8 in the amino-terminal portion of desmin protein. The phosphorylation of serine 6,7, and 8 might be instrumental for the commitment and the proliferation of cardiomyocytes. In contrast to that, the expression of the mutant desminS31,32A variant, had no significant effect on the onset of the cardiomyogenesis. The desminS31,32A EBs showed a very mild phenotype with smaller fields of beating cardiomyocytes as compared to the wild-type desmin EBs of the same developmental stage. Thus, the serine residues 31 and 32, which are not targeted by kinases, seem to be less important for the proper function of desmin. In the past, the phosphorylation of analogous serine or threonine residues in other intermediate filament proteins was shown to regulate the disassembly of the intermediate filament proteins during mitosis *(Izawa et al.2005)*. Thus, it is most likely, that the mutant desminS6,7,8A variant elicits the phenotype, which was described above, due to the irreversible aggregation of desmin intermediate filament protein. Indeed, Höllrigl and colleagues reported that they had found aggregated desmin in immunofluorescence microscopy of EBs, which express the mutant desminS6,7,8A variant. The cardiomyocytes of these EBs were completely lacking myofibrils and typically assembled intermediate filament protein. These cardiomyocytes were also reported to show aberrant morphology and to contain irregularly shaped cytoplasmic material, which was reminiscent of the aggresomes, which were observed in desmin-related cardiomyopathies *(Goldfarb et al.2008)*.

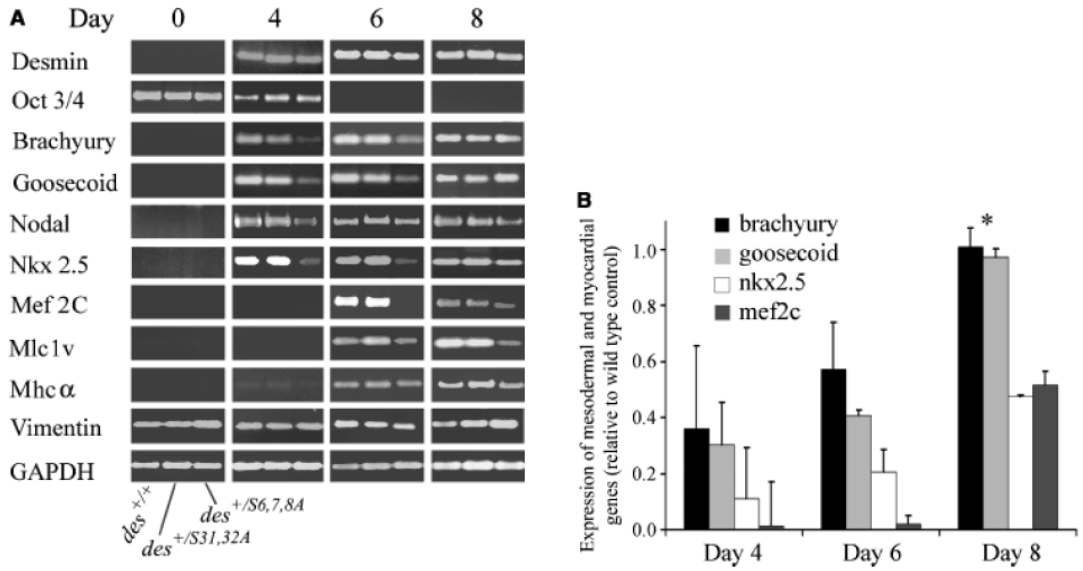


Figure 1.5.4: *The molecular consequences of the expression of the mutant desmin variants (desS6,7,8A and desS31,32A).(from Höllrigl et al., 2007)*
(A) Results of semi-quantitative RT-PCR (Höllrigl et al.2007).
(B) Timing of the expression of the TF genes brachyury, goosecoid, nkx2.5 *and* mef2C. *The presented values are set relative to their expression in wild-type* $des^{+/+}$ *EBs(1.0). The degree of the delay in the expression of these TFs is visualized.*

From the data of Höllrigl and colleagues work *(2007)* and previous experiments, it was concluded that desmin in general and in particular its amino-terminus, plays a vital role in the commitment of the mesoderm to the cardiomyogenic lineage, in the early differentiation of cardiomyocytes and in the generation of myofibrils. The embryoid body model of *in-vitro* differentiation again was proven to be a powerful tool to study early developmental processes.

1.6. Desminopathy

In the past years, a subset of myopathies was found, which is characterized by the presence of abnormal extensive accumulations of granular and filamentous aggregates in the contractile elements of a muscle cell, the sarcomeres. Because of the pre-dominant occurance of desmin in these aggregates, the described myopathies were named “desminopathies” (DM) or “desmin-related myopathies” (DRM). Affected patients exhibit a slowly progressive skeletal muscle weakness associated with cardiomyopathy. The cardiomyopathy is manifested by conduction blocks, arrhythmias, and chronic heart failure resulting in sudden premature death. First symptoms appear in the second or third decade of life. Usually, skeletal muscle weakness is progressive, often ascending from distal to proximal limb muscles, finally affecting the trunk, and eventually leading to respiratory failure. However, the intrasarcoplasmic aggregates, which are characteristic of DRM, do not only contain desmin, but also αβ-crystallin, dystrophin, actin, ubiquitin, synemin, syncoilin, nestin, plectin, and

vimentin. The aggregates are found as multifocal cytoplasmic inclusions or spheroid bodies, or as disseminated granulo-filamentous material. Additionally, histological observation showed myofibrillar disarray and loss of sarcomere integrity in affected tissue. This ultimately leads to muscle cell death in an apoptotic or necrotic pathway, further causing fatty degeneration and calcification.

Disease-associated Mutations

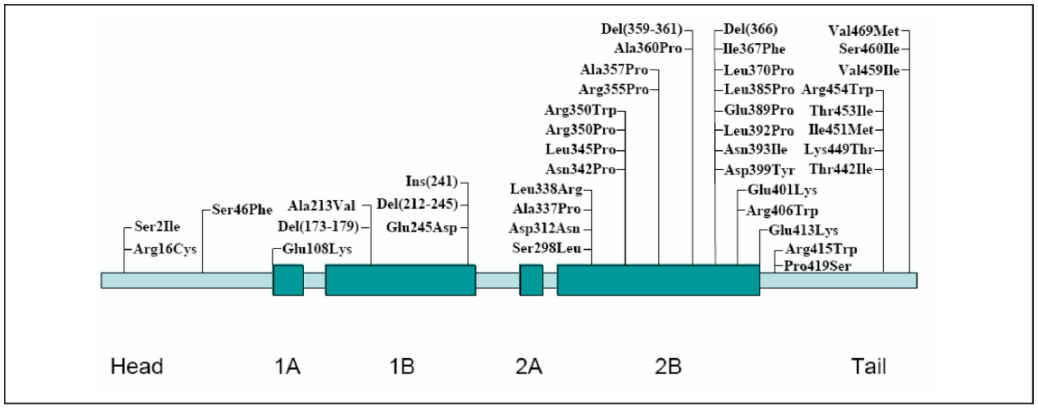


Fig.1.6.1: *Schematic representation of* ***structural organization of desmin protein*** *and predicted positions of disease-causing mutations. (Goldfarb et al. 2008)*

To date 42 pathogenic mutations in the *desmin* gene could be identified *(Goldfarb et al. 2008)*. The number of patients is rapidly increasing because of the rising awareness of this frequently miss-diagnosed disease. DRM is associated with either autosomal dominant or recessive inheritance. So far 37 miss-sense mutations, three small in-frame deletions, and deletion of exon 3, which is caused by mutated donor and acceptor splice sites in the *desmin* gene, have been described as disease-causing. The majority of the mutations (22) were found in the conserved 2B α-helical segment, some additional mutations (10) could be identified in the tail domain of desmin protein, and five mutations were located to the head domain. Interestingly, so far no mutations in 1A and 2A segments were found. However, in some cases of DRM the *desmin* gene was found unaffected, but instead a mutation in αβ-crystallin, which serves as a chaperone for the desmin protein, could be identified. Filament and network assembly studies indicate that most, but not all disease-causing mutations make desmin assembly-incompetent and able to disrupt a pre-existing filamentous network in a dominant negative way. However, it is remarkable that even point mutations often lead to severe alterations in desmin filament structure and assembly, finally giving way to diseased tissue. To date there are three different mechanisms discussed, which may lead to disturbed desmin homeostasis and therefore to a pathologic phenotype. The first is a compromised assembly of the mutant protein. The second mechanism is summarized as “altered biophysical properties”

of the mutant protein, which eventually leads to an increased susceptibility to stress-induced filament break-down and increased proteolysis. Both kinds of disturbance are caused by mutations in the α-helical segments, which are crucial to proper folding of desmin units and assembly of the filaments. The third mechanism regards the ability of the assembled protein filaments to interact with cellular binding partners. The interaction ability of mutant protein may be severely impaired, which in consequence may lead to degradation of the impaired protein (in our case desmin) and its segregation into protein granules. This was shown to be the case with desmin mutant in the non-helical tail segment. In DM or DRM most probable it is a combination of all three mechanisms, not a single cause, which leads to a diseased phenotype in individual patients. This suspicion is supported by increasing evidence that most severe cases of desminopathies are caused by a combination of mutations in two or more relevant genes (e.g. αβ-crystallin, desmin, and lamin A/C). The interference of mutant desmin in filament-formation was tested in *in vitro* filament formation experiments, generation of ordered cytoskeletal arrays and assays for the ability to integrate into an existing cytoskeletal network in various cell lines. Analysis of filament assembly behaviour demonstrates that some desmin mutants do not prevent filament assembly. Some other mutations interfere with the assembly process at distinct stages and these are classified into three groups: 1) mutations that compromise the longitudinal annealing properties; 2) mutants with enhanced adhesiveness leading to filament aggregation; and 3) mutants showing rapid disintegration of assembly precursors. However, mutations in *desmin* are responsible for inadequate supply of normal functional desmin. Toxic aggregates containing mutant misfolded desmin and debris of other myofibrillar and ectopically expressed proteins accumulate in the myofibers and eventually lead to their destruction. In addition to the toxic accumulation of mutant protein species, mutant desmin was shown to hamper normal interactions with other cytoplasmic proteins.

The genetic background of desminopathy: autosomal recessive inheritance

So far, three autosomal recessive (AR) *desmin* mutations are known. Patients who are carriers had the earliest onset of disease and the fastest progression of illness. A patient homozygous for a deletion of seven amino acids in the 1B helix (Arg173-Glu179del) developed generalized muscle weakness and atrophy, predominantly in the distal muscles of the upper extremities. In the same patient atrioventricular block required urgent implantation of a permanent pacemaker. In addition to that, the patient suffered from intestinal malabsorption.

The progression of disease led to cardiac and respiratory failure and intestinal pseudo-obstruction at the patient's age of 28 years.

In another family three siblings were compound-heterozygous for the Ala360Pro and Asn393Ile substitution mutations in the *desmin* gene. In all three, a complete heart block required implantation of a permanent pacemaker at the patient's ages of two, nine and ten years. Between the ages 20 and 24 years, all three developed progressive muscle weakness and wasting in the extremities and trunk, weakness in the neck and facial muscles, swallowing and breathing difficulties. Finally, all three died due to congestive heart failure at the age of 28, 30 and 32 years. Histopathologic analysis revealed intramyofiber accumulation of amorphous desmin immunoreactive material with characteristic subsarcolemmal distribution. Interestingly, several older members of the same family carrying either the Ala360Pro or the Asn393Ile mutation did not show any signs of muscle or heart disease. *(Goldfarb et al.2008)* Resuming, there is increasing evidence that certain mutations in the *desmin* cause serious problems in individuals who carry that mutation.

In Vitro studies

In vitro studies of *desmin* knock down in C2C12 myoblasts gave some insight into desmin's function. The C2C12 myoblasts were treated with antisense RNA of *desmin*. The use of antisense RNA interfered with *desmin* gene expression in the C2C12 myoblasts and led to blockade of myoblast fusion and formation of myotubes. In addition, *desmin* antisense RNA treatment interfered with the normal induction of muscle specific gene expression, including the myogenic helix-loop-helix (HLH) regulators of transcription myoD and myogenin *(Capetanaki et al., 1997).*

Another *in vitro* experiment was done with embryonic stem cells (ESC), which differed in their *desmin* alleles. In this study ESC with wild type *desmin* allele and heterozygously or homozygously mutated *desmin* alleles were allowed to differentiate in an embryoid body (EB) model. These EBs normally recapitulate the *in vivo* skeletal, smooth and cardiac myogenesis. This *in vitro* study revealed that *desmin* null EBs showed totally inhibited skeletal myogenesis, which was manifested by the absence of myotube formation and the absence of myogenic marker expression, such as myogenin, myoD, myf5, and myosin heavy chain. In addition to that, formation of smooth muscle was also completely absent in the *desmin* null EBs *(Weitzer et al. 1995).* In contrast to that, cardiac differentiation occurred and fields of beating cardiomyocytes could be observed. In summary, the data of the EB *in vitro* study suggests that desmin is indispensable for skeletal and smooth muscle development,

but in contrast seems to be negligible for cardiac differentiation *(Capetanaki et al., 1997).* However, the *in vitro* data is not supported by *in vivo* experiments.

The animal model

Desmin null mice were generated in 1996 by Milner D.J. and colleagues. The knock-out mice are viable, fertile and develop all three types of muscle tissue. In comparison to wild type litter mates the *desmin* null mice appear normal and do not show any anatomical defects. Though the mice appear normal, they have defects in their muscle structural organization. In the second week after birth, the *desmin* null mice appear more lethargic than their wild type and heterozygous litter mates. Dissections from these mice showed extensive calcium deposits in the myocardium and signs of degeneration. Histological and electron microscopic analysis revealed the appearance of a general loss of cardiomyofiber tension, with areas of disruption and disorganization of cardiac myofibers. In contrast to that, dissections from neonates did not show the same signs of degeneration and calcification. However, electron microscopy of *desmin* null ventricular muscle revealed that several myofibers demonstrated severely disrupted architecture. In contrast to wild type fibers, which display laterally aligned myofibrils and packed mitochondria, the *desmin* null myofibrils have lost their lateral alignment and often show disrupted myofibrils, particularly in the position of the intercalated discs. The loss of myofibrillar anchorage to the plasma membrane is one of the hallmarks of the observed defects in *desmin* null mice. Furthermore, the mitochondria have lost their shape and organization, and often appear to be "swollen". *(Capetanaki et al.1997)*

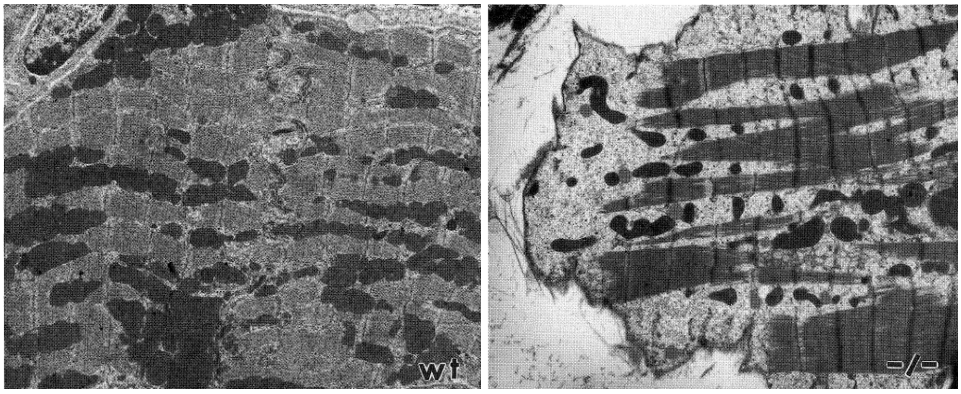


Fig.1.6.2: The muscle architecture in wild type (wt) and desmin null (-/-) mice *(Capetanaki et al.1997). Transmission electron microscopy of cardiac muscle from desmin wt and null mice is shown above. Wt muscle (left) shows aligned arrays of myofibrils terminating and inserting into intercalated discs. Myofibrils are occasionally interrupted by packed strands of mitochondria. In desmin null muscle (right) myofibrils are disorganized and separated from the sarcolemma, hypercontracted, and damaged. The mitochondria are disorganized and abnormal in size and shape.*

Histological examination of *desmin* null mice skeletal muscle of the tongue, leg and diaphragm revealed similar defects in the integrity and organization of the myofibers, but to a lesser extent than it was seen in the cardiac muscle. The abnormalities that were observed ranged from loss of myofiber tension, ragged and disorganized myofibers with less discernible striation to loss of stable nuclear positioning and indications of degeneration. Cross sections of skeletal muscle from *desmin* null and wild type mice also demonstrate that in mice lacking desmin, many fibers are ragged in appearance, of smaller diameter, and are less closely packed, when compared to wild type muscle. Among the different skeletal muscles, tongue is most affected by myofiber disruption and disintegration. The diaphragm also is severely affected. Less affected are muscles of the thigh and the long muscles of the back. This pattern shows that in *desmin* null mice more active skeletal muscles are more affected by myofiber disruption than less active ones. Histological analysis of smooth muscle revealed similar defects. The abnormalities in the smooth muscle of *desmin* null mice include loose organization of smooth muscle cells, and loss of the normal elongated nuclear shape. In summary the effects of the absence of desmin are most severe in the myocardium, with smooth muscle seemingly least affected and skeletal muscle exhibiting damage that is intermediate in severity. However, only a fraction of the cell population in a given muscle tissue is defective. Together, the findings of Y.Capetanaki, D.J. Milner, G.Weitzer and colleagues *(Capetanaki et al. 1997; Milner et al. 1996; Weitzer et al. 1995)* suggest that *in vivo* desmin is not absolutely necessary for the formation and assembly of myofibrils, but they rather indicate that it is required for maintenance of cellular integrity and possibly for repair and regeneration after mechanical damage. Muscle that is deficient in desmin seems to be more fragile than wild type muscle and seems to be more easily damaged by contractile activity.

Another animal model brought more insight into formation of protein aggregates. Transgenic mice expressing a human mutation p.Arg173-Glu179del in the *desmin* gene show accumulation of chimeric intracellular aggregates of desmin and other cytoskeletal proteins in the myocardium. Such inclusions are not seen in knock-out mice, supporting the hypothesis that mutant misfolded proteins may act as seeds in the formation of these protein aggregates in desminopathies. The protein aggregates disrupt the continuity and overall organization of the desmin network throughout the cell. These aggregates appear as electron-dense granulofilamentous structures proximal to the nucleus and in the inter-myofibrillar space. Missfolded desmin protein seems to escape proteolytic breakdown and attracts other

cytoskeletal proteins into insoluble high molecular weight chimeric aggregates that grow and finally have toxic effects on the tissue *(Goldfarb et al. 2008).*

Conclusions from the two existing models

The *in vitro* and *in vivo* studies introduced above indicate that desmin and in general the IF network is vital for the maintenance of cellular integrity and architecture of muscle cells, but not for myogenic commitment, differentiation or fusion of myoblasts *in vivo*. The IFs together with extracellular- and nuclear matrix proteins build up a network that extends throughout the muscle fibers. This network is thought to serve in coordination of contraction and relaxation, in strengthening the muscle, in transmission of mechanical forces and mechano-chemical signals throughout the muscle fiber, to the nucleus, other cellular organelles and the entire muscle in a coordinated fashion *(Capetanaki et al. 1997).* Ablation of the *desmin* gene expression demonstrated that functional desmin protein is crucial to the architectural and functional integrity of striated muscle. Mice lacking desmin showed a multisystem disorder, involving cardiac, skeletal, and smooth muscle. Histological and electron microscopic analysis in both heart and skeletal muscle tissue revealed severe disruption of muscle architecture and degeneration. Structural abnormalities included loss of lateral alignment of myofibrils and abnormal mitochondrial organization. These defects appear most severe in the heart, which exhibited progressive degeneration and necrosis of the myocardium, accompanied by extensive calcification. The consequences range from cardiomyocyte hypertrophy and chamber dilatation to heart failure. Chamber dilation is characterised by extensive myocyte death, fibrosis and calcification of the myocardium *(Li et al.1996; Milner et al.1996).* Abnormalities of smooth muscle included hypoplasia and degeneration. However, the detailed mechanism by which desmin deficiency leads to heart failure remains elusive, but one important hint may be the finding of disturbed mitochondrial behaviour, distribution and function in desmin-deficient mice *(Capetanaki et al.2002).* According to a study from Milner D.J. *(Milner et al.2000)* mitochondrial abnormalities appear prior to any other obvious effect in desmin-deficient mice. Mitochondria are despite their important role in a cells respiratory cycle, the central executioners in apoptotic and non-apoptotic cell death. Activation of pro-death signal transduction cascades or damage pathways can perturb the mitochondrial membrane integrity. This may lead to the activation of the mitochondrial permeability transition pore (mtPTP) complex and/or to the activation of Bcl-2-Bax complex or the activation of caspases. In both cases, the cells “decision to die”, directly affects its mitochondria and is executed by these organelles. In *des*-deficient mice, “swollen”

mitochondria were observed. This might be a consequence of permeabilization of the mitochondrial inner and outer membrane (IM/ OM). The permeabilization of the OM will release proteins from the intermembrane space, such as cytochrome C, certain pro-caspases, adenylate kinase 2, and apoptosis-inducing factor. The permeabilization of the IM leads to dissipation of the intra-mitochondrial proton gradient, release of small solutes, such as calcium and glutathione, and to an influx of water and sucrose, that eventually may cause swelling of mitochondria. The permeabilization of the OM and IM can be triggered by a variety of apoptotic second messengers, including different members of the Bcl-2-family, like Bax, Bac, and Bid. The function and the localization of the different Bcl-2-family members is further influenced by signaltransduction molecules, including phosphatases, kinases, and caspases. Another non-protein factor, which mediates important "decisions" in mitochondria and concomittant in the cell, is calcium (Ca^{2+}). Ca^{2+}-homeostasis is mainly executed by mitochondria. On one hand, excessive intracellular Ca^{2+} contributes to a final common pathway of cytotoxic events, leading to formation of reactive oxygen species (ROS) and cell death. On the other hand, Ca^{2+} is a major signaling molecule, that stimulates for instance Ca^{2+}-sensitive enzymes in the Krebs cycle (pyruvate dehydrogenase (DH), isocitrate DH, and oxoglutarate DH), which allow the cell to adapt to increased need of energy. However, studies on the IF cytoskeleton have shown its importance for normal mitochondrial behaviour and function. Whether the abnormal behaviour of mitochondria is triggered by the lack of a functional IF network and the disturbance of Ca^{2+}-homeostasis is a consequence, or the latter is the leading cause, remains elusive.

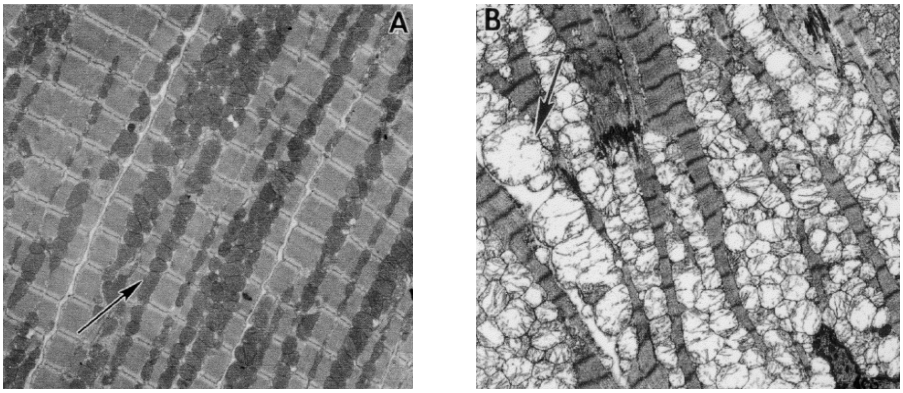


Fig.1.6.3: Desmin- null cardiac muscle. *TEM pictures of the extensive mitochondrial proliferation and degeneration in desmin- null cardiac muscle (Capetanaki Y., 2002)* ***(A)*** *Cardiac muscle of wild type mice, with normal ultrastructure, laterally aligned myofibrils, and strands of mitochondria between myofibrils.* ***(B)*** *Cardiac muscle of exercised desmin-null mice, with severe mitochondrial proliferation and swelling.*

Amino acid deletions or substitutions due to miss-sense mutations in the *desmin* gene may lead to aberrant filament assembly and function or to changes in filament turnover rates. Unfortunately, the detailed mechanisms, which lead to pathologic phenotypes, are still unclear. It is speculated that, compromised assembly of desmin protein and altered biophysical properties of the mutated protein may lead to increased susceptibility to stress-induced filament break down and increased proteolysis. Another speculation is that altered desmin protein may be impaired in its interaction options with potential cellular binding partners, and thus is prone to degradation or segregation into protein granules *(Bär et al. 2004)*.

A *desmin* null mutation in mouse model gives rise to a cardiomyopathy which early in post-natal life is seen as lysis of individual cardiomyocytes. This lysis is followed by invasion of the affected region by macrophages, and development of varying degrees of calcification and fibrosis. Disruption of the sacolemma, probably at the intercalated discs, seems to be a main event preceding cardiomyolysis, whereas myofibrillar disorganization due to a lack of intermyofibrillar desmin filaments seems to be of less significance *(Thornell et al.1997)*.Once regional myolyis has occurred, the entire myocardium will be weakened, because in mammals the cardiomyocytes do not regenerate. The heart lacks the equivalents of skeletal muscle satellite cells. Therefore, as fibroblasts invest, replace dying cardiomyocytes, and secrete extra-cellular matrix components, fibrosis occurs *(Thornell et al.1997)*. Thus, the advanced degeneration of the myocardium in *des*$^{-/-}$ mice, the calcification and fibrosis do not seem to be specific for the *des*$^{-/-}$ cardiomyopathy, but rather seem to be a result of a general repair process. This explains why the same hallmarks of myolysis, myocyte degeneration, calcification and fibrosis, as they were observed in the *des*$^{-/-}$ animals, were also found in animals that express normal *desmin (Reichenbach et al. 1969)*.

1.7. Clinical manifestation of desminopathy

Desminopathy originally was described as skeletal and cardiac myopathy. It is characterized by bilateral weakness in distal leg muscles spreading proximally, or as proximal muscle weakness spreading distally and eventually leading to weel-chair-dependence. The weakness may involve extremities, trunk, neck flexors and facial muscles. In some variants of the disease patients experience dizziness, syncopal (short loss of consciousness) and fainting episodes associated with conduction blocks. These conditions require implantation of a permanent pace maker. Since the desminopathy ten years ago was defined as an independent pathology, many new affected families were identified. In a review from Goldfarb and

colleagues *(Goldfarb et al.2008)* 65 affected families with 98 patients are mentioned. Constantly increasing knowledge and rising awareness enables a more precise definition of clinical manifestations and diagnostic criteria.

The pattern of inheritance was reliably established in 52 families with desminopathy. It was autosomal dominant in 32 of them and recessive in three families. Seventeen patients had sporadic onset with *de novo* mutations. The age of onset in autosomal dominant inherited desminopathy is between 14 and 48 years, while patients with recessive mutations develop the disease in their childhood or adolescence. The analysis of the clinical manifestations led to characterization of distinct, sometimes overlapping forms of desminopathy:

Progressive skeletal myopathy

A progressive uncomplicated form of skeletal muscle weakness was observed in 13 patients from six families. Initial symptoms were bilateral distal muscle weakness in the lower extremities, which later spread to proximal leg muscles, upper extremities, and neck flexors. Muscle biopsy showed abnormal accumulation of desmin-immunoreactive deposits in muscle fibers. As causative agent two different mutations were identified. The first was an amino acid substitution from asparagine to aspartate at residue 342. The second was a deletion of three amino acids from Glu359 to Ser361 *(Goldfarb et al.2008).*

Respiratory dysfunction

Respiratory dysfunction causes nocturnal hypoventilation with oxygen desaturation and eventually day time respiratory failure. The context between a disorder of a muscle specific protein and respiratory dysfunction is made by the diaphragm. The diaphragm, unlike other skeletal muscles, is functioning in an environment in which forces can be transmitted in both, longitudinal and transversal, directions in each respiratory cycle. Here desmin can serve as visco-elastic element to dissipate forces in both planes, because it is the only (known) molecule that has dual orientation. The critical significance of desmin in the respiratory cycle is most likely reflected by the fact that in the diaphragm the content of desmin is 38% higher compared to biceps femuris muscle. However, respiratory dysfunction combined with skeletal muscle weakness was observed in 10 patients from 5 families. The illness was caused by a missense mutation in desmin (i.e. a shift from alanine 357 to proline). Cardiac abnormalities were not observed in these patients. Muscle biopsies showed variation in fiber size and furthermore, intra-cytoplasmic eosinophilic patches, which in immuno-cytochemistry turned out to be desmin deposits. Moreover, deposits of desmin were seen in the subsarcolemmal space between myofibrils in electron microscopy. *(Goldfarb et al.2008)*

Cardiomyopathy

Primary cardiomyopathies are classified pathophysiologically as dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), restrictive cardiomyopathy (RCM), and arrythmogenic right ventricular cardiomyopathy (ARCM). DCM is characterized by an increased ventricular chamber size and reduced systolic output. The disease pattern of RCM results from all processes that lead to stiffening of the myocardium. Infiltration and fibrosis lead to impaired ventricular filling and reduced diastolic volume, although wall thickness and cavity size appear normal. Out of 34 patients with cardiomyopathy, 18 were diagnosed with DCM, 10 with RCM, and 6 with HCM. As the heart conduction system is rich in desmin, a frequent feature in desminopathy patients is atrioventricular conduction abnormality. This condition often requires urgent implantation of a permanent pacemaker. Skeletal myopathy followed by cardiomyopathy was observed in 32 patients from 20 families. Cardiomyopathy alone with no signs of skeletal myopathy was seen in 19 patients from 15 families. *(Goldfarb et al.2008)*

Correlation between a genotype and a phenotype in desminopathy

Routine genetic screening gave convincing evidence that distinct clinical phenotypes of desminopathy resulted from different mutations. Cardiomyopathy, smooth muscle myopathy, respiratory dysfunction, neuropathy, facial paralysis or cataracts may be present in some cases, but absent in others. Phenotypic manifestations in members of the same family were concordant, with few exceptions. This observation suggests that the type and location of the mutation within the *desmin* gene most likely influences the resulting phenotype. For instance, patients carrying mutations in the 2B segment tend to show primarily skeletal muscle pathology, while those carrying mutations in the 1B and tail domains predominantly develop a more ominous cardiac disease. Moreover, there is a clear tendency for the age of onset to be four to ten years earlier and with faster progression in patients with cardiomyopathy as an early feature of disease. *(Goldfarb et al., 2008)*

Table1.7: *Clinical phenotypes of desminopathy caused by mutations located in the different functional domains of desmin protein. (Goldfarb et al. 2008)*

	Desmin Domain		
Phenotypes	**1B**	**2B**	**Tail**
Isolated Progressive Skeletal Myopathy	1	11	3
Skeletal Myopathy with Respiratory Insufficiency	1	6	3
Skeletal Myopathy Followed by Cardiomyopathy	3	24	5
Cardiomyopathy Followed by Skeletal Myopathy	4	10	3
Isolated Cardiomyopathy	6	4	8
Total number of patients	15	55	22

1.8. Working hypothesis and design of this thesis

From previous studies, we knew that the proper expression of the desmin protein is vital for cardiomyogenesis, although transgenic *des*$^{-/-}$ mice develop a functional heart and are viable. To further investigate desmin's influence on cardiomyogenesis, two major experimental branches were designed. The first branch serves to prove a potential physical interaction of the desmin protein and regulatory elements in the genomic DNA. Therefore, a chromatin immuno-precipitation (ChIP) was performed with EBs of different developmental stages. In ChIP, residual proteins are reversibly cross-linked to the genomic(g) DNA. The gDNA is shredded by sonication. The proper sonication of the gDNA is an important step, because finally the ChIP samples are analysed by PCR for the presence of specific bands. Incomplete sonication of the ChIP samples might result in false-positive signals in later PCR. After the sonication, the samples are incubated with antibodies, which are specific for the protein of interest. Then protein A-coupled sepharose beads are added to hold the Fc-portion of the antibodies. The samples undergo a series of washing steps with different buffers to wash away unbound DNA, residual salts, and traces of RNA. In parallel, a "whole-amount" (input) sample is prepared. The input samples reflect the total amount of DNA, which is present in the sample before the series of washing steps. Finally, the crosslink between DNA and protein is reversed and the DNA is purified by phenol-chloroform extraction. The samples are used for ChIP-PCRs. These PCRs allow for amplification and therefore for visualization of the genomic regions, which physically interact with the protein of interest. Therefore, as it is mentioned above, the sonication is a vital step in this proecedure, because incomplete sonication may lead to the purification of a DNA fragment, which had bound the protein of interest at another site than the genomic element of interest.

As the second experimental model we chose a luciferase reporter assay system. This reporter assay system was designed to see if desmin is not only able to bind to DNA, but also to interact with regulatory genomic elements, such as the *nkx 2.5* promoter and the minimal cardiac enhancer *(Lien et al.1999).* Therefore, desmin was co-expressed with plasmids, which contain the *firefly luciferase* reporter gene under the control of the *nkx 2.5* promoter or the minimal cardiac enhancer element additionally. The Dual Luciferase Reporter Assay® (Promega) provides a system, which allows for validation of such interactions. To introduce the luciferase reporter plasmids and other plasmids into eukaryotic cells, two different methods of transfection were engaged. First, the calcium phosphate precipitation-method was used. This method is useful for transient transfections. It works by a mechanism, in which the DNA associates with calcium-phosphate crystals and is taken up by endocytosis. Second,

Lipofectamin 2000 (Invitrogen) was used to transiently transfect eukaryotic cells. The Lipofectamine 2000 seems to give better transfection efficiencies and the results are more reproducible.

These two methods may demonstrate that desmin interferes with cardiomyogenesis on the transcriptional level by binding to promoter and enhancer elements of transcription factor genes, which are crucial in cardiomyogenesis, and that desmin alters the expression of such TF genes.

2. Results and discussion

2.1. The interaction of desmin with the 5'UTR of nkx 2.5

2.1.1 The *nkx 2.5* gene

The murine *nkx 2.5* gene is located on chromosome 17. The *nkx 2.5* promoter region and the minimal cardiac enhancer region are critical to murine heart development *(Searcy et al.1998; Lien et al.1999).* The locations of the *nkx 2.5* gene, its promoter region and a minimal cardiac enhancer region are shown in figure 2.1.1. The arrows indicate the binding sites of specific primer pairs, which were used in ChIP PCRs. The specific primer pairs were chosen to amplify the nkx 2.5 promoter region and the minimal cardiac enhancer region. Primer pairs, which bind to regions adjacent to these two well characterized genomic elements, were used as negative controls, as shown in figure 2.1.4.

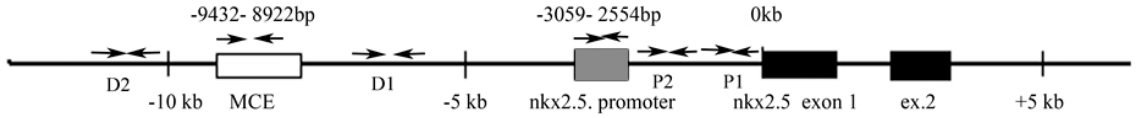


Fig.2.1.1*: Schematic drawing of the mouse* nkx 2.5 *gene and its upstream regions. Primer binding sites are indicated as arrows. Minimal cardiac enhancer (white box),* nkx2.5 *promoter (grey box),* nkx 2.5 *exons 1 and 2 (black boxes). Primer pairs: distal 2 (D2), distal 1 (D1), proximal 2 (P2) and proximal 1 (P1).*

2.1.2 Interaction of desmin with the nkx2.5 promoter region

2.1.2.1 Desmin is able to bind to the *nkx 2.5 promoter*

To assess the binding of desmin to the *nkx 2.5* promoter region during early cardiomyogenesis, $des^{+/+}$ embryonic stem cells (ESC) were aggreagted to form embryoid bodies (EBs). In EBs some cells autonomously undergo cardiomyogenesis starting with the development of *brachyury*-positive primitive mesoderm around day 3 after aggregation. Finally, the autonomous cardiomyogenesis in EBs results in the development of fully differentiated and functional cardiomyocytes between day 7 and 11. The EBs of different developmental stages (day 5 to 9) were subjected to chromatin-immuno-precipitation. The

method is described in detail in the methods chapter 4.2.8. Input samples (In) reflect the whole gDNA, which is present in the sample prior to the selective purification in the immuno-precipitation (IP). Desmin (des) samples contain gDNA fragments, which were bound by desmin and were selectively purified in presence of desmin-specific antibodies. Mock samples were prepared in presence of pre-immune sera from mice or without the addition of specific antibodies, respectively. The Mock samples should serve as negative controls. Histone H3 samples were prepared in presence of specific antibodies to histone H3. The Histone H3 samples should serve as positive controls. In all ChIP PCRs an internal PCR negative control was included, with sterile distilled water as "template" (data not shown). In the following demonstrations only PCR results were included, when the internal negative control was free of contamination.

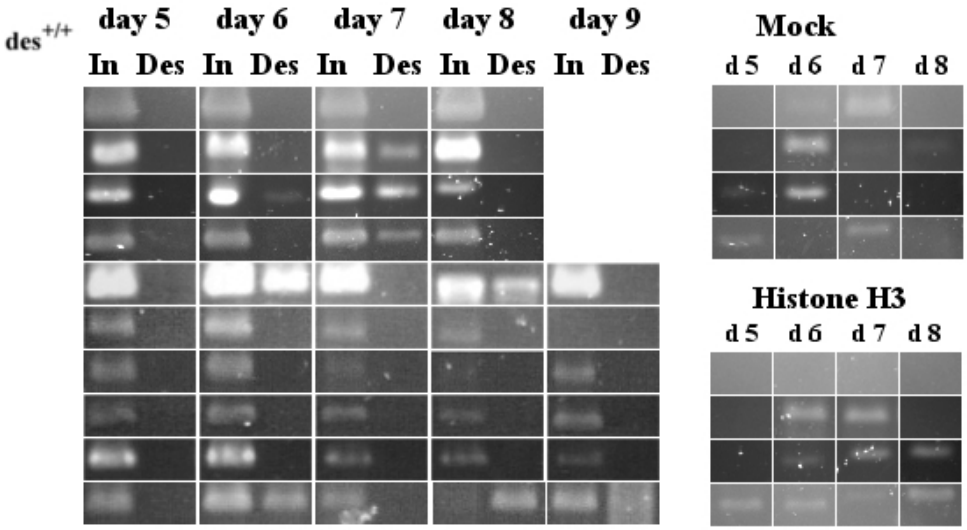
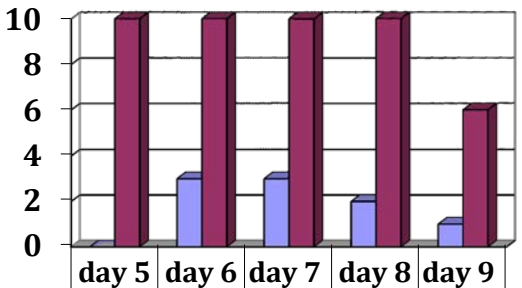


***Fig.2.1.2.1A**: Summary of ChIP PCR results from* des$^{+/+}$ *EBs.*
The ChIP PCR samples were prepared from desmin wild type (des$^{+/+}$) embryoid bodies of different developmental stages (days 5-9 of EB development, as indicated). (In) Input samples; (Des) desmin samples; Mock: negative control; Histone H3 (Hist)



	day 5	day 6	day 7	day 8	day 9
desmin bound	0	3	3	2	1
total number of replicates	10	10	10	10	6

***Fig.2.1.2.1B**: Columnar diagram of the nkx 2.5 promoter ChIP PCR results with desmin wild type EBs.*

By performing ChIP PCRs with des $^{+/+}$ embryoid bodies, we found that desmin binds to the *nkx 2.5* promoter, at day 6, day 7, day 8 and once at day 9, but not at day 5 of *in-vitro* differentiation. Interestingly, the binding of desmin to the *nkx 2.5* promoter at day 6 corresponds to the timing of *in vitro* cardiomyogenesis. Usually, in $des^{+/+}$ EBs first signs of beating cardiomyocytes may be observed around day 6.

The histone H3 samples were chosen as positive controls, because in theory the histone H3 component is ubiquitously and unselectively bound to transcriptionally and synthetically inactive chromatin. Therefore, we expected to find PCR bands in all the histone H3 samples. As it is shown above this expectation was not fulfilled. One might speculate that the lack of bands could be due to the onset of transcriptional activity, which is enabled by loosening of the tight histone packing. This loss of histone packing prior to the observation of first beating cardiomyocytes in day 6 embryoid bodies would perfectly go along with the onset of *nkx 2.5* gene expression.

A striking throwback of the presented results is the presence of PCR bands in the mock samples. In the beginning of this thesis, these samples were prepared without specific antibodies, later a pre-immune serum was used instead. They were included as negative controls and were expected to show no PCR bands. The reason why the Mock samples showed PCR bands might be found in the use of protein A-coupled sepharose beads. These protein A-coupled sepharose beads need to be blocked with salmon or herring sperm DNA, as we found out after a couple of experiments with positive mock samples. However, as the time frame of a diploma thesis is too short to repeat all experiments, here the complete data set is shown, including the results of sub optimal procedures.

2.1.2.2 Amino-terminally truncated desmin fails to bind to the *nkx 2.5* promoter

The N-terminus of desmin is known to be vital for cardiomyogenesis and for DNA-binding *(Tolstonog et al.2005; Höllrigl et al 2007, Hofner et al. 2007).* The expression of an N-terminally truncated variant of desmin protein was shown to severly interfer with cardiomyogenesis in the EB model. When $des^{\Delta 1\text{-}48}des^{\Delta 1\text{-}48}$ ESC are *in-vitro* differentiated in the EB model, they develop delayed smaller and less interconnected fields of beating cardiomyocytes compared to $des^{+/+}$ EBs *(Hofner et al.2007).* The $des^{\Delta 1\text{-}48}des^{\Delta 1\text{-}48}$ EBs were included in the ChIP experiments to demonstrate the absence of desmin binding to chromatin, when the N-terminal portions, which are vital for DNA-binding and interaction, are missing. Therefore, *in-vitro* differentiated ESC, which ectopically express N-terminally truncated desmin *($des^{\Delta 1\text{-}48}des^{\Delta 1\text{-}48}$)*, were subjected to ChIP procedure. The ChIP samples were used for

PCR with primers, which serve to specifically amplify the *nkx 2.5* promoter. Input samples (In) reflect the whole gDNA, which is present in the sample prior to the selective purification in immuno-precipitation (IP). Desmin (des) samples contain selectively purified gDNA fragments, which were bound by desmin-specific antibodies. Mock samples were prepared in presence of pre-immune sera from mice or without the addition of specific antibodies, respectively. The mock samples are negative controls. Histone H3 samples were prepared in presence of specific antibodies to histone H3. The Histone H3 samples serve as positive controls. In all ChIP PCRs an internal PCR negative control was included, with sterile distilled water as "template" (data not shown). In the following demonstration only PCR results were included, when the internal negative control was free of contamination.

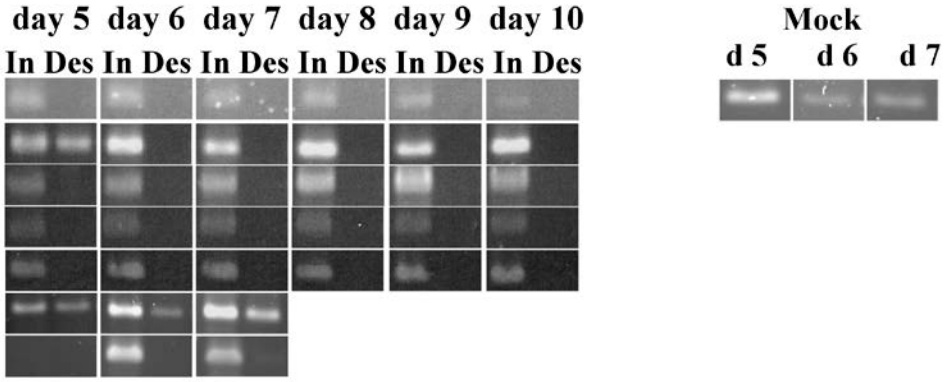
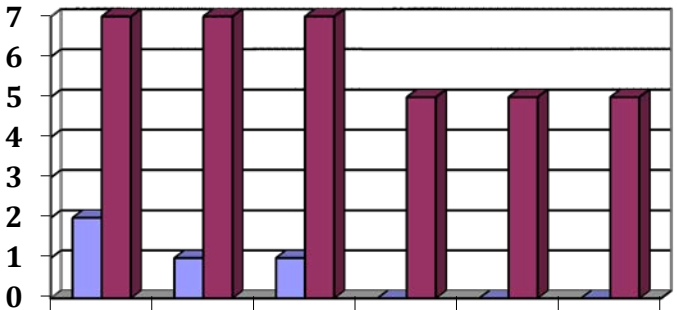


***Fig.2.1.2.2A**: Summary of ChIP PCR results from des$^{\Delta 1\text{-}48}$des$^{\Delta 1\text{-}48}$EBs.*
(In) input samples; (Des) desmin samples; Mock: negative control. Histone H3 (Hist) are positive controls.



	day 5	day 6	day 7	day 8	day 9	day 10
desmin bound	2	1	1	0	0	0
total number of replicates	7	7	7	5	5	5

***Fig.2.1.2.2B**: Shows a columnar diagram of the nkx 2.5 promoter ChIP PCR results with des $^{\Delta 1\text{-}48}$ des $^{\Delta 1\text{-}48}$ EBs.*

Due to the findings of my colleagues A.Höllrigl and M.Hofner in 2007, we expected the embryoid bodies, which ectopically express an N-terminally truncated desmin variant, to be unable to bind to DNA. The N-terminus of desmin is known to be vital for cardiomyogenesis and for DNA-binding *(Tolstonog et al.2005; Höllrigl et al 2007, Hofner et al. 2007).*

To explain the observed DNA-binding from desΔ1-48, there may be two theories. The first would be that desmin harbours an alternative DNA-binding site despite the N-terminal. The second would be that something went wrong in the ChIP procedure and that the PCR bands derive from contamination of the ChIP samples themselves. From the ChIP results, it is impossible to say if there is an alternative DNA-binding site in the desmin protein apart from the N-terminal, which leads to the unexpected rare positive result. Most likely, the bands in the desmin samples of day 5, day 6 and day 7, are artefacts, because this result occurred only once in all the ChIP samples. Likewise as the positive bands in the mock samples, this is most likely the result of the improper blocking of protein A-coupled sepharose beads or DNA contamination of the ChIP samples. In conclusion, we doubt that the positive signal from the N-terminally truncated desmin is an image of the real condition during the differentiation of des $^{\Delta 1\text{-}48/\Delta 1\text{-}48}$ embryonic stem cells. We further assume that the truncated version of desmin (des $^{\Delta 1\text{-}48/\Delta 1\text{-}48}$) is unable to bind to the *nkx 2.5* promoter.

2.1.2.3 Ectopically expressed desmin binds to the *nkx 2.5* promoter earlier during *in-vitro* differentiation of cardiomyocytes

Embryonic stem cells, which ectopically express desmin (des $^{+/+/ect}$), underwent *in-vitro* differentiation in the embryoid body model. In addition to the input, desmin, histone H3 and the mock samples, c-Myc samples were included as positive controls. C-Myc binds to E-boxes, which are numerous in the 5'UTR of the nkx 2.5 gene (at least eight). E-boxes are common elements in promoter and enhancer sequences. The consensus sequence of E-boxes is CANNTG. The E-boxes serve as binding sites for transcription factors, which contain a basic-helic-loop-helix structural motif.

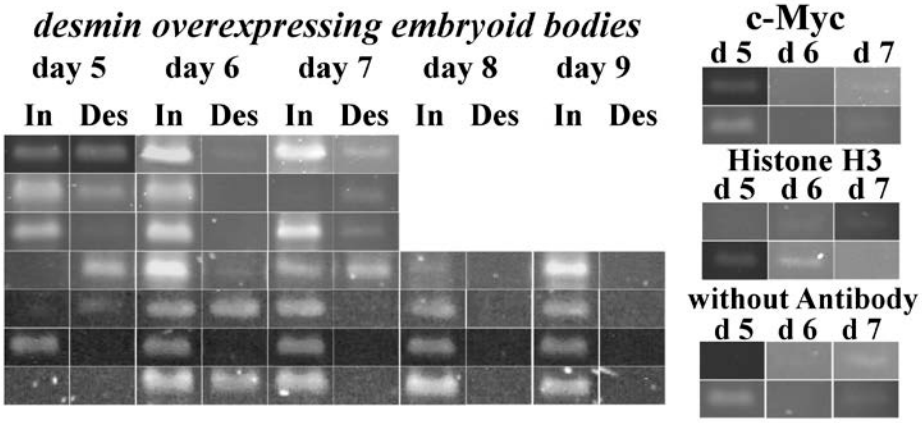
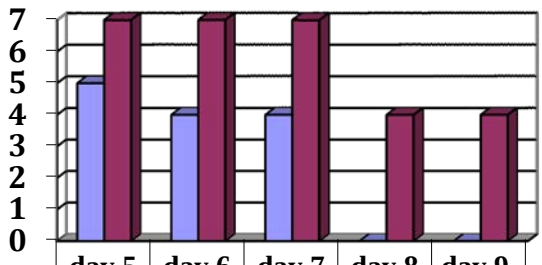


***Fig.2.1.2.3A**: Summary of ChIP PCR results from des$^{+/+}$ des^{ect} EBs of different developmental stages (as indicated). Input samples (In), Desmin (Des), C-Myc samples were included as positive controls. "Without antibody" (negative control). Histone H3(positive control)*



	day 5	day 6	day 7	day 8	day 9
desmin bound	5	4	4	0	0
total number of replicates	7	7	7	4	4

***Fig.2.1.2.3B**: Shows a columnar diagram of the nkx 2.5 promoter ChIP PCR results with des $^{+/+}$ des ^{ect} EBs.*

The ChIP PCR from *des* $^{+/+/ect}$ EBs revealed that desmin is bound to the *nkx 2.5* promoter at day 5, 6, and 7 but not at day 8 of EB development. The binding of ectopically expressed desmin at day 5 is perfectly in line with the fact that *des* $^{+/+/ect}$ EBs develop beating cardiomyocytes one day earlier compared to wild type *des* $^{+/+}$ EBs.
The bands in the "without antibody" samples are most probably derived from unspecific bindings between un-blocked sepharose beads and gDNA. As it was already mentioned above, the un-blocked beads were used accidentally in the beginning of this thesis. Later, the experiment was repeated with blocked sepharose beads. This helped to reduce unwanted bands, but sometimes even led to the complete lack of bands in PCR from specifically purified samples.

2.1.2.4 Desmin-deficient embryoid bodies as a negative control

The ChIP experiment dataset is rounded up by the inclusion of embryoid bodies from desmin-deficient (*des* $^{-/-}$*)* ESC. These *des* $^{-/-}$ EBs develop beating cardiomyocytes, but several days later, compared to the desmin wild type EBs. In addition to that, the *des* $^{-/-}$ EBs develop smaller fields of beating cardiomyocytes, which are also less interconnected. On one hand, the following dataset was included for the means of completeness. On the other hand, it was a helpful tool to prove the specificity and the reliability of the ChIP method.

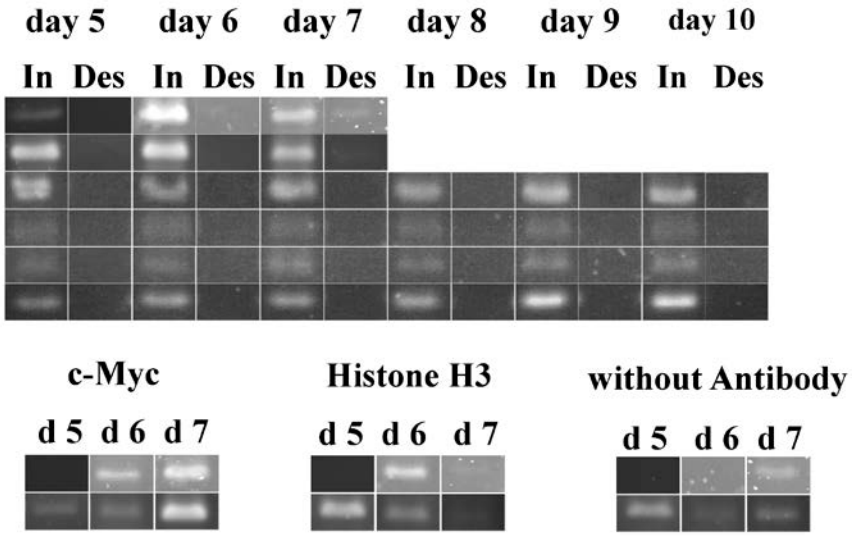
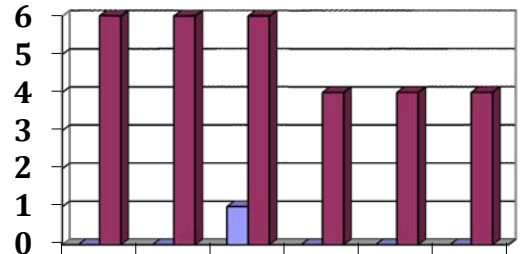


***Fig.2.1.2.4A**: Summary of ChIP PCR results from des$^{-/-}$ embryoid bodies.*
Input samples (In), Desmin (des) samples, C-Myc samples as positive controls, "Without antibody" as negative controls. Histone H3 as positive controls.



	day 5	day 6	day 7	day 8	day 9	day 10
desmin bound	0	0	1	0	0	0
total number of replicates	6	6	6	4	4	4

***Fig.2.1.2.4B**: Shows a columnar diagram of the nkx 2.5 promoter ChIP PCR results with des$^{-/-}$ EBs.*

The figures 2.1.2.4AB nicely show the presence of the *nkx 2.5* promoter DNA in the input samples, but the lack of bands in the anti-desmin samples. The weak positive result from day 7 *des* $^{-/-}$ EBs is most probably derived from a contamination of unknown source. The bands from the "without antibody" samples again most probably result from the use of unblocked sepharose beads, which can lead to unspecific binding of gDNA. Fortunately, un-specific bindings were not found in the anti-desmin samples.

2.1.3 Interaction of desmin with a „minimal cardiac enhancer" region

The minimal cardiac enhancer was characterized in experiments from Lien and colleagues in 1999 *(Lien et al.1999).* The scientists cloned sequences of the 5' untranslated region of the *nkx 2.5* gene upstream of the *lacZ* gene to find important *cis-acting* elements. The cloned sequences were used to generate transgenic mice, in which the expression of the 5'UTR-*lacZ*

transgene could be monitored by β-galactosidase staining. The transgenic mice were screened for β-galactosidase staining. Further, organ-specific expression of the *lacZ* reporter in the transgenic mice was matched to the different cloned sequences. The cardiac-specific region of the 5'UTR of the *nkx 2.5* gene was named the "cardiac enhancer". To further delimit the boundaries of the cardiac enhancer, a series of 5'and 3'deletion was created and tested for cardiac-activity in transgenic mice. This study revealed that two separable regions of the *nkx 2.5* 5'UTR positively influence cardiac activity. The first is located between positions -9435 and -8922 of the *nkx2.5* 5'UTR , whereas the second is located between positions -8039 and -7353. The cardiac specific region -9435 to -8922 was defined as the minimal cardiac enhancer, because it alone exhibited cardiac activity. In contrast, the second region (-8039 to -7353) was shown to be unable to confer cardiac expression alone, but was able to enhance the expression of the minimal cardiac enhancer (-9435 to -8922) *(Lien et al.1999)*.

To test the potential of desmin to interact with this minimal cardiac enhancer region, we designed ChIP primers (MCE1107 2 fwd and rev). The sequence of these primers is shown in a list in chapter 3.5.

2.1.3.1 Desmin is able to bind to the minimal cardiac enhancer

Wild type ESC were aggregated to EBs, as it is described above and in the methods chapter 4.1.6. The EBs were subjected to chromatin immune-precipitation as described in detail in chapter 4.2.8. Input samples (In) reflect the whole gDNA amount, which is present in the sample prior to the selective purification in the immune-precipitation (IP). Desmin (des) samples contain selectively purified gDNA fragments, which were bound by desmin-specific antibodies. Mock samples were prepared in presence of pre-immune serum or without the addition of specific antibodies. The mock samples serve as negative controls. Histone H3 samples were prepared in presence of specific antibodies to histone H3. The Histone H3 samples serve as positive controls. Transcriptionally and synthetically inactive chromatin is tighly wrapped around histones. Therefore, histone H3 should be unseclectively bound to the minimal cardiac enhancer, unless this region is highly transcriptionally active.

In all ChIP PCRs an internal PCR negative control was included, with sterile distilled water as "template" (data not shown). In the following demonstration only PCR results were included, when the internal PCR negative control was free of contamination.

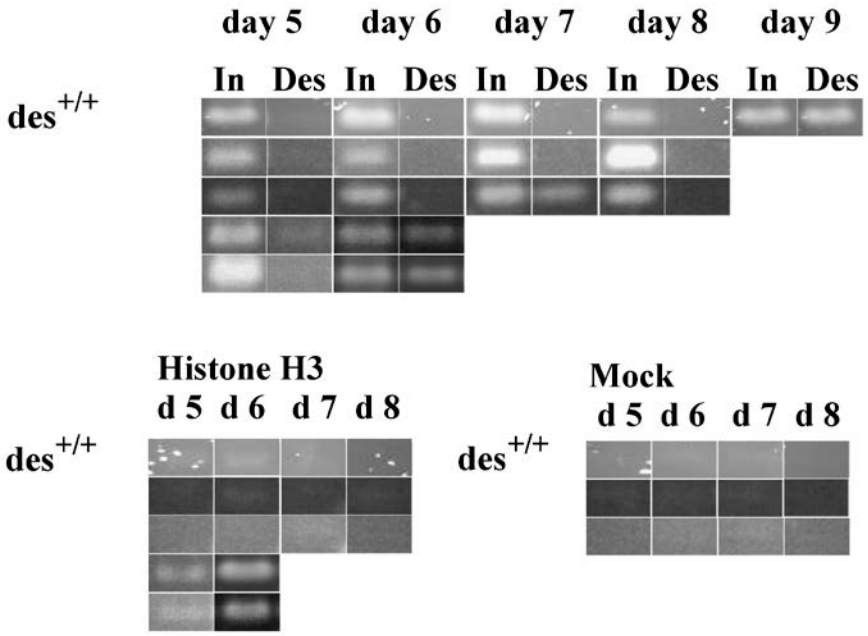
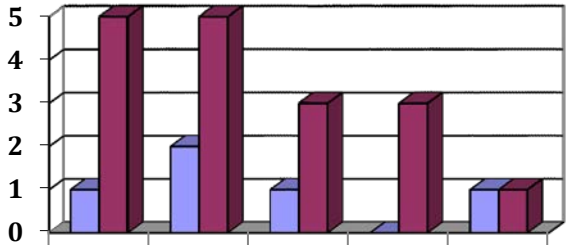


***Fig.2.1.3.1A**: Summary of ChIP PCR results from des$^{+/+}$ embryoid bodies. Input samples (In). Desmin (Des) samples. Mock samples serve as negative controls. Histone H3 samples as positive controls.*



	day 5	day 6	day 7	day 8	day 9
desmin bound	1	2	1	0	1
total number of replicates	5	5	3	3	1

***Fig.2.1.3.1B**: Shows a columnar diagram of the minimal cardiac enhancer ChIP PCR results with des$^{+/+}$ EBs.*

In the results which are presented in figures 2.1.3.1AB, wild type desmin (des $^{+/+}$) is bound to the minimal cardiac enhancer at day 5, day 6, and day 7 of EB development. As the day 9 EBs were included only once in the experiment, it may be doubted that the positive signal depicts the real state during EB development, but however, this result was included for the means of data completeness. In case of the data, which is shown here, the mock negative controls of ChIP procedure prove to be satisfying.

2.1.3.2 Amino-terminally truncated desmin might harbour an alternative DNA binding site

Embryonic stem cells, which ectopically express an amino-terminally truncated variant of the *desmin* gene (*des* $^{\Delta 1\text{-}48/\Delta 1\text{-}48}$) were aggregated to form embryoid bodies (EBs). These EBs were subjected to ChIP as described in detail in chapter 4.2.8. Input samples (In) reflect the whole gDNA amount, which is present in the sonicated sample prior to selective purification in presence of specific antibodies. Desmin (Des) samples were selectively purified in the presence of specific antibodies to desmin protein. Histone H3 samples were purified in the presence of specific antibodies to the histone H3 component. The histones are bound to transcriptionally and synthetically inactive chromatin, and therefore serve as positive controls.

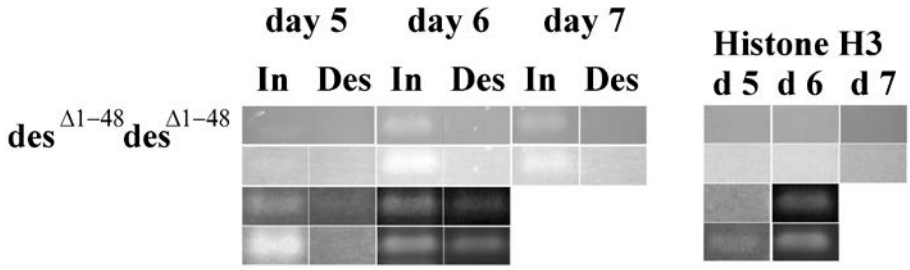
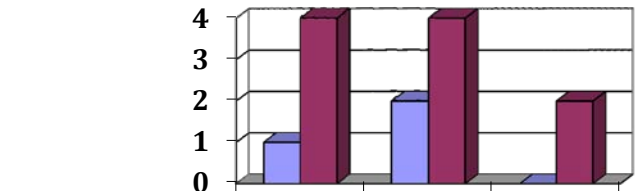


***Fig.2.1.3.2A**: Shows a representative summary of ChIP PCR results. The ChIP PCR samples were prepared from des $^{\Delta 1\text{-}48/\Delta 1\text{-}48}$ embryoid bodies.*



	day 5	day 6	day 7
desmin bound	1	2	0
total number of replicates	4	4	2

***Fig.2.1.3.2B**: Shows a columnar diagram of the minimal cardiac enhancer ChIP PCR results with des $^{\Delta 1\text{-}48/\Delta 1\text{-}48}$ EBs.*

In general it is known that the amino-terminal region of the desmin protein harbours domains, which are capable of interacting with other proteins or DNA *(Tolstonog et al.2005)*. Therefore, we expected the *des* $^{\Delta 1\text{-}48/\Delta 1\text{-}48}$ EBs to reveal no binding of desmin to the minimal cardiac enhancer region. Nevertheless, the results from ChIP of *des* $^{\Delta 1\text{-}48/\Delta 1\text{-}48}$ EBs are quite similar to the results from the *des* $^{+/+}$ EBs. This implicates that there might be an alternative DNA binding domain within the desmin protein, besides the already known one. Unfortunately, the data, which is presented here, is far too small to confirm this suspicion. To

draw any serious conclusions, the experiments would have to be repeated several times to create a greater data set.

2.1.3.3 Ectopically expressed desmin shows strong binding to the minimal cardiac enhancer

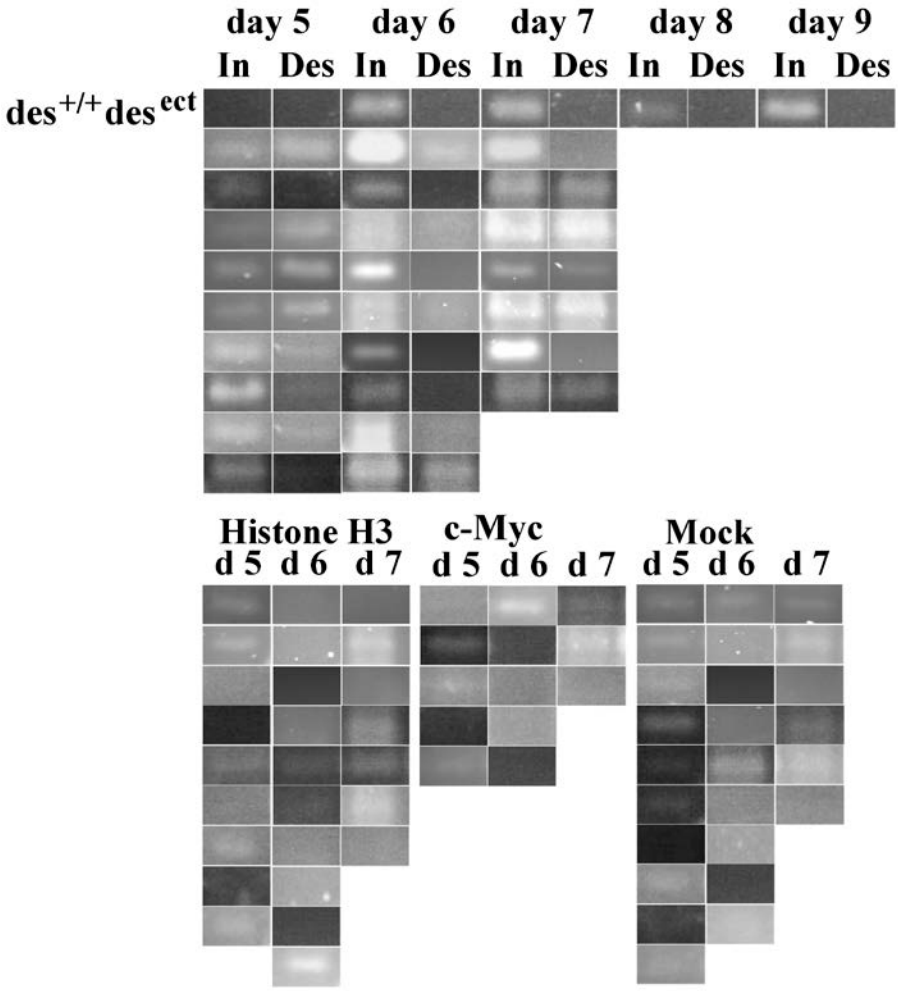
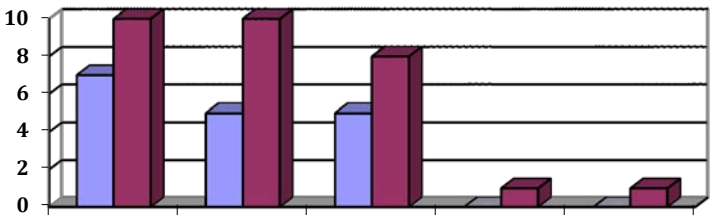


***Fig.2.1.3.3A**: Summary of ChIP PCR results from des$^{+/+/ect}$ embryoid bodies.*



	day 5	day 6	day 7	day 8	day 9
desmin bound	7	5	5	0	0
total number of replicates	10	10	8	1	1

***Fig.2.1.3.3B**: Shows a columnar diagram of the minimal cardiac enhancer ChIP PCR results with des$^{+/+/ect}$ EBs.*

Ectopically expressed desmin (des $^{+/+/ect}$) shows strong presence at the minimal cardiac enhancer region. Unfortunately, due to a mistake in data filing the quality of some of the gel pictures is really bad, and the bands are hardly visible. Another throwback in the presented data is again the high prevalence of bands from mock samples. So in summary it is hard to say if the interaction of desmin with the minimal cardiac enhancer is an artefact or an image of the real situation in developing EBs.

Finally, I want to state that the samples from *des* $^{+/+/ect}$ EBs and *des*$^{-/-}$ EBs were prepared in the very beginning of this thesis. By and by I was able to improve my ChIP skills, which is visible in form of the results presented above from the *nkx 2.5* promoter ChIP. The data from *des* $^{+/+/ect}$ EBs and *des*$^{-/-}$ EBs minimal cardiac enhancer ChIP is shown anyway, although it might distort the overall impression.

2.1.3.4 Samples from desmin-deficient embryoid bodies demonstrate inaccuracy in sample processing

ESC, which do not express desmin (*des*$^{-/-}$), were aggregated to form EBs. The EBs were subjected to ChIP as it is described in detail in chapter 4.2.8. The *des*$^{-/-}$ EBs were included as negative controls of the ChIP experiment. By the inclusion of ChIP samples from *des*$^{-/-}$ EBs we wanted to demonstrate the specifity of the ChIP results, which were shown so far. The samples from *des*$^{-/-}$ EBs, which were selectively purified in presence of desmin-specific antibodies, were expected to be free of DNA. As these cells do not express desmin, the desmin (Des) ChIP samples should not reveal any bands from ChIP PCR.

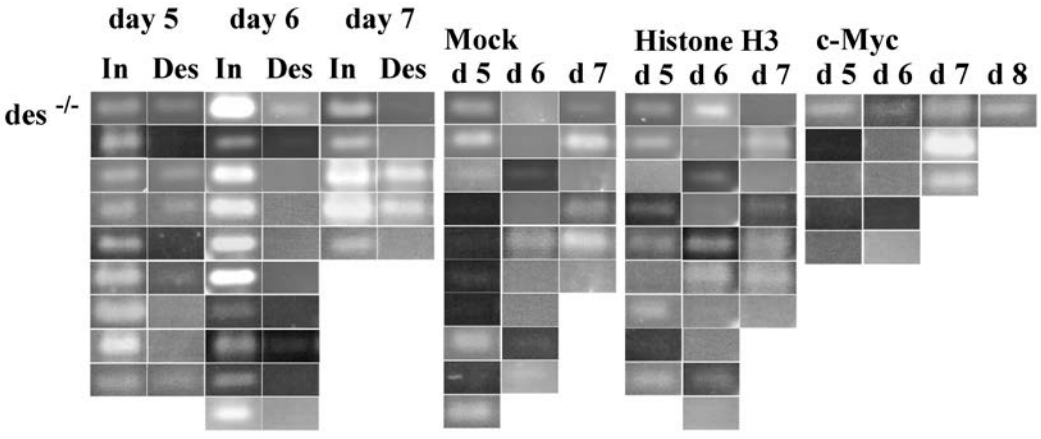
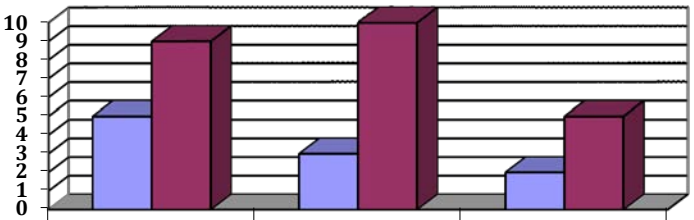


Fig.2.1.3.4A*: ChIP PCR results from des*$^{-/-}$ *embryoid bodies. Input (In) samples: gDNA prior to IP, Desmin (Des), Mock samples: pre-immune serum or without the addition of specific antibodies (negative control). Histone H3 (positive control), c-Myc (positive controls).*



	day 5	day 6	day 7
desmin bound	5	3	2
total number of replicates	9	10	5

***Fig.2.1.3.4B**: Shows a columnar diagram of the minimal cardiac enhancer ChIP PCR results from des $^{-/-}$ EB samples.*

The results which are shown in figure 2.1.3.4AB, demonstrate that the ChIP protocol still contained some inaccuracy. Besides the use of unblocked protein A-coupled sepharose beads, the sonication of the samples could be a major source for DNA contamination of the original samples. As the samples were all sonicated at the same sonicator it might be that some gDNA remained on the sonicator after cleaning it with distilled water and 70% ethanol. Especially in the beginning of this thesis, the samples were treated in a way, which makes this very likely. The samples of different genotypes with respect to their desmin alleles were sonicated one after the other. The sonicator was cleaned in between, but as repeatedly desmin bands appeared in the desmin-deficient (*des*$^{-/-}$) samples, the sonication protocol was changed. While in the beginning the samples were cooled after one cycle of sonication and another sample was sonicated, later the samples of one genotype were sonicated to the end, with continuous cooling in a ice-water bath. This reduced the chance of contamination and the efforts of cleaning in between each sonication of the samples of different genotypes and saved a lot of time. Also the sonication series was accomplished in the following sequence: *des* $^{-/-}$ EBs of all developmental stages were sonicated first, then the *des* $^{\Delta 1\text{-}48/\Delta 1\text{-}48}$ EBs of all developmental stages were sonicated, then the *des* $^{+/+}$ EBs were sonicated and finally the *des* $^{+/+/ect}$ EBs were sonicated. In between the sonicator was extensively cleaned with distilled water and 70% ethanol, by immersing the sonicator in the liquids.

However, due to the presence of many bands in anti-desmin samples from *des* $^{-/-}$ EBs, this data set is not satisfying as negative control, but rather leads to question the ChIP procedure.

2.1.4 Proof of specificity of desmin interaction with the 5'UTR of the *nkx 2.5* gene

The same ChIP samples, which were used in the ChIP experiment to test desmins ability to bind to the *nkx 2.5* promoter and the minimal cardiac enhancer, were used to challenge the specificity of desmins interaction with these genomic elements. Therefore, ChIP primer pairs, which specifically bind to adjacent regions of the *nkx 2.5* promoter and the minimal cardiac

enhancer (MCE), were designed. These primers were generated for the specific amplification of a region located distally of the MCE (D2), a region in between the MCE and the *nkx 2.5* promoter (D1), a region proximally of the *nkx 2.5* promoter (P2) and a region proximally of the *nkx 2.5* exon 1 (P1). The primer pairs P2 and P1 both serve to specifically amplify distinct regions between the *nkx 2.5* promoter and the *nkx 2.5* exon1. The binding sites of the primers are indicated in figure 2.1.1.

The ChIP PCRs with these primers are included to test the specificity of desmin binding to the MCE and the *nkx 2.5* promoter. If desmin specifically binds these regulatory gDNA elements, the ChIP PCRs with primers D1, D2, P1, and P2 are expected to be free of any PCR product.

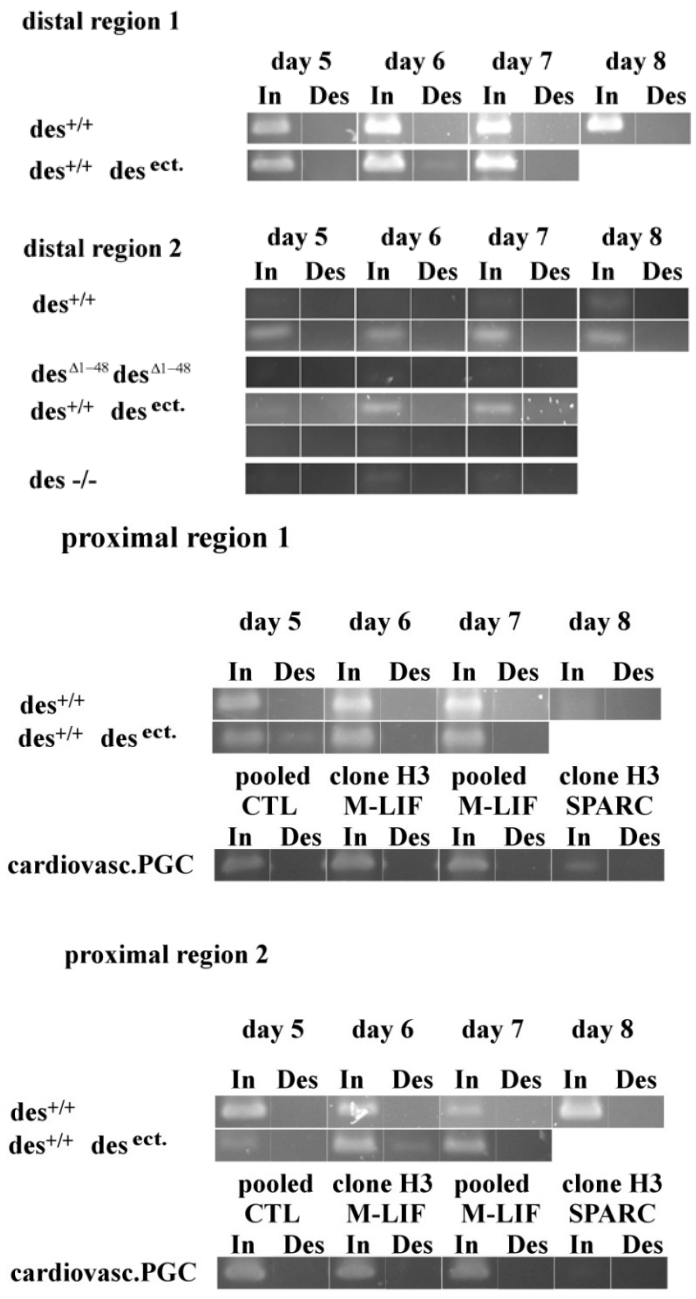


***Fig.2.1.4**: Summary of ChIP PCR results from the regions adjacent to the nkx 2.5 promoter and the minimal cardiac enhancer.The binding sites of the primers are indicated in figure 2.1.1.* des$^{+/+}$*: wild type EBs,* des$^{+/+/ect}$*: ectopic desmin expression,* des$^{\Delta 1\text{-}48/\Delta 1\text{-}48}$ *: N-terminally truncated desmin,* des$^{-/-}$ *: desmin null EBs. Cardiovasc.PGC: cardiovascular progenitor cells (diploma thesis W.Weber 2006). CTL: untreated control, M-LIF: CVPGC treated with M-Leukemia Inhibitory Factor, SPARC: secreted protein, acidic, rich in cysteine.*

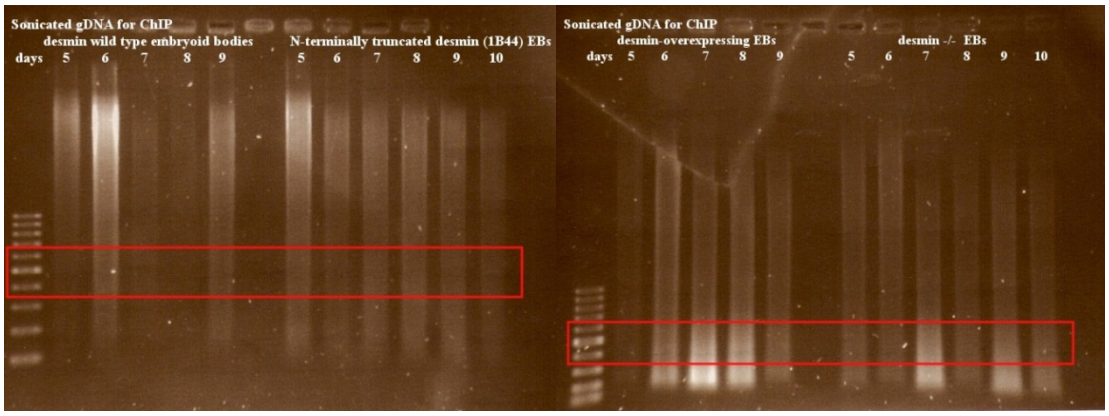
The ChIP PCRs with primers, which specifically amplify genomic regions adjacent to the *nkx 2.5* promoter and the minimal cardiac enhancer, revealed that desmin is not bound to these gDNA regions, as it is shown in figure 2.1.4. These negative results show that desmin binding to the *nkx 2.5* promoter and the MCE is specific and restricted to these regulatory elements in the 5'UTR of the *nkx 2.5* gene.

2.1.5 Selected issues of ChIP

In the following, some problems that occurred during the ChIP sampling will be discussed. First, the importance of proper sonication will be outlined. Second, as the summarized ChIP results show all results that were revealed regardless of their output, a summary of the best ChIP results of the *nkx 2.5* promoter and the minimal cardiac enhancer (MCE) will be presented and discussed.

2.1.5.1 Sonicated gDNA

The following figures show the analysis of sonicated genomic DNA (gDNA) on a 0.8% agarose gel. The samples were prepared from embryoid bodies of different developmental stages, as indicated in the figure.



A) *des$^{+/+}$ and des $^{\Delta 1\text{-}48/\Delta 1\text{-}48}$ EBs* ***B)*** *des$^{+/+/ect}$ and des$^{-/-}$ EBs*

Fig.2.1.5.1AB*: The analysis of the sonication of gDNA from EBs. These samples are later used as the "input" templates for ChIP PCRs. The rectangle marks the ideal degree of sonication, which results in gDNA fragments of 400-600 bp.*

Prior to the selective purification of genomic DNA fragments, the gDNA is fragmented by sonication. After the sonication, the majority of the gDNA fragments should be between 400-600 base pairs in size to guarantee for specificity of signals in ChIP PCR, which are obtained due to the binding of the protein of interest and selective purification of the samples in presence of specific antibodies to the protein of interest. The sonicated samples, which are shown in Fig.2.1.5.1A and B were treated the same way. They were all sonicated 18 times (90% cycle, 45% output), with the same intensity and duration of the sonication cycles. The

pictures above illustrate, that the samples were very inconsistent in their resistance to the sonication. While the samples from *des* $^{+/+}$ and *des* $^{\Delta 1\text{-}48/\Delta 1\text{-}48}$ EBs, which are shown in Fig.2.1.5.1A, show insufficient fragmentation of the gDNA, the samples from *des* $^{+/+/ect}$ and *des* $^{-/-}$ EBs, which are shown in Fig.2.1.5.1B, show sufficient fragmentation of the gDNA. In the latter, the majority of the gDNA is fragmented to 400-600 base pairs and below. This means, that the same number of cycles, with the same settings of output and duration of sonication, resulted in a different grade of fragmentation in the samples. The fact, that the same sonication treatment of the samples resulted in very inconsistent fragmentation, puts emphasis on the throwbacks of this method. To obtain good results, it is critical that the majority of the gDNA is fragmented to 400-600 bp. Otherwise the immune-precipitation would result in the co-purification of a gDNA fragment, which is bound by the protein of interest at another location, than the region of interest. This could lead to "false" positive results. As it is demonstrated by the different grades of sonication in figure 2.1.5.1AB, it is difficult to find the repetition of sonication cycles, which results in optimal but not excessive fragmentation. The samples, which were prepared from different stages of embryoid body development, were inconsistent in their resistance to sonication.

One of the striking limitations of the ChIP procedure is that the analysis of the proper fragmentation of the sonicated ChIP samples has to wait until the "input" samples are prepared. After a phenol-chloroform extraction, the "input" samples may be analysed on a 0.8% agarose gel. If the fragmentation turns out to be incomplete for the following procedure, the ChIP sample has to be sonicated again. This was omitted in the presented experiments. So the gDNA fragments in the samples were partly longer than 600 base pairs. This might contribute to false positive results, if one of these oversized fragments was bound by desmin or another protein, which was targeted in the immune-precipitations.

2.1.5.2 Desmin at the nkx 2.5 promoter

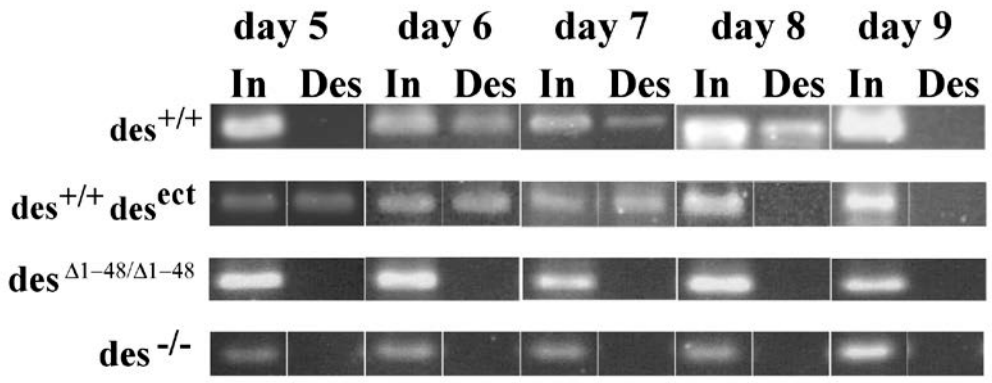


Figure 2.1.5.2*: Shows the best results from nkx 2.5 promoter ChIP PCRs.*

The data, which is shown in figure 2.1.5.2, show a selection of the best results from the *nkx 2.5* promoter ChIP PCRs. The data demonstrate that desmin is bound to the *nkx 2.5* promoter at day 6, day 7, and day 8 in wild type (*des* $^{+/+}$) embryoid bodies. In embryoid bodies, which ectopically express an additional desmin allele (*des* $^{+/+/ect}$), desmin is bound to the *nkx 2.5* promoter at day 5, day 6, and day 7. These results are in line with the development of beating cardiomyocytes in embryoid body (EB) model of *in-vitro* differentiation. In *des* $^{+/+}$ (wild type) embryoid bodies, the expression of desmin begins at day 3 and it was detected in semi-quantitative real time PCR in low levels at day 4 of EB development *(Hofner et al.2007)*. The first beating cardiomyocytes in *des* $^{+/+}$ (wild type) EBs appear at day 7.

In *des* $^{+/+/ect}$ EBs the expression of desmin was detected at day 0 of EB development in a semi-quantitative real-time PCR *(Hofner et al.2007)*. The first beating cardiomyocytes appear at day 6 of EB development in *des* $^{+/+/ect}$ EBs.

The results from the ChIP PCRs implicate that desmin is able to bind to the *nkx 2.5* promoter. The binding of desmin to the *nkx 2.5* promoter was proven to be specific in ChIP PCRs with primers, which serve to specifically amplify genomic regions adjacent to the *nkx 2.5* promoter, by the absence of PCR products from these ChIP PCRs, as shown in figure 2.1.4. The binding of desmin disapears at day 8 of EB development in *des* $^{+/+/ect}$ EBs and at day 9 in *des*$^{+/+}$ EBs. This suggests that desmin binds to the *nkx 2.5* promoter from the onset of its expression as long as the majority of this protein is not fully assembled into intermediate filaments of the type III. As soon as the intermediate filaments begin to assemble from desmin subunits, these subunits are sequestered from the nucleus to take over their role in the maintenance of the structural integrity of cardiomyocytes. Therefore, after the development of fully differentiated and functional beating cardiomyocytes, the desmin protein no more binds to the *nkx 2.5* promoter, as the ChIP PCRs revealed. As in the desmin ectopically expressing EBs, the desmin protein is available from the very beginning of development, these EBs are able to develop beating cardiomyocytes one day prior to the wild type EBs. From this, we concluded that the desmin protein units are sequestered one day earlier from the nucleus. Therefore, the desmin protein cannot be detected at the *nkx 2.5* promoter at day 8 in the majority of the *des* $^{+/+/ect}$ EBs.

2.1.5.3 Minimal cardiac enhancer region

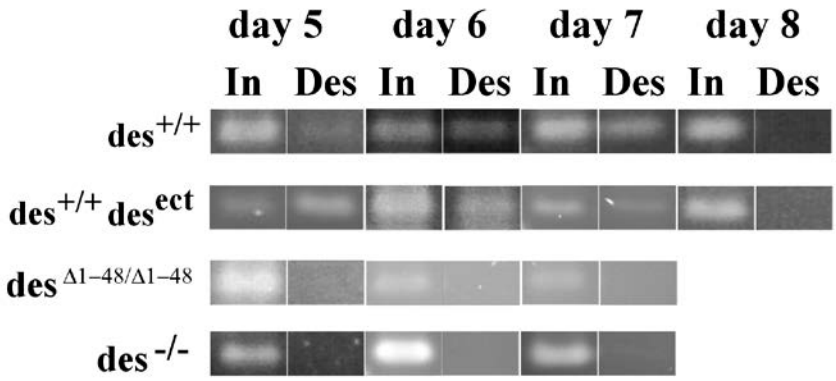


Figure 2.1.5.3*: Shows the best results from the ChIP PCRs of the minimal cardiac enhancer region.*

From the data, which is shown in figure 2.1.5.3, we concluded that the desmin protein more or less equally binds to the *minimal cardiac enhancer* region *(Lien et al.1999)* as it binds to the *nkx 2.5* promoter. This means that in wild type (*des*$^{+/+}$) EBs desmin binds to the minimal cardiac enhancer region at day 6 and at day 7 of EB development. From the ChIP PCR results of the minimal cardiac enhancer we found that desmin binding already disappeared at day 8. It might be that this absence of desmin binding at day 8 of EB development results from earlier sequestering from the MCE, to fullfill its natural role in the maintenance of the structural integrity of cardiomyocytes. In line with the results from the *nkx 2.5* promoter, *des* $^{+/+/ect}$ EBs revealed that desmin is bound to the minimal cardiac enhancer region at day 5, day 6, and day 7. The binding of desmin at day 5 of EB development in *des* $^{+/+/ect}$ EBs, is again one day ahead of the *des*$^{+/+}$ EBs, which is in line with the observation of earlier development of beating cardiomyocytes in these EBs.

2.2 The cloning strategy of MCE-cis

The MCE-cis plasmid is a reporter plasmid, which is based on the NKE24 reporter plasmid. The NKE24 plasmid was cloned by K.Yutzey (Cincinnati). It is a pGL3b-derived reporter plasmid, with the firefly *luciferase* gene under the control of the *nkx 2.5* promoter. The MCEcis reporter plasmid contains the sequence of the minimal cardiac enhancer *(Lien et al. 1999)* upstream of the firefly *luciferase* under the control of the *nkx 2.5* promoter. It was cloned to prove the hypothesis of a physical interaction between desmin and the minimal cardiac enhancer region, which arose from the ChIP experiments.

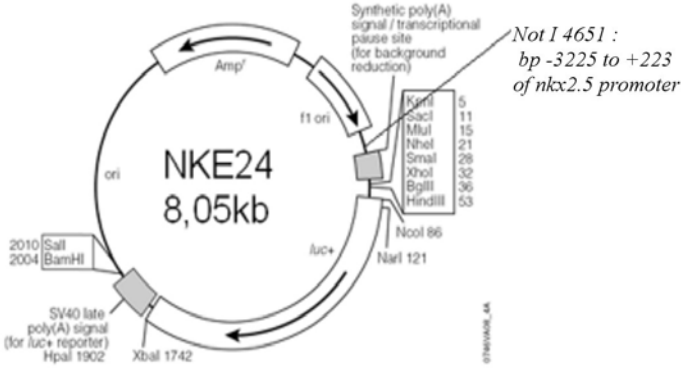


Fig.2.2A*: NKE24, the firefly luciferase reporter plasmid.*

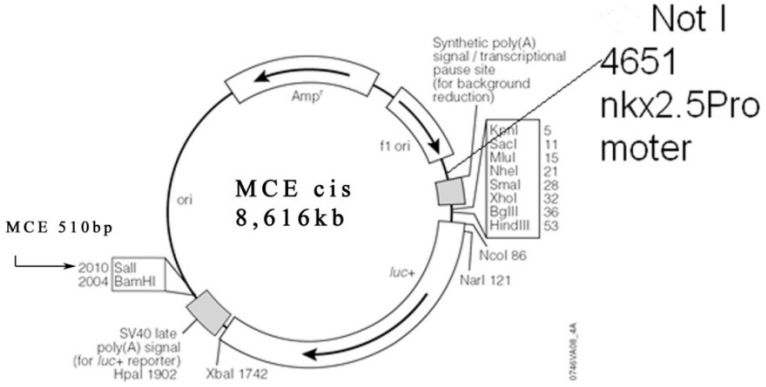


Fig.2.2B: *The NKE24-derived MCEcis-plasmid containing 510bp of a minimal cardiac enhancer sequence upstream of the nkx2.5 promoter and the luciferase reporter gene.*

The minimal cardiac enhancer region was cloned in maximal possible distance to the *nkx 2.5* promoter to enable alignment of the enhancer with the promoter without destroying important regulatory elements within the plasmid. This was reported in various publications to be crucial in the enhancement of gene expression.

2.2.1 Design of primers for the production of the DNA insert

PCR primers were designed for the production of the minimal cardiac enhancer insert, which is flanked by SalI restriction sites:

SalI fwd: 5'-taatgtcgactctgggtcctaatgc-3'
SalI rev: 5'-tattgtcgactcaaggtgcacatga-3'

2.2.2 Production of the insert for cloning

To produce the insert of the minimal cardiac enhancer region for cloning, a PCR with the *Pyrococcus furiosus* polymerase was set up. This type of polymerase harbours proof-reading activity and its use reduces the frequency of point mutations in the sequence of the PCR product. As a template the genomic DNA of C3H mouse was used. The PCR conditions were as indicated in table 2.2.2. The PCR product was analysed on a 1.2% agarose gel. The 530 base pair PCR product was cut out with a clean and sharp scalpel under short UV-

illumination. The cut gel slices were dissolved and the PCR products were extracted with a Promega Wizard SV Gel extraction kit.

Temperature	Duration	Cycles
95°C	4 min.	
95°C	1 min. 30 sec.	x 34
56.5°C	45 sec.	x 34
72°C	2 min. 30 sec.	x 34
72°C	5 min.	
4°C	Infinitely	

Table 2.2.2: The PCR conditions

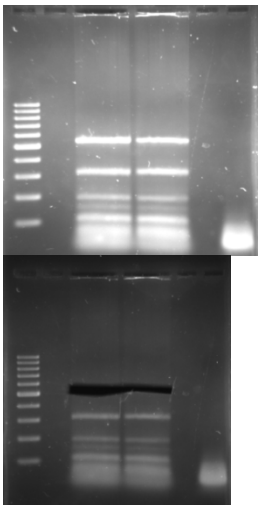


***Figure 2.2.2**: The analysis of the PCR product.*

2.2.3 Restriction of the vector backbone and the DNA insert

The NKE24 backbone vector was cut with *Sal I*. In a 40µl reaction mix, 4µg vector DNA was incubated at 37°C for two hours. Then the vector was dephosphorylated with 2 Units of alkaline phosphatase from calf intestine (CIP) at 37°C for 40 minutes. The restriction enzyme and the CIP was heat inactivated at 65°C for 20 minutes.

In parallel, the DNA insert was cut with Sal I to produce compatible ends for later ligation with the vector backbone. In a 40µl reaction mix 90ng of the insert were incubated at 37°C for three hours. The restriction enzyme was heat inactivated at 65°C for 20 minutes. The cut DNA fragments were purified from buffers and inactivated enzymes by phenol-chloroform-isopropanol extraction. The purified DNA was precipitated with a double volume of 96% ethanol and 1/10 1M sodium chloride solution at -20°C overnight. The precipitated DNA was pelleted by centrifugation at 4°C and 14.000 rpm for 30 minutes. The DNA pellet was washed with 70% ethanol and re-hydrated in 30µl sterile distilled Millipore water.

2.2.4 The ligation of the vector and the insert

The cut vector and insert were ligated in four different molar ratios: (A) 1:1, (B) 1:3 and (C) 1:0, where the last (1:0) served as a negative control. Another negative control, were the addition of T4 ligase was omitted, was included (D). 100ng cut NKE 24 plasmid DNA was combined with the respective amount of insert DNA and T4 ligase. The reaction mixtures were incubated at room temperature for three hours and then the ligase was heat inactivated at 65°C for 30 minutes.

2.2.5 The transformation of competent E.coli and screening of transformants

Competent E.coli XL-1 were transformed with 10µl of each ligation mixture.
From each transformation 50µl of the suspension was plated to sterile LB+Amp-agar plates. On the next day the plates were examined for colonies. From ligations (A) and (B) for clones were picked and used to inoculate 4ml LB+amp overnight cultures. The plasmids of the selected clones were retrieved by a mini plasmid preparation with the Promega kit.
The plasmids of the different clones were subjected to restriction analysis with *SalI*.

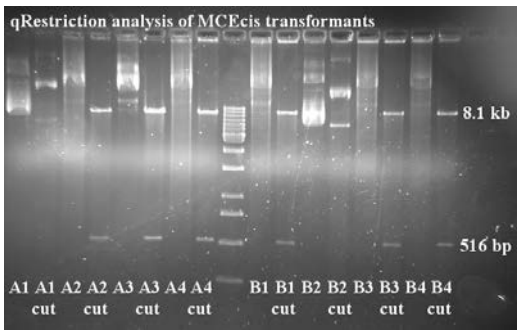


***Figure 2.2.5A**: Restriction analysis of MCEcis transformants on a 1% agarose gel.*

To screen the clones for the right orientation of the insert, a PCR with specific primers was set up. As primers SalI forward and MCEcis reverse were used:

Sal I fwd	5'-TAATGTCGACTCTGGGTCCTAATGC-3'
MCEcis rev	5'-TAAGTGCGGCGACGATAGTCATGC-3'

Originally, the Sal I fwd primer was designed to generate the MCE insert for cloning as a PCR product. Therefore, this primer anneals inside at the beginning of the cloned sequence. The MCEcis rev primer was designed for later sequencing, it anneals outside of the cloned sequence. By choosing a primer that anneals inside of the sequence and one that finds complementarity in the vector backbone, one can see if the DNA was inserted in the desired orientation. If it is oriented the desired way, the PCR gives a product, if the DNA is inserted the wrong way, there is no PCR product, because the primers both face the same direction.
The plasmids served as templates and the PCR conditions were as follows:

Temperature	Duration	Cycles
95°C	5 minutes	
95°C	1 minute	x 33
57°C	30 seconds	x 33
72°C	1 minute	x 33
72°C	2 minutes	
4°C	Infinitely	

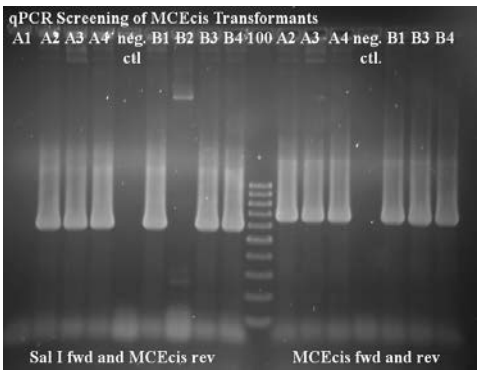


***Figure 2.2.5B**: Shows the analysis of the PCR products from PCR screening. Left band: 597bp, right: 681 bp.*

The PCR screening of the clones was done in two different set ups: the first was done to check the orientation of the insert. Therefore the primers Sal I fwd and MCEcis rev were used (as it is indicated in the picture). The use of these primers resulted in a 597 bp product in positive clones and in the lack of any product in clones that were negative in the previous screening of transformants. The second screening was done to check if the sequencing PCR works out. Therefore the primers MCEcis fwd and rev were used. They resulted in positive clones in the production of a 681bp product. The PCR product for sequencing has to be somewhat larger than the inserted sequence itself, because otherwise the sequence is un-readable at its borders. Unfortunately, from both PCR reactions the product band is somewhat blurred. This might have a couple of reasons, like the too long extension time, acidic hydrolysis of DNA in water, or the use of a circular template in these PCRs.

However, clone MCEcisA3 was chosen for sequencing. Therefore, a 50ml LB+Amp liquid overnight culture was inoculated. The plasmids were retrieved by the midi plasmid preparation kit from Promega. The plasmid and the primers MCEcis fwd and rev were sent to VBC genomics for sequencing. The sequencing result is shown in figure 2.2.6C aligned (by BLAST http://blast.ncbi.nlm.nih.gov/Blast.cgi) to the sequence of the minimal cardiac enhancer as it was published by Lien et al 1997. The alignment of the two sequences revealed that the sequence of MCE cis contains five additional bases. These additional bases are indicated as red letters in figure 2.2.6C.

```
Score =  918 bits (497),  Expect = 0.0
 Identities = 510/515 (99%), Gaps = 5/515 (1%)
 Strand=Plus/Plus

MCEcis 1    TCTGGGTCCTAATGCGGGTGGCGTCTCTCTTGACAGGCAGCGTTTGGGGACAACAGCGGG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MCElien1    TCTGGGTCCTAATGCGGGTGGCGTCTCTCTTGACAGGCAGCGTTTGGGGACAACAGCGGG  60

MCEcis 61   GAAGGGAGATAAGATGACATACCAGAGCAGATTTGGTGTGCGCGCTGATACTCCTGGCCC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MCElien61   GAAGGGAGATAAGATGACATACCAGAGCAGATTTGGTGTGCGCGCTGATACTCCTGGCCC  120

MCEcis 121  GACAGGAAACTCGGAGCTATTTAAAAAGGCCCTATCGATTACTTTATCTTCCCCGGAGGA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MCElien121  GACAGGAAACTCGGAGCTATTTAAAAAGGCCCTATCGATTACTTTATCTTCCCCGGAGGA  180

MCEcis 181  AAACTTCTTGCCGAGAGACAAAAGATGTCCCCCTACCTAAAGATACAAGGCCACACAGCA  240
            ||  | ||||||||||||||||||||||||||||||||||||||||||||||||||||
MCElien181  AA--T-CTTGCCGAGAGACAAAAGATGTCCCCCTACCTAAAGATACAAGGCCACACAGCA  237

MCEcis 241  GAGGGTTTGTACAGGCGACGCGCGAGTAGATCCCTGGGAGCGCAGAGACGCCTTTCTACC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MCElien238  GAGGGTTTGTACAGGCGACGCGCGAGTAGATCCCTGGGAGCGCAGAGACGCCTTTCTACC  297

MCEcis 301  GGCAGAGACTGAAGTTTGACTGGAGCGAGGCGGGGCGGAGCCACCTCCCGGTCCCCGTGC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MCElien298  GGCAGAGACTGAAGTTTGACTGGAGCGAGGCGGGGCGGAGCCACCTCCCGGTCCCCGTGC  357


MCEcis 361  ACTCCGGAATTGTGAACGCTCCCGCAAAGTCCCCGCGAGTGTTGTGTTCACACTAATTGG  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
MCElien358  ACTCCGGAATTGTGAACGCTCCCGCAAAGTCCCCGCGAGTGTTGTGTTCACACTAATTGG  417
```

```
MCEcis 421  TGTGCCTCTGAGTTCCATCCGTACTccccccccccccAAGTTTAAATGCTCCTTTTAAG  480
            ||||||||||||||||||||||||||||||||||  ||||||||||||||||||||
MCElien418  TGTGCCTCTGAGTTCCATCCGTACTCCCCCCCCCCC--AAGTTTAAATGCTCCTTTTAAG  475

MCEcis 481  GGCTTGAGTGTCTGCAGCCGTCATGTGCACCTTGA  515
            ||||||||||||||||||||||||||||||||||
MCElien476  GGCTTGAGTGTCTGCAGCCGTCATGTGCACCTTGA  510
```

***Figure 2.2.5C**: Shows the sequencing (VBC gemomics) result of the MCEcis insert. **Red** letters indicate additional bases in the cloned MCE sequence compared to Lien's MCE. **Light blue** letters highlight GATAA-binding sites.*

The pairwise alignment of the MCE-cis sequencing result and the published MCE *(Lien et al.1999)* sequence revealed that the MCE-cis insert harbours several additional basepairs. At position 183 an adenin was inserted, at position 184 a cytosin was inserted and at position 186 a thymine was inserted into the MCEcis sequence. At the positions 457 and 458 of MCEcis two cytosines were inserted. The MCE sequence as it is pulished by Lien et al. 1999 was revealed from SV129 mice, whereas the MCE-cis insert for cloning was produced from a gDNA template from C3H mouse. As gDNA sequences from C3H mouse are not available from the NCBI-gene bank, it remains elusive if the differences in the MCE sequence from SV129 mice *(Lien et al.1999)* and the MCE sequence from C3H mouse results from errors in the PCR reaction, from sequencing mistakes or most likely from polymorphism in the different mouse genotypes. Fortunately, the crucial GATAA-binding sites were not affected by these deviations from the published sequence of MCE. This unaffectedness of vital TF binding sites affirms the suspicion that the additional basepairs in the cloned MCE sequence derive from genomic polymorphisms. Although, the MCE-cis sequence did not completely match the published minimal cardiac enhancer sequence a QIAGEN endotoxin-free plasmid preparation was made, because we were not sure about the effect of the base insertions and to have a result for the cloning in this thesis.

2.2.6 Restriction of clone MCE-cisA3 plasmids compared with NKE24

The MCE-cis plasmid from the maxi prep and NKE24 were cut with the restriction enzymes *XhoI* and *BamHI*. This results in the production of three different DNA fragments in both plasmids: Restriction of the MCEcis results in one 4.2 kb fragment, in one 2.4 kb fragment and in one 1.9 kb fragment, if the sequence of the minimal cardiac enhancer is cloned successfully. If the MCE sequence was not cloned successfully into the MCE-cis plasmid, the pattern of restriction bands would be the same as from the restriction of NKE24. The restriction of the NKE24 plasmid results in one 3.7 kb fragment, one 2.4 kb fragment and one 1.9 kb fragment.

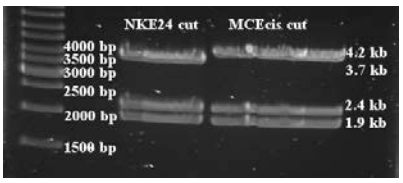


***Figure 2.2.6A**: Restriction analysis of the NKE24 and the MCEcis luciferase reporter plasmids on a 0.8% agarose gel*

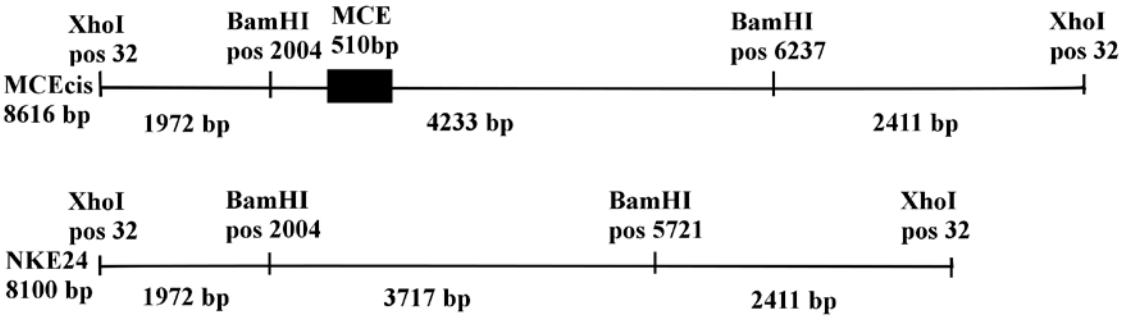


***Figure 2.2.6B**: Shows a physical map of the two luciferase reporter plasmids: MCEcis black box: sequence of minimal cardiac enhancer at SalI site (2010bp) and NKE24. The linear illustration was chosen to point out the differences in the position of the restriction sites.*

2.3 The cloning strategy of the MCEtrans vector

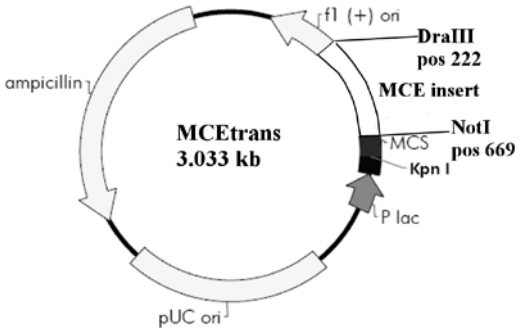


***Figure 2.3**: The hypothetical MCEtrans vector.*

We tried to clone the MCEtrans plasmid to see if the minimal cardiac enhancer preferentially acts in "trans" meaning that it is able to activate the expression of a luciferase reporter from another plasmid *(Farr et al.1992)*. The MCEtrans plasmid itself is not a reporter plasmid, as it does not carry the firefly *luciferase* gene. It is based on a pBluescriptKS+/- vector from Stratagene. The pBluescriptKS contains a pUC origin of replication, an F1 origin of replication, an *ampicillin resistance* gene and a *lacZ* gene. The plasmid was retrieved from XL-1 E.coli from the glycerol stocks in a -80°C freezer. Therefore, a mini plasmid preparation with the SV Wizard kit from Promega was done from an overnight culture.

2.3.1 Restriction of pBluescriptKS

To prove the identity of the retrieved plasmid, a qualitative restriction was set up. The restriction enzymes *DraIII* and *BamHI* were used:

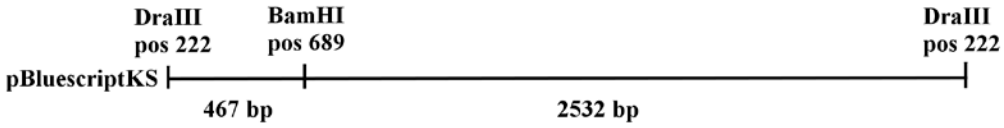


Figure 2.3.1A*: Shows a physical map of the pBluescriptKS vector.*

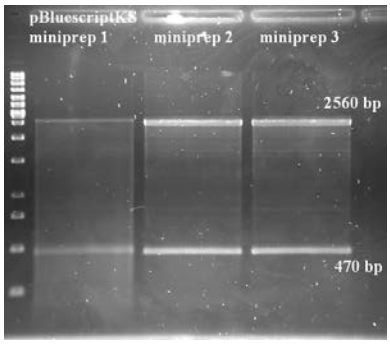


Figure 2.3.1B*: Shows the analysis of the restriction of pBluescript on a 0.8% agarose gel.*

2.3.2 Production of the minimal cardiac enhancer insert for cloning

The MCE DNA insert for cloning was produced by PCR with *Pyrococcus furiosus (Pfu)* polymerase and C3H mouse gDNA. To generate compatible ends for cloning specific primers, which introduce a *DraIII* and a *NotI* restriction site into the PCR product, were used:

DraIII fwd: 5'- TAA TCA CGT AGT GTC TGG GTC CTA ATG C- 3'

NotI rev: 5'- TAT TCG CCG GCC GCT CAA GGT GCA CAT GA-3'

The PCR conditions were as follows:

Temperature	Duration	Cycles
95°C	5 min.	
95°C	1 min. 30 sec.	x 34
63°C	1 min. 30 sec.	x 34
72°C	2 min. 30 sec.	x 34
72°C	5 min.	
4°C	Infinitely	

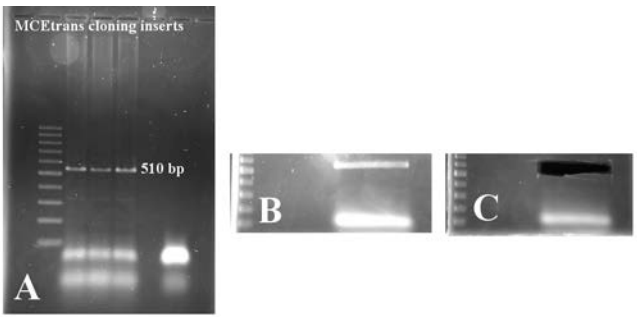


Figure 2.3.2*: The analysis of 10µl of the Pfu-polymerase PCR products on a 1.2% agarose gel (A) and subsequent extraction of the PCR product(B, C).*

One of these PCR products (40µl) was purified from a 1.2% preparative agarose gel, as shown in figure 2.3.2B. As shown in figure 2.3.2C, the PCR product was cut out of the gel and the DNA insert was purified with the SV Wizard Gel extraction kit from Promega.

2.3.3 Restriction of pBluescript and the DNA insert

Therefore, 1000ng of pBluescript and 100ng of the MCE DNA insert were used to set up to different double digests with the enzymes *DraIII* and *NotI*. The restriction mixes were incubated at 4°C overnight. On the next day the mixtures were incubated at 37°C for two hours. Then the enzymes were heat inactivated at 65°C for 25 minutes.

Although, the sticky ends, which are produced in the restriction with *DraIII* and *NotI* enzymes are incompatible, the vector was de-phosphorylated with calf intestine dephosphatase (CIP) to eliminate the chance of self-re-ligation of the vector alone. This was done because the cloning of the MCE-trans vector turned out to be more complicated than it was expected, in order to eliminate more complications.

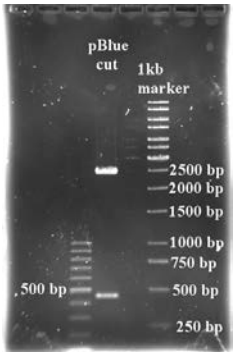


Figure 2.3.3*: The analysis of pBluescript after the digestion with DraIII and NotI. The vector is completely cut.*

2.3.4 The ligation of the vector and the DNA insert

The cut pBluescriptKS vector and the MCE insert DNA were mixed in a molar ratio of 1:3. After several failed trials the ligation mixture was finally prepared with 100mM dATP in addition to T4 ligase from Promega. The ligation reaction was incubated at room temperature for two hours. After the heat inactivation at 65°C for 30 minutes, the reaction was shortly cooled on ice. Thereafter, 12µl of the ligation mix was transformed into competent E.coli XL-1. 50µl and 100µl of the transformant suspension was plated onto LB+Amp agar plates.

2.3.5 Screenig of the MCEtrans transformants

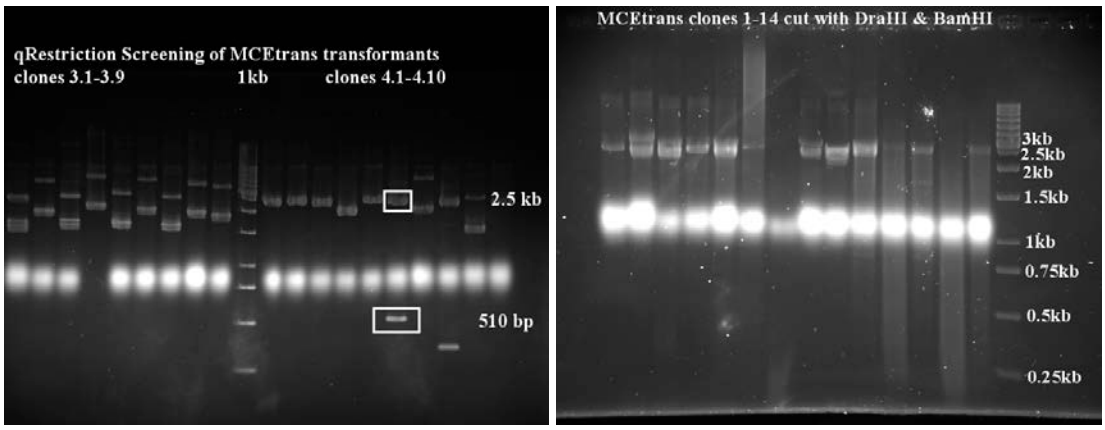


Figure 2.3.5.1*: Shows the analysis of MCEtrans clones after digestion with DraIII and NotI restriction enzymes on a 1.2% agarose gel.*

The outcome of this analysis was that none of the clones carried the MCE insert. Most of the plasmids were only linearized (e.g clones 1, 3, 4, 12, and 14). Some suffered from DNase degradation (e.g. clones 6, 7, 11, and 13). The bright and diffusive bands in the analysed samples in figure 2.3.5.1 results from the use of impure bovine serum albumin (BSA), which was needed in the restriction.

After the first attempt of cloning, the screening of the MCEtrans transformants revealed a single clone (MCEtrans 4.6), which seems to have a MCE insert of 510 base pairs, as it is shown in figure 2.3.5.1 on the left. On the right in figure 2.3.5.1 the analysis of the clones from the second attempt is shown. The second attempt of cloning did not reveal a clone, which is positive for the MCE sequence.

However, the clone MCEtrans 4.6 and later the clones from the second cloning trial (MCE-trans clones 1-14), were further subjected to a qualitative PCR screening, with primers, which were designed for later sequencing of the MCEtrans insert:

MCEtrans fwd: 5'- GAT AGG GTT GAG TGT TGT TCC AGT TT-3'

MCEtrans rev: 5'- ATT ACC GCC TTT GAG TGA GCT GAT A-3'

The PCR conditions were as follows:

Temperature	Duration	Cycles
95°C	5 min	
95°C	1 min	x 29
65°C /alt. 66°C	45 sec/ alt. 30 sec.	x 29
72°C	1 min 30 sec	x 29
72°C	2 min	
4°C	Infinitely	

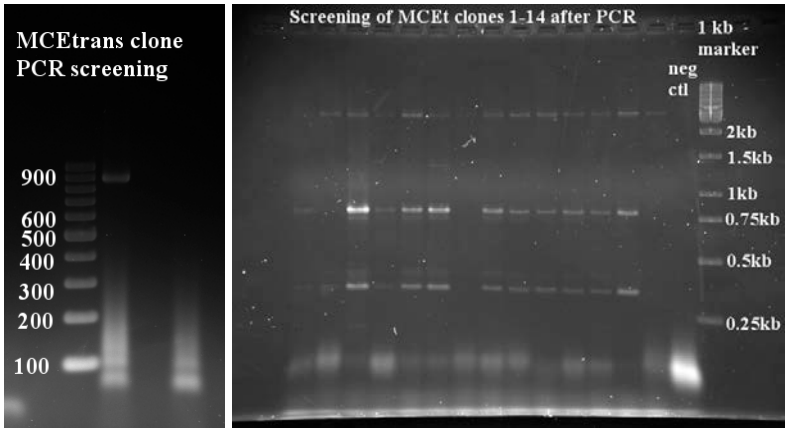


Figure 2.3.5.2*: Shows the analysis of the PCR screening of MCE-trans clones on a 1.2% agarose gel. Left: Screening of MCE-trans clone 4.6. Right: Screening of MCE-trans clones 1-14, which were anyway already shown to be negative for the MCE insert in figure 2.3.5.1*

The expected size of the PCR product was for plasmids with MCE insert a fragment of 1066 bp. For plasmids, that had self-re ligated the expected products size was a fragment of 994 bp. For a successfully cut plasmid, which re-ligated without the *DraIII-NotI* sequence, we expected a 551 bp band.

The only clone that was obtained from the first MCEtrans cloning did not carry the MCE sequence. The left picture shows the PCR product, which is produced from the empty vector. The initial restriction with *DraIII* and *NotI* seems to have been incomplete, so the whole cloning procedure was repeated from the very beginning. The right picture in figure 2.3.5.2 shows the PCR screening after repeated cloning. Unfortunately, the MCEtrans vector could not be cloned successfully.

2.4 The influence of desmin on the expression of an nkx 2.5-luciferase reporter gene

From the ChIP experiments we found that desmin binds to the *nkx 2.5* promoter and to the minimal cardiac enhancer region between day 5 and 8 of EB development. To address the question why desmin binds to the *nkx 2.5* promoter, we choose the Dual Luciferase Reporter System (Promega). Therefore, three different cell types were transfected with *luciferase* reporter plasmids and a desmin-expression vector (pBK-RSVcDesmin). The first cell type, which was used in transfection, was the 10T ½ fibroblasts. Naturally, the 10 T ½ fibroblasts do not express desmin, as it is shown in figure 2.5.6.1. These cells were used to assess the changes in luciferase reporter activity upon desmin co-expression. The successful ectopic expression of desmin in 10T ½ fibroblasts is also demonstrated in figure 2.5.6.1. The second cell type, which was transfected, was the C2C12 myoblasts. These cells express moderate levels of desmin, which can be further increased upon differentiation into myotubes in the presence of horse serum. In the following experiments undifferentiated C2C12 myoblasts were transfected. The third cell type, which was transfected, was primary cardiomyocytes from new born mice. The isolation of primary cardiomyocytes is described in chapter 4.1.7.

As luciferase reporter plasmids the NKE24-reporter vector was used. The NKE24 (*nkx 2.5* pro::*luc*) contains the firefly *luciferase* gene downstream of the *nkx 2.5* promoter (by K.Yutzey; *Searcy et al.1998 describes a lacZ variant of the nkx 2.5 promoter reporter*). The MCEcis vector is based on the NKE24, but carries the minimal cardiac en-hancer sequence *(Lien et al. 1999)* in addition to the *nkx 2*.5 promoter upstream of the *firefly luciferase* reporter gene. In all transfections the phRL-TK plasmids was included as internal control of transfection efficiency. It codes for the *renilla reniformis luciferase* gene downstream of the *thymidine kinase* (TK) promoter. The plasmids are described in detail in chapter 3.6 and are depicted in figure 3.6.1-7.

Further to assess the impact of desmin knock down at mRNA level on the activity of the *nkx 2.5* pro::*luc* reporter and the minimal cardiac enhancer sequence, short hairpin RNA expressing plasmids were co-transfected.

2.4.1 Plasmid-based RNAi

To study the effect of a desmin knock-down on RNA level in luciferase reporter assays, we purchased a plasmid-based RNA interference (pshRNA) set from SA Biosciences. The plasmids in this set express short hairpin RNAs (shRNA) and act by RNA interference (RNAi) on the cellular gene expression.

RNA interference (RNAi) is a term that sums up a variety of processes, which naturally occur inside a cell. RNAi processes are initiated by the presence of double stranded RNA (dsRNA) molecules. RNAi leads to altered gene expression within the affected cell and even in neighbouring cells. RNAi is sequence specific. It can lead to transcriptional silencing, mRNA slicing or translational repression of target sequences. In the last years, the use of RNAi techniques became a powerful tool in science. RNAi enables a sequence specific, transient or conditional knock down of a gene of interest. RNAi experiments in cultured cells have become a very fast, easy and clean model to study the function of a gene in transient conditional knock down experiments. RNAi effects are mediated by double stranded RNA molecules (dsRNA). They naturally occur as a consequence of viral infection or endogenous sources. Nowadays, plasmid-based RNAi approaches are used as the vehicle of choice to induce RNAi in eukaryotic cells. The RNAi-plasmids code for a 21-nucleotide sequence which matchs the sense strand of a target mRNA. Upon the transcription of the RNAi-sequence, which is encoded on the plasmid, hairpin-structured RNAi molecules, named short hairpin RNAs (shRNAs) are formed. A helicase, named Dicer cleaves the shRNAs into duplexes of small interfering RNA molecules (siRNAs). The cleaved siRNA duplexes are incorporated into the effector complex RISC (RNA-induced silencing complex). The RISC basically consists of one siRNA, the complementary target mRNA and a member of Argonaute (Ago) protein family. The Ago family proteins contain two major domains, the PAZ-domain and the PIWI-domain. The first domain, the PAZ, is also conserved in dicer and is an RNA-binding domain (RBD). In consideration of the PAZ domain´s conservation in dicer and Ago proteins, it is assumed that the PAZ serves to direct the passing off the siRNA strands from dicer to Ago effectors *(Song et al.2004)*. The second domain of Ago proteins, the PIWI, shows high similarity to members of RNase H family, which cleave the RNA strand of DNA/RNA duplexes.

However, the shRNAs, microRNAs (miRNAs), and small interfering RNAs (siRNAs) function as guide molecules in RNAi and serve for target specificity. Ultimately, RNAi processes can lead to various effects, ranging from RNA cleavage, post transcriptional gene silencing (PTGS) to long lasting translational repression and chromatin silencing. The miRNAs are a naturally existing large gene-family. The members of miRNA gene family normally have key roles in diverse regulatory pathways, including the control of developmental timing, haematopoietic cell differentiation, apoptosis, cell proliferation and organ development *(Kim 2005)*.

2.4.1.1 The murine desmin short hairpin RNA plasmids (pshRNA)

The pshRNA plasmids were bought from SA Biosciences. The pshRNA are provided as a set of four different silencing clones and one negative control plasmid. Each of the silencing plasmids carries a sequence that specifically targets the mRNA of the gene of interest and expresses GFP. The negative control contains a random scrambled DNA sequence, which does not target the gene of interest. The following sequence shows the murine desmin mRNA *(NCBI nucleotide database; Accession numbers: NM 010043,* GI:33563249*).* The target sites of the pshRNA clones within the desmin mRNA are indicated by bold coloured letters.

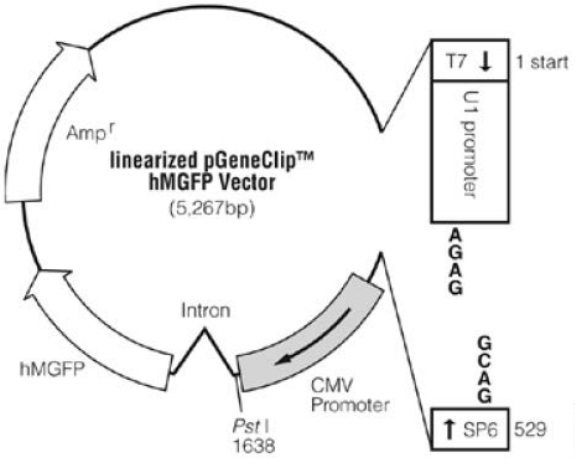
```
   1 atccactcca gccggctgcc cgcccgctgc ctcctctgtg cgtccgccca gccagcctcg
  61 tccacgccgc caccatgagc caggcctact cgtccagcca gcgcgtgtcc tcctaccgcc
 121 gcaccttcgg cggcgccccg ggcttctctc tgggctcccc gctgagctct cccgtgttcc
 181 ctcgagcagg cttcggtacc aagggctcct cgagttcaat gacatcccgc gtgtaccagg sh3
 241 tgtcgcgcac gtcgggcggg gctggaggct tggggtcgct gcggtctagc cggctgggga
 301 ccacccgagc gccatcctat ggcgcgggcg agctgctgga cttctccctg gccgacgctg
 361 tgaaccagga gttcctggcc acgcgcacca acgagaaggt ggagctgcaa gagctcaatg
 421 accgcttcgc caactacatc gagaaggtgc gcttcttgga gcagcagaac gccgcgctcg
 481 ccgccgaggt caaccggctc aagggccgcg aaccgactcg ggtcgccgag ctctacgagg
 541 aggagatgcg cgagctgcgg cgccaggtgg aggtgctcac caaccagcgc gcccgggtcg
 601 acgtggagcg tgacaacctg atagacgacc tgcagaggct caaggccaaa ctacaggagg
 661 aaatccaact aagagaagaa gcagagaaca acttggctgc cttccgagcg gatgtggatg
 721 cagccactct agctcgtatt gacctggagc gcagaatcga atccctcaac gaggagatcg sh2
 781 cgttccttaa gaaagtgcat gaagaggaga tccgtgagct tcaggcccag cttcaggaac
 841 agcaggtcca ggtggagatg gacatgtcca agccggacct cacagctgcc ctcagggaca
 901 tccgggctca gtatgagacc atcgcggcta agaacatctc tgaggctgaa gaatggtaca sh4
 961 agtccaaggt ttcagacttg actcaggcag ccaataagaa caacgatgcg ctgcgccaag
1021 ccaagcagga gatgatggaa taccgacacc agatccagtc ctacacctgc gagattgatg sh1
1081 ccctcaaggg caccaacgac tccctgatga ggcagatgag ggagctggag gatcgctttg
1141 ccagcgaggc caatggctat caggacaaca ttgcgcgcct ggaggaggag atccgacacc
1201 taaaggatga gatggcccgc catctgcgcg agtaccagga cctgctcaat gtgaagatgg
1261 ccttggatgt ggagatcgcc acctaccgga agctactgga gggcgaggag agcaggatca
1321 accttcctat ccagaccttc tctgctctca acttccgaga aaccagcccc gagcaaaggg
1381 gttctgaagt ccataccaaa aagacagtga tgatcaagac cattgagacc cgggatggag
1441 aggttgtcag cgaggctaca cagcaacaac atgaagtgct gtaagccagg aattcagtgt
1501 cctggccccg tcctcactgc ctcctgaagc cagcctcttc cactctcgga tatcacaccc
1561 agccactttt ctccactcac aggctctgac ccccccctcac cgatcacccc tttgtggtct
1621 tcatgctgcc caaccccagg gaacccctca gccacctctg cagaccctcc catgagccct
1681 ggctattggc aggtgtcaaa gctggctctt aagagagaac ccagctcaag tcatcgccct
1741 tccccttcca cctttgtgac ccctggctta ggagagggta ccagagaggg tgttgggatc
1801 tgcagggtca ggaccgagtt tgtggacatc cccagcctgg gtcagagaca gaatgaagcc
1861 tcagcgagct gagatggaga gtgggggggcc tgaaaactgc cctcatggcc cctctctttc
1921 ccatcgcagc ccaggatggc cttggaaagc gggggctgta agagggaagc ggaaggtgct
1981 ggatgtggga gcaggagcta cagaaggaga gaggatgggt gaggagctgg agaggaagga
2041 agagagaggc agagagtggg ctcaggttgg tgggagggta ccacctcccc tgcctgcccc
2101 tcccaccgca ggggcctgga cagaaacaat aataaagaga caagcacaaa aaaaaaaaaa
2161 aa
```

pshRNA sequences:

Clone 1 : TCCTACACCTGCGAGATTGAT
Clone 2 : GATCGCGTTCCTTAAGAAAGT
Clone 3 : CAAGGGCTCCTCGAGTTCAAT
Clone 4 : GACCATCGCGGCTAAGAACAT

Negative control: ggaatctcattcgatgcatac → no match in murine Desmin mRNA (RefSeq. NM_010043)

Figure 2.4.1.1A*: Shows the 2162 bp sequence of the murine desmin mRNA (NCBI nucleotide database; Accession numbers: NM 010043,* GI:33563249*).The target sequences of the different pshRNA clones are highlighted in different colours and in bold letters. The random sequence of the negative control has no match in the desmin mRNA sequence.*



2.4.1.1B: *Shows a map of the backbone of pshRNA with green fluorescent protein (GFP) under the control of a CMV-promoter (as a tracer). The targeting sequences, which form the short hairpin RNA, are incorporated in the vector between positions 438 and 439 (not shown in the map) under the control of the human U1 promoter. (U1 is a multigene family of small nuclear RNAs).*

Figure

2.4.1.1.1 The transformation of pshRNA into competent E.coli

The pshRNA plasmids were introduced into competent E.coli XL-1 by heat-shock transformation. Thereafter, 100µl of each reaction was plated on LB+Amp agar plates. (The rest was stored at -80°C.) On the next day, one colony of each plate was picked to inoculate a 5ml liquid LB+Amp starter culture. The starter culture was used to inoculate a 250ml liquid LB+Amp overnight culture for QIAGEN endotoxin-free maxi plasmid preparation.
On the next day, the liquid cultures were used to generate glycerol stocks of the pshRNA clones 1-4. The remaining culture was centrifuged for endo-toxin-free maxi prep. The plasmids from this preparation are of transfection grade and were subsequently used to transfect different eukaryotic cells.
DNA concentration of the plasmids after maxi prep:

pshRNA clone1: [852.80 ng/µl]
pshRNA clone 2: [910.17 ng/µl]
pshRNA clone 3: [1591.71 ng/µl]
pshRNA clone 4: [1432.07 ng/µl]
pshRNA neg.Ctl: [1110.74 ng/µl]

2.4.1.1.2 The Quality control and restriction of pshRNA

To verify the identity of the pshRNA plasmids a diagnostic restriction with *PstI* was performed. The pshRNA-GFP vector is cut in two fragments by *PstI* if it carries a hairpin insert: 4130 base pairs and 1197 base pairs.

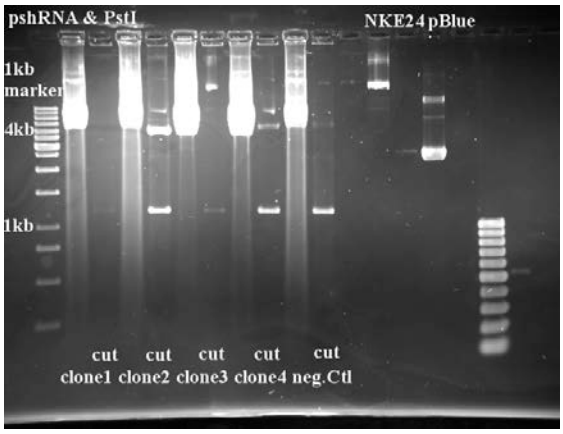


Figure 2.4.1.1.2: *The restriction analysis of the pshRNA plasmids.*

2.4.2 *Nkx2.5* promoter-driven Luciferase Expression in transfected 10 T ½ Fibroblasts

First of all we wanted to see if desmin not only binds to the *nkx 2.5* promoter and the minimal cardiac enhancer (MCE), but also interferes with the activity of these regulatory elements in fibroblasts, which have the potential to become myoblasts upon forced expression of MyoD *(Weintraub et al.1989).* Therefore, 10T ½ fibroblasts were transfected with several different reporter plasmids. The transfected cells were assayed with Promega's Dual Luciferase Assays. The vectors, which were used in transfections, are listed and explained in the material and methods chapter (3.6). In all the different transfections the *Renilla reniformis* luciferase expression plasmid (phRL-TK) from Promega was co-transfected. It served as an internal control of transfection efficiency. The data which are listed under "Ratio F/R" come from the measured activity of the firefly luciferase divided by the measured activity of the renilla luciferase. This procedure is meant to correct possible errors, which arise from different transfection efficiency in the samples. The data which is listed under "Renilla luciferase" always refers to the activity of the *Renilla reniformis* luciferase alone. In all the transfections the amount of phRL was chosen constant (1:10 of the total DNA concentration), but the values of the renilla luciferase activity showed great deviation from sample to sample. Therefore, we decided to explore if the expression of the renilla luciferase from the internal control phRL-TK (Promega) itself is influenced by the other co-expressed plasmids.
The activity of firefly luciferase and renilla luciferase was assayed with the Promega Dual Luciferase Assay components and was measured in a Berthold Luminometer 48 hours after the transfection. The measured values of the same transfection set up were compared with each other and were summarized as smaller (<) , equal (=) , or greater (>) than a value obtained in presence of the promoter-less pGL3b or NKE24, which carries the firefly luciferase under the control of the *nkx 2 .5* promoter. A set of data was considered as "equal" (=), if the division of the two raw values of reporter activity resulted in a ratio smaller than

1.6, because this means that the increase or decrease in reporter activity was less than 0.6. A transcription factor, an enhancer or a promoter element should cause a certain x-fold increase (factor) in the reporter activity otherwise it does not influence the reporter activity significantly. Therefore, a factor of 1.6 was chosen as the minimum, which represents "unequal" data. To reveal this factor of increase or decrease, the greater value was always divided by the smaller and then related to the context. The following table shows an example of the raw data from Luciferase Assay with transfected 10T ½ fibroblasts and the calculation of the factor, which is the basis for the classification of the results as "smaller" (<), "equal" (=), or "greater" (>):

Plasmid identity	**Firefly Luc**	**Renilla Luc**	**Ratio F/R*1000**
pGL3b	1400	407990	3.43
NKE24	3650	179160	20.37
Ratio >/< (factor)	3650/1400= **2.607**	407990/179160= **2.277**	20.37/3.43= **5.939**
NKE24	68740	18277850	3.76
NKE24+Des	50520	9341940	5.41
Ratio >/< (factor)	68740/50520= **1.361**	18277850/9341940= **1.957**	5.41/3.76= **1.439**
NKE24+Des	123120	110853300	1,111
NKE24+Des+Kd4	189390	89763600	2,110
Ratio >/< (factor)	189390/123120= **1.538**	110853300/89763600= **1.235**	2.11/1.111= **1.899**

2.4.2.1 The nkx 2.5 promoter drives luciferase expression

Firefly luciferase (∑ 13)	**Renilla luciferase (∑ 13)**	**Ratio F/R (∑ 13)**
13 pGL3b< NKE24	8 pGL3b< NKE24	11 pGL3b< NKE24
0 pGL3b= NKE24	1 pGL3b= NKE24	2 pGL3b= NKE24
0 pGL3b> NKE24	4 pGL3b> NKE24	0 pGL3b> NKE24

Table 2.4.2.1: Comparison of reporter activities from promoter-less pGL3b firefly luciferase reporter vector, with the nkx 2.5 *promoter-driven firefly luciferase expression (NKE24). NKE24:* nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity.*

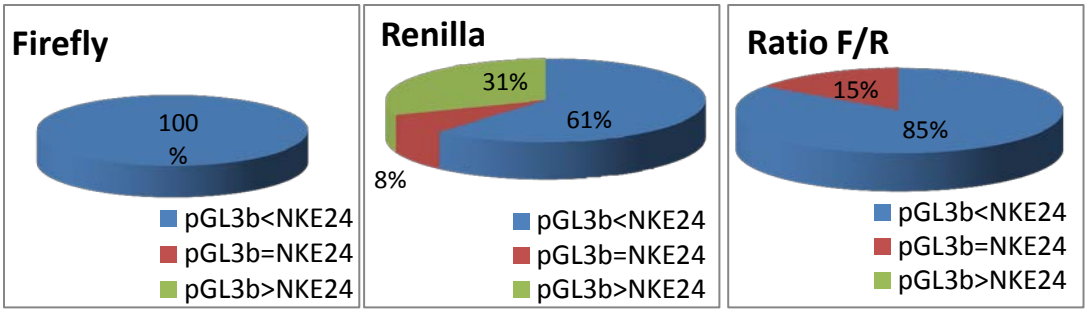


***Figure 2.4.2.1**: The results of the luciferase reporter activities in circular diagrams.*

The results , which are shown in figure 2.4.2.1, demonstrate that the *nkx 2.5* promoter in the NKE24 plasmid is able to drive the expression of downstream *firefly luciferase* reporter gene in 10T ½ fibroblasts. The results from *Renilla reniformis luciferase* expression show that the transfection efficiency in NKE24-transfections was greater, than in pGL3b transfections. This is rather a random result than a specific. The data from the ratio of firefly luciferase and renilla luciferase (Ratio F/R) serves to correct a possible error in the system, which might arise from different transfection efficiencies. The ratio F/R data confirms that the expression of the firefly luciferase reporter is activated in presence of the *nkx 2.5* promoter. The mean fold of luciferase reporter activation from NKE24 is 3.74 with a standart deviation of 1.85. The peak fold-increase was found one time with 7.28-fold increase in reporter activity. The lowest ratio of NKE24 and pGL3b was found to be 1.14 in one experiment, which obviously did not work.

2.4.2.2 The influence of desmin expression on the promoter-less pGL3b

The impact of desmin expression on the *nkx 2*.5 prom::*luc* reporter (NKE24 and MCE-cis) was in the main focus of this thesis. To be sure that any observed effect is due to a specific interaction of desmin with the *nkx 2.5* promoter, we included a series of co-expression of desmin from the pBK-RSV-cDesmin vector with the promoter-less pGL3b. If the expression of desmin has no influence on the basic activity of the firefly luciferase from pGL3b the results are expected to show a random distribution of pGL3b<pGL3b+Des, pGL3b= pGL3b+Des, pGL3b> pGL3b+Des.

Firefly luciferase (13)	**Renilla luciferase (13)**	**Ratio F/R (13)**
0 pGL3b< pGL3b+Des	3 pGL3b< pGL3b+Des	5 pGL3b< pGL3b+Des
11 pGL3b= pGL3b+Des	6 pGL3b= pGL3b+Des	3 pGL3b= pGL3b+Des
2 pGL3b> pGL3b+Des	4 pGL3b> pGL3b+Des	5 pGL3b> pGL3b+Des

Table 2.4.2.2: Shows the results of the luciferase reporter activities from the promoter-less pGL3b vector (firefly luciferase) and phRL (renilla luciferase) upon desmin co-expression (pBK-RSVcDesmin).

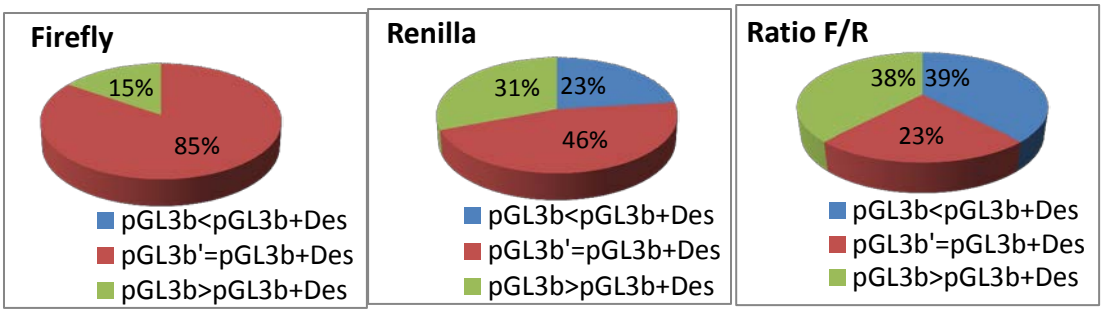


***Figure 2.4.2.2**: Shows the results of the luciferase reporter activities in circular diagrams.*

As we expected, the co-expression of desmin has no impact on the basic firefly luciferase from pGL3b. This is demonstrated by the great portion of values which are summarized as pGL3b= pGL3b+Des. In addition to that, the renilla luciferase activity is neither influenced by the co-expression of desmin nor is there any difference in the transfection efficiency with or without desmin. The data from the ratio F/R nicely demonstrates the lack of any specific effect of desmin on the promoter-less pGL3b, by a distribution of the results close to 1:1:1.

2.4.2.3 The influence of desmin expression on nkx 2.5 promoter-driven luciferase reporter

The main focus of this thesis was to test the impact of desmin expression on regulatory elements, such as the *nkx 2.5* promoter. Therefore, the *nkx 2.5* pro::*luc* reporter vector (NKE24) was transfected alone or in combination with the desmin-expression vector pBK-RSV-cDesmin into 10T ½ fibroblasts. The reporter activity, which resulted from the transfections, was assayed in the Dual Luciferase Assay system (Promega).

Firefly luciferase (∑15)	**Renilla luciferase (∑15)**	**Ratio F/R (∑15)**
1 NKE24< NKE24+Des	0 NKE24< NKE24+Des	2 NKE24< NKE24+Des
5 NKE24= NKE24+Des	6 NKE24= NKE24+Des	10 NKE24= NKE24+Des
9 NKE24> NKE24+Des	9 NKE24> NKE24+Des	3 NKE24> NKE24+Des

Table 2.4.2.3: Shows the results of the luciferase activities from nkx 2.5 promoter-driven reporter (NKE24) compared to luciferase reporter activity from the same vector in co-expression with desmin. NKE24: nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity. Des: ectopic expression of desmin from pBK-RSVcDesmin.*

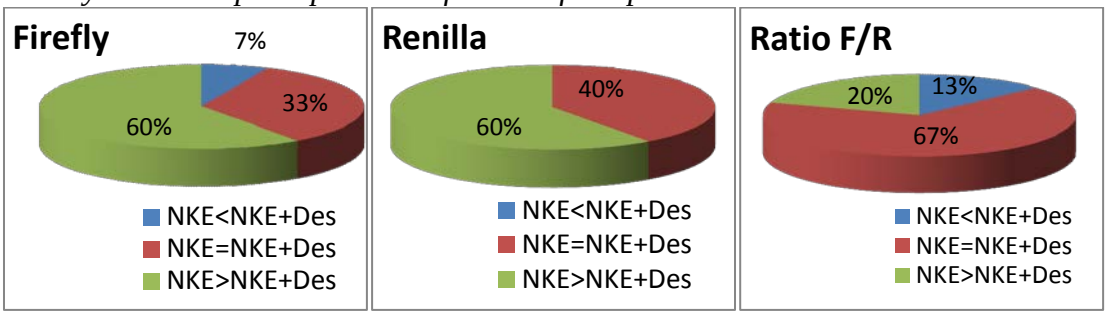


***Figure 2.4.2.3**: Show circular diagrams of the results of nkx 2.5 promoter-driven reporter activities, compared to the co-expression with desmin.*

The data from above demonstrates that the co-expression of desmin from pBK-RSV-cDesmin plasmid either influences both luciferase reporters in an inhibitory manner or simply that the transfection efficiency was greater from the NKE24 transfections alone, than in combination with desmin. The underlying mechanism remains unknown. However, the firefly luciferase alone just gives values without any reference to the efficiency of transfection, and is therefore useless. Only the normalized luciferase results (ratio F/R) are valueable. The normalized results from luciferase activity (ratio F/R) indicate that in 67% of the samples the co-expression of desmin from pBK-RSVcDesmin does not influence the firefly reporter activity under the control of the *nkx 2.5* promoter. In one of the compared reporter activities from NKE24 and NKE24+desmin we found an eight-fold increase of the reporter activity after the co-expression of desmin, but most of the other results show that the reporter activity slightly decreases (ranging from 1 to 0.5-fold) or stays at the same range. By the term "the same range" is meant that the values did not increase or decrease more than 1.6-fold in the reporter activity.

2.4.2.4 The influence of desmin co-expression on the minimal cardiac enhancer reporter plasmid

To test if desmin co-expression results in changes of reporter activity, 10T ½ fibroblasts were co-transfected with the MCEcis reporter vector. The MCEcis reporter vector carries the firefly *luciferase* reporter gene under the control of the *nkx 2.5* promoter downstream of the minimal cardiac enhancer *(Lien et al. 1999).*

Firefly luciferase (4)	Renilla luciferase (8)	Ratio F/R (4)
0 MCE<MCE+Des	1 MCE<MCE+Des	0 MCE<MCE+Des
1 MCE=MCE+Des	0 MCE=MCE+Des	4 MCE=MCE+Des
3 MCE>MCE+Des	7 MCE>MCE+Des	0 MCE>MCE+Des

Table 2.4.2.4: Shows the results of luciferase reporter activity, which contains the minimal cardiac enhancer upstream of the nkx 2.5 promoter-driven firefly luciferase (MCE), under the influence of ectopic desmin expression. Renilla luciferase: from phRL-TK, serves as internal control of transfection efficiency. Ratio F/R: normalized reporter activity (normalized to renilla luc, to correct deviations from differential transfection efficiency)

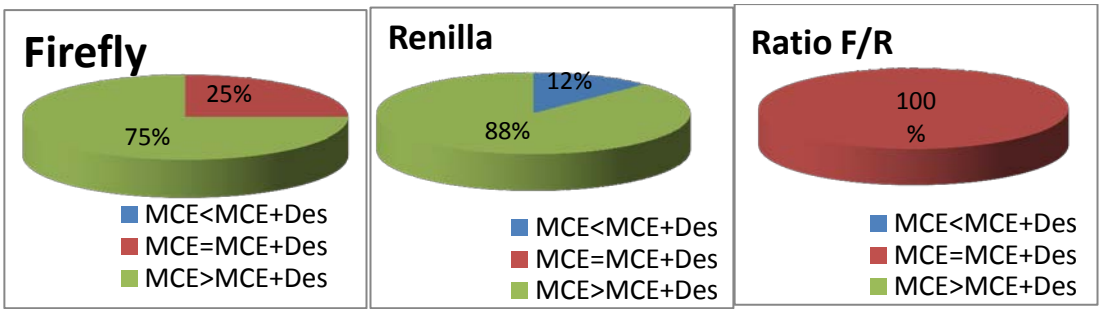


Figure 2.4.2.4*: Shows circular diagrams of the results shown above. MCE:* mce:: nkx 2.5 pro:: luc.

Despite the fact that this data set is too small to draw any serious conclusions, it shows the same inhibitory effect of desmin co-expression on the luciferase reporters, as we observed from NKE24 and NKE24+Desmin at the level of the separate luciferase reporters (firefly and renilla). This also might be a consequence of reduced transfection efficiency in combination with desmin, than from MCE alone. However, the normalized reporter activity (ratio F/R) shows that the co-expressed desmin is not able to serve for addiditional activation of the *nkx 2.5* promoter, which controls the firefly luciferase reporter gene.

2.4.2.5 The influence of a short hairpin RNA negative control on the luciferase reporters

The short hairpin RNA plasmids were included in this experiment to explore the impact of desmin knock down at mRNA level on the expression of the luciferase reporter. We ordered pshRNA-GFP constructs from SA Biosciences, to evaluate the transfection efficiencies. Due to the fact that so far there was no possibility to see if the transfection has worked, we estimated the pshRNA-GFP variant as the most useful to us. The idea behind was to see the degree of transfection efficiency already before the luciferase activity was assayed with the Dual Luciferase System of Promega.

The pshRNA clones express an hMGFP- variant, which is an improved synthetic version of GFP. The green fluorescent protein (GFP) was originally cloned from *Monastrea cavernosa*. The *hMGFP* is a codon optimized variant of this naturally existing protein. From *hMGFP* commonly known transcription factor (TF) consensus sites were elimi-nated (Promega) from the *gfp* gene. This reduces or even abolishes the chance that TFs interfere with the expression of GFP. The peak excitation wavelength of hMGFP occurs at 505nm and the peak emission occurs at 515nm.

During the process of Dual Luciferase Assay, an ATP-containing beetle luciferin substrate (LAR II) is brought in contact with the firefly luciferase. The luciferase enzyme oxidizes the luciferin in the substrate to oxyluciferin. This is a reaction, which is accompanied by the release of energy in form of light. The emitted light has a wavelength of 530-635nm. Although the wavelengths of the light, which is emitted from the firefly luciferase reaction, does not match the peak excitation of the hMGFP and although this is very improbable, we can not exclude that the emitted light from luciferin oxidation might lead to the excitation of GFP or that the presence of GFP interferes somehow with the performance of the Dual

Luciferase Assay. This effect is commonly refered to as “crosstalk”. To exclude this threat of the experiments, we contacted an agent of Promega, who stated that such interference was not observed when they tested this (personal correspondence).

However, upon the automatic addition of the Stop+Glo® reagent, the light emission from firefly luciferase is quenched at least by a factor of 10^5. The Stop+Glo® reagent not only serves to quench the firefly luciferase light reaction, but also contains the renilla luciferase substrate (coelenterazine). The renilla luciferase catalyzes the decarboxylation of coelenterazine to coelenteramide, which is accompanied by the release of CO_2 and energy in form of a light shot of 482 nm. The wavelength, which is emitted from the renilla luciferase reaction, is quite close to the peak excitation wavelength of hMGFP (505nm). Due to the proximity of the wavelengths from renilla luciferase reaction and the GFP excitation, interference of the GFP and the renilla signal (crosstalk) might occur. Another consideration about the crosstalk of luciferase and GFP is that the overall amount of photons should stay the same, regardless if they make the long way round from luciferase to GFP before they reach the detector. At the detector of the luminometer single photons with a spectral range of 340-630 nm are counted. It is unclear if the fluorescence from GFP interferes with the luciferase signal and if this further leads to a potentiation of the firefly luciferase signal or to a reduced detection of the firefly luciferase signal due to the absorbance of photons by GFP. Therefore, the consequence of pshRNA-GFP co-expression was assessed empirical. The results are shown in figures 2.4.2.5- 2.4.2.7ABC.

Firefly luciferase (3)	**Renilla luciferase (3)**	**Ratio F/R (3)**
1 NKE<NKE+Kdneg	1 NKE<NKE+Kdneg	1 NKE<NKE+Kdneg
1 NKE=NKE+Kdneg	2 NKE=NKE+Kdneg	1 NKE=NKE+Kdneg
1 NKE>NKE+Kdneg	0 NKE>NKE+Kdneg	1 NKE>NKE+Kdneg

Table 2.4.2.5: The compared results of Dual Luciferase Assays after transfection of 10 T ½ fibroblasts. NKE24: nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity. Kdneg: negative control shRNA-GFP.*

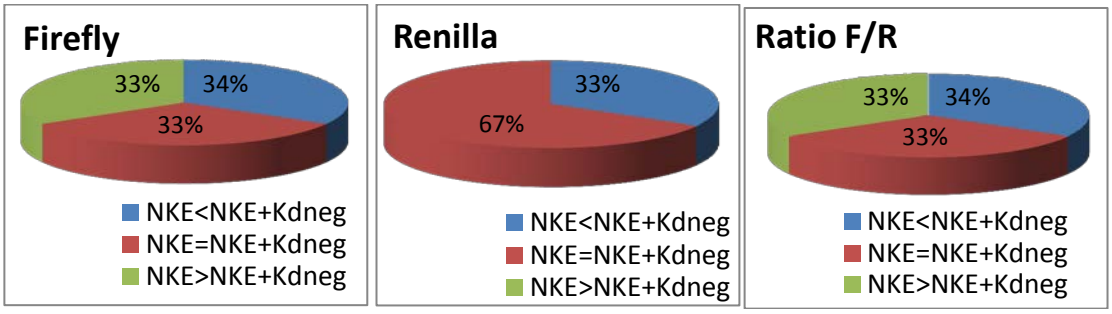


Figure 2.4.2.5*: Reflects the results, which are shown in the table, as circular diagrams.*

The results shown in figure 2.4.2.5 demonstrate that the expression of the short hairpin RNA negative control plasmid does not influence any of the luciferase reporters. The distribution of NKE24< NKE24+Kdneg, NKE24= NKE24+Kdneg, and NKE24> NKE24+Kdneg is equal in the firefly as well as in the ratio F/R data. The data from renilla luciferase show 67% of equal renilla luciferase activity in presence of NKE24 and NKE24+Kdneg, which resembles equal transfection efficiencies. The "crosstalk" of GFP and the luciferase signals, which is discussed above, was not observed here.

2.4.2.6 The influence of short hairpin RNA negative control on desmin and luciferase reporters

The pshRNA-GFP negative control was transfected into 10T ½ fibroblasts to prove that this short hairpin RNA neither targets desmin, nor results in changed activity of the *nkx 2.5* pro::*luc* reporter (NKE24). To prove that pshRNA-GFP negative control does not interfere with the reporter activity is the basis for subsequent emperiments with short hairpin RNAs, which target the desmin mRNA.

Firefly luciferase (4)	**Renilla luciferase (4)**	**Ratio F/R (4)**
1 N+Des<N+Des+Kdneg	0 N+Des<N+Des+Kdneg	0 N+Des<N+Des+Kdneg
3 N+Des=N+Des+Kdneg	3 N+Des=N+Des+Kdneg	4 N+Des=N+Des+Kdneg
0 N+Des>N+Des+Kdneg	1 N+Des>N+Des+Kdneg	0 N+Des>N+Des+Kdneg

Table 2.4.2.6: Shows the compared luciferase reporter activities from NKE24, which is co-expressed with the short hairpin RNA negative control (Kdneg), with desmin and without desmin. N(KE24): nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity. Des: ectopic expression of desmin from pBK-RSVcDesmin.*

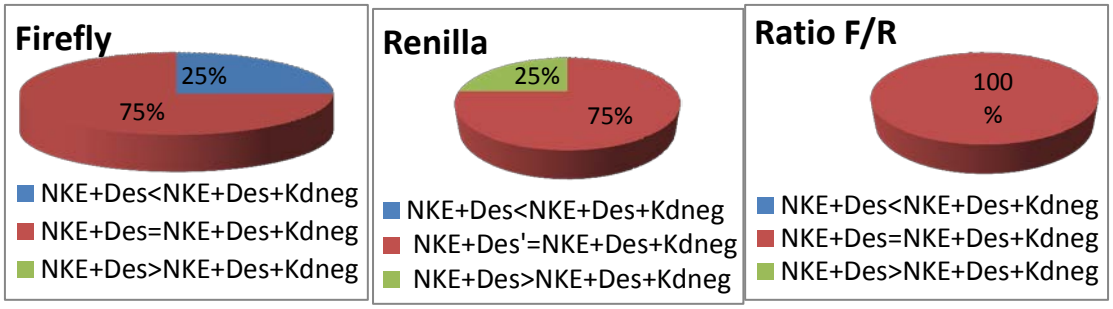


Figure 2.4.2.6*: Depicts the results shown above in circular diagrams.*

The co-expression of pshRNA-GFP negative control (Kdneg) with desmin from pBK-RSVcDesmin and the NKE24 (*nkx 2.5* pro::*luc*) reporter revealed no significant change in the luciferase reporter activity.

2.4.2.7 The influence of the knock down of desmin at RNA level on the luciferase reporters

The short hairpin RNA-GFP plasmids from SA Bioscience target desmin mRNA, as it is demonstrated in figure 2.4.1. Semi-quantitative real time PCR, as described in chapter 2.4.7, was included to demonstrate successful knock down. The knock down of desmin mRNA was included to test the impact of the knock down on the expression of the luciferase reporters. If desmin acts as an activator of transcription, its knock down at mRNA level should result in decreased expression of the *nkx 2.5* pro::*luciferase* reporter. In contrast, the knock down of desmin at mRNA level by RNAi should lead to increased reporter activity of the *nkx 2.5* pro::*luc*, if desmin is an inhibitor of transcription.

A) Short hairpin RNA clone 1

Firefly luciferase (2)	Renilla luciferase (2)	Ratio F/R (2)
0 NKE24< NKE24+Kd1	0 NKE24< NKE24+Kd1	0 NKE24< NKE24+Kd1
2 NKE24= NKE24+Kd1	2 NKE24= NKE24+Kd1	2 NKE24= NKE24+Kd1
0 NKE24> NKE24+Kd1	0 NKE24> NKE24+Kd1	0 NKE24> NKE24+Kd1

Table 2.4.2.7.A1: The compared activities of the luciferase reporters with the short hairpin RNA clone 1 (Kd1) and without the shRNA clone 1. NKE24: nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity.*

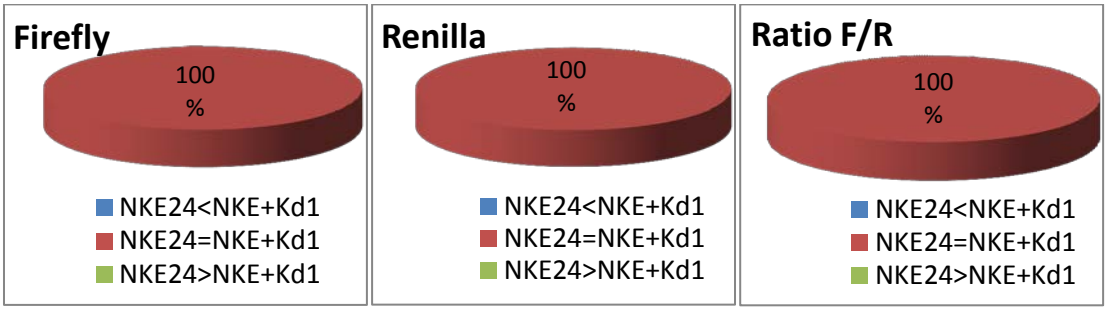


Figure 2.4.2.7.A1*: The relative difference in luciferase activities under the influence of the shRNA clone 1.*

Two replicates of this transfection set up are too little to draw any serious conclusion. Besides that, the data from this transfections indicate that the co-expression of pshRNA-GFP clone 1 (Kd1) does not influence the luciferase reporter activity at all. If desmin was able to activate the *nkx 2.5* promoter , we would have expected that the luciferase reporter activity decreases, upon the co-expression of the short hairpin RNA.

Firefly luciferase (2)	Renilla luciferase (2)	Ratio F/R (2)
1 N+Des< N+Des+Kd1	0 N+Des<N+Des+Kd1	2 N+Des<N+Des+Kd1
1 N+Des= N+Des+Kd1	1 N+Des=N+Des+Kd1	0 N+Des=N+Des+Kd1
0 N+Des> N+Des+Kd1	1 N+Des>N+Des+Kd1	0 N+Des>N+Des+Kd1

Table 2.4.2.7.A2: Shows the compared activities of the luciferase reporters in combination with desmin and under the influence of the shRNA clone 1(Kd1). N(KE24): nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity. Des: ectopic expression of desmin from pBK-RSVcDesmin.*

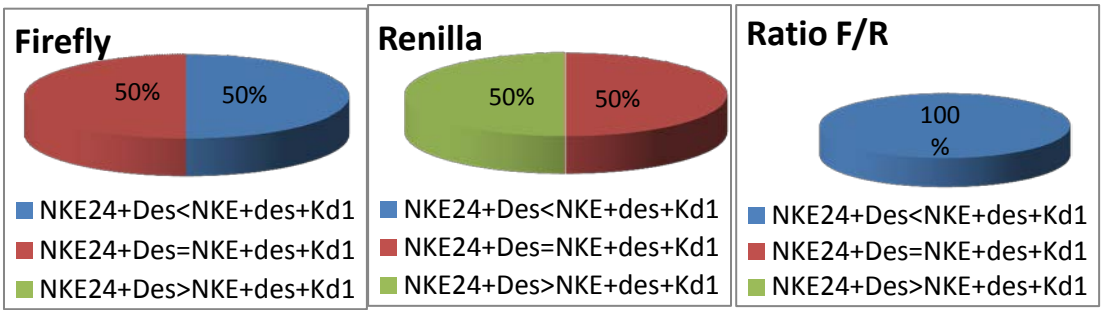


Figure 2.4.2.7.A2*: Depicts the relative difference in luciferase activities under the influence of desmin and the shRNA clone 1.*

This experimental set up was only used twice, so the results are not really significant. Besides the insignificance due to the low number of replicates, the data shows that there is no influence on the reporter activities of both luciferases. The renilla reporter data shows that the efficiency of transfection was slightly greater in the transfections without the pshRNA-GFP clone 1 (Kd1).The normalized reporter values (ratio F/R) demonstrate the consequence of the deviation of transfection efficiency, as two 50:50 results finally changed to a 100% result (NKE+Des< NKE+Des+Kd1). The data indicates that the knock down of desmin at mRNA level might increase the reporter signal. The mean value of increase in reporter activity is 1.89 with a standart deviation of 0.04.

B) Short hairpin RNA clone 3

Firefly luciferase (2)	Renilla luciferase (2)	Ratio F/R (2)
0 NKE24< NKE24+Kd3	0 NKE24< NKE24+Kd3	0 NKE24< NKE24+Kd3
2 NKE24= NKE24+Kd3	2 NKE24= NKE24+Kd3	2 NKE24= NKE24+Kd3
0 NKE24> NKE24+Kd3	0 NKE24> NKE24+Kd3	0 NKE24> NKE24+Kd3

Table 2.4.2.7B: Shows the compared activities of the luciferase reporters alone or in combination with the shRNA clone 3(Kd3). NKE24: nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity.*

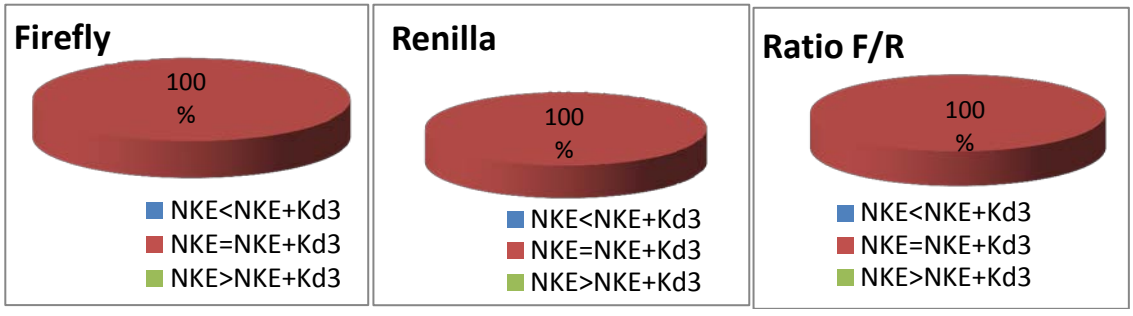


Figure 2.4.2.7B*: Depicts the relative difference in luciferase activities under the influence of the shRNA clone 3.*

In the 10T ½ fibroblasts, which do not express desmin, the co-expression of pshRNA-GFP clone 3 (Kd3) does not influence the luciferase reporter activity. Concomitant, the knock down of desmin at mRNA level, which is anyway absent in 10T ½ fibroblasts , does not change the reporter signal significantly.

Firefly luciferase (2)	**Renilla luciferase (2)**	**Ration F/R (2)**
0 N+Des< N+Des+Kd3	0 N+Des< N+Des+Kd3	2 N+Des< N+Des+Kd3
2 N+Des= N+Des+Kd3	0 N+Des= N+Des+Kd3	0 N+Des= N+Des+Kd3
0 N+Des> N+Des+Kd3	2 N+Des> N+Des+Kd3	0 N+Des> N+Des+Kd3

Table 2.4.2.7B: Shows the compared activities of the luciferase reporters in combination with desmin and under the influence of the shRNA clone 3(Kd3). N(KE24): nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity. Des: ectopic expression of desmin from pBK-RSVcDesmin.*

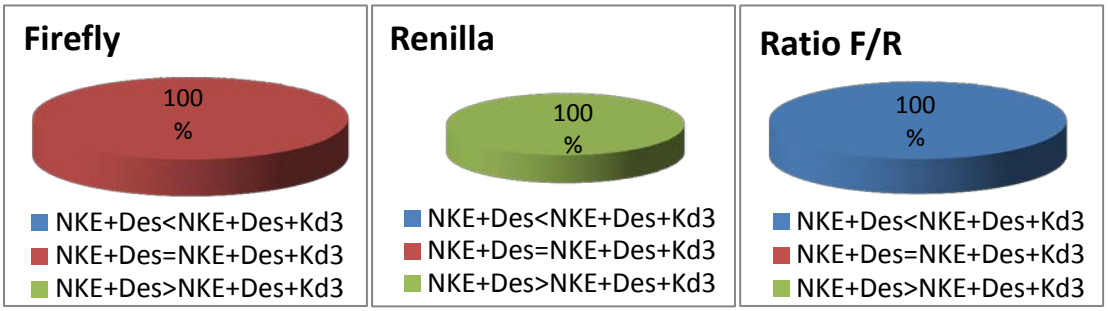


Figure 2.4.2.7B*: Depicts the relative difference in luciferase activities under the influence of desmin and the shRNA clone 3.*

At the level of the firefly luciferase alone, the co-expression of the pshRNA-GFP clone 3 (Kd3) with desmin from pBK-RSV-cDesmin and the *nkx 2.5* pro::*luc* reporter (NKE24) seems to leave the reporter activity unchanged. In contrast, the activity of the renilla luciferase is lower when the pshRNA-GFP clone 3 is co-expressed. This reflects a sudden decrease in the efficiency of the transfection. However, the mean value of decrease in renilla reporter activity from co-transfection of pshRNA-GFP clone 3 and pBK-RSVcDesmin compared to NKE24 and pBK-RSVcDesmin alone was 3.49 with a standart deviation of 1.1. From the experiment, which is presented here, it is impossible to draw any conclusions from this observation. This set up can not elucidate if the decrease in renilla reporter results from decrease in transfection efficiency or from an effect, which is promoted by the co-expression of pBK-RSVcDesmin. The Dual Luciferase Assay system is designed to rely on the normalization of the first reporter, which is controlled by a regulatory element of choice, to the second, which is the renilla luciferase downstream of the *thymidine kinase (TK)* promoter. To evaluate the reporter activity, the values can not be separated. The normalized values of reporter activity (ratio F/R) indicate that the co-expression of the pshRNA-GFP clone 3 “increased” the luciferase reporter activity compared to NKE24 in combination with desmin. The mean value of fold increase in

luciferase reporter activity was 3.15 with a standart deviation of 0.03. Here it seems appropriate to mention that in different reporter studies it was observed that reporter vectors, especially their promoters, might interfere with each other *(Farr et al. 1992)*. The pshRNA-GFP clones express the hMGFP from a CytoMegaloVirus (CMV) promoter, which is known as one of the strongest promoter elements. In contrast to this theory, the same effect of promoter crosstalk would have occured in the co-expression of pshRNA-GFP negative control, which was not the case. So in conclusion, desmin seems to act as an inhibitory molecule on the *nkx 2.5* promoter in 10T ½ fibroblasts. This is demonstrated by the increase in luciferase reporter activity upon co-expression of short hairpin RNA clone 3, which targets desmin at mRNA level.

C) Short hairpin RNA clone 4

Firefly luciferase (4)	Renilla luciferase (4)	Ratio F/R (4)
0 NKE24< NKE24+Kd4	0 NKE24< NKE24+Kd4	0 NKE24< NKE24+Kd4
0 NKE24= NKE24+Kd4	0 NKE24= NKE24+Kd4	4 NKE24= NKE24+Kd4
4 NKE24> NKE24+Kd4	4 NKE24> NKE24+Kd4	0 NKE24> NKE24+Kd4

Table 2.4.2.7C: The compared activities of the luciferase reporters from NKE24 and phRL-TK upon the co-expression of the short hairpin RNA clone 4(Kd4). NKE24: nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity.*

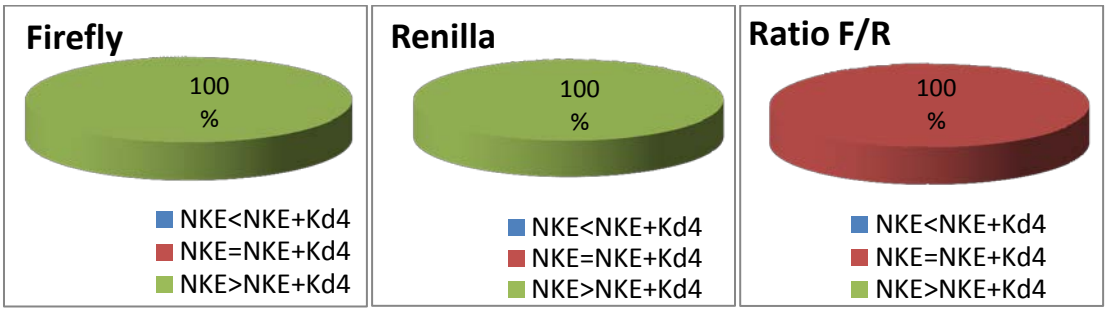


***Figure 2.4.2.7C**: The relative difference in luciferase activities under the influence of the shRNA clone 4.*

The firefly luciferase activity was found greater from NKE24 alone than in combination with pshRNA-GFP clone 4. The reporter activity from renilla luciferase shows that the transfection efficiency was greater from NKE24 transfections than in combination with pshRNA-GFP clone 4. In consequence, the normalized reporter activity (ratio F/R) demonstrates that the knock down of desmin at mRNA level does not influence the luciferase reporters. As the 10 T ½ fibroblasts do not express desmin, this result fulfils our expectations. A knock down of a genes mRNA, which is not expressed, should result in unchanged reporter activity, as it is demonstrated in figure 2.4.2.7C.

Firefly luciferase (6)	Renilla luciferase (6)	Ratio F/R (6)
3 N+Des< N+Des+Kd4	2 N+Des< N+Des+Kd4	3 N+Des< N+Des+Kd4
3 N+Des= N+Des+Kd4	3 N+Des= N+Des+Kd4	3 N+Des= N+Des+Kd4
0 N+Des> N+Des+Kd4	1 N+Des> N+Des+Kd4	0 N+Des> N+Des+Kd4

Table 2.4.2.7C: The compared activities of the luciferase reporters in combination with desmin and under the influence of the short hairpin RNA clone 4(Kd4). N(KE24): nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity. Des: ectopic expression of desmin from pBK-RSVcDesmin.*

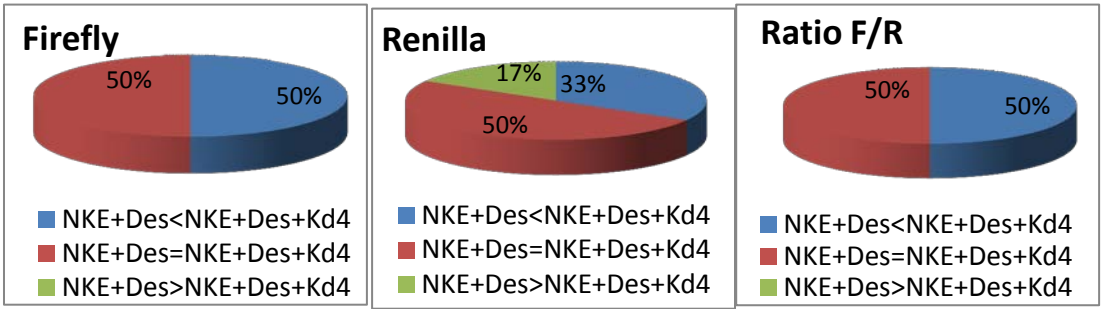


Figure 2.4.2.7C*: The relative difference in luciferase activities under the influence of desmin co-expression and simultaneous desmin mRNA-targeting by the shRNA clone 4 (Kd4).*

The results from the transfection of 10T ½ fibroblasts with the short hairpin RNA-GFP clone 4 (Kd4), in NKE24 and pBK-RSV-cDesmin co-expression background revealed that the knock down of desmin lead to increased reporter activity in 50% of the compared samples. As the knock down of desmin leads to increase in reporter activity from *nkx 2.5* pro::*luc*, desmin might act inhibitory on the *nkx 2.5* promoter in 10T ½ fibroblasts. The renilla luciferase depicts the influence of this plasmid combination on the transfection efficiency. The values are relatively balanced, except for a slightly greater transfection efficiency when pshRNA-GFP clone 4 was co-transfected. This is for sure not a specific effect. The results of the firefly reporter normalized to the renilla reporter (ratio F/R) resembles the result from the firefly raw data. It indicates that desmin might act inhibitory on the *nkx 2.5* promoter, because the knock down of desmin at mRNA level lead to increased *nkx 2.5* pro::*luc* reporter activity. The mean increase in reporter activity was 2-fold with a standard deviation of 0.43.

The finding that desmin might act inhibitory on the *nkx 2.5* promoter, seems to be in conflict with the theory that desmin expression positively influences heart relevant genes and TFs *(Hofner et al. 2007, Höllrigl et al. 2007).* The underlying mechanism of desmins interference with genomic regulatory elements, for sure does not fit in a black-white scheme, which means that it might be both an inhibitor and an activator, depending on the situation in the cell and its state of development. However, the 50% of data with increased reporter activity from desmin knock down is opposed by another 50% of equality in reporter activity before and after knock down of desmin.

2.4.3 Luciferase assays of transfected C2C12 myoblasts

Undifferentiated C2C12 myoblast naturally express desmin. The level of desmin expression is further increased when the C2C12 myoblasts are driven into differentiation by the addition of 5-10% horse serum to the growth medium. Upon the activation of differentiation these cells form myotubes. For the transfection and later use in Dual Luciferase Assay the C2C12 were used undifferentiated.

2.4.3.1 The *nkx 2.5* promoter drives luciferase reporter expression

To test if the *nkx 2.5* pro::*luc* reporter plasmid (NKE24) results in greater reporter activity than it is derived from the promoter-less pGL3b in C2C12 myoblasts, the C2C12 myoblasts were transfected with these plasmids in different approaches. The luciferase reporter activity was assayed in the Dual Luciferase Reporter Assay (Promega) 48 hrs after transfection.

Firefly luciferase (∑ 12)	**Renilla luciferase (∑ 12)**	**Ratio F/R (∑ 12)**
7 pGL3b< NKE24	1 pGL3b< NKE24	8 pGL3b< NKE24
3 pGL3b= NKE24	7 pGL3b= NKE24	2 pGL3b= NKE24
2 pGL3b> NKE24	4 pGL3b> NKE24	2 pGL3b> NKE24

Table 2.4.3.1: Shows the results of the reporter activities of the promoter-less pGL3b vector and the nkx 2.5 promoter-driven luciferase expression (NKE24) compared to each other. NKE24: nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity.*

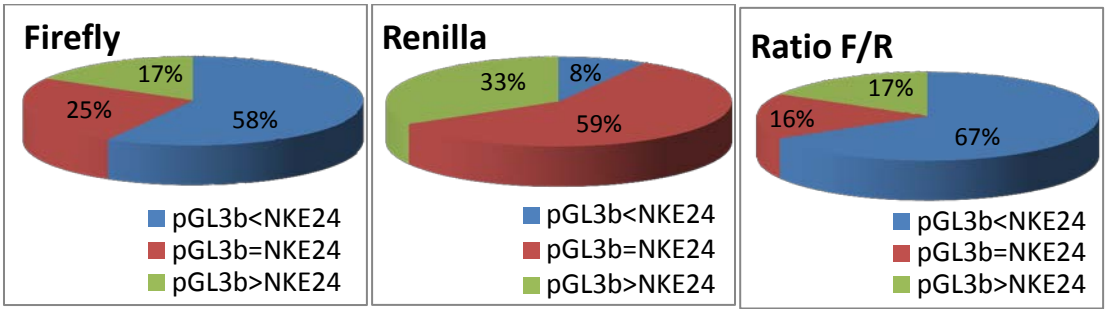


Figure 2.4.3.1*: Shows the results of the luciferase reporter activities in circular diagrams.*

The results from C2C12 myoblast transfection with either pGL3b or NKE24 show that the *nkx 2.5* promoter is able to activate the expression of the firefly luciferase reporter. The activity of the renilla luciferase co-reporter from phRL-TK, served to assess the individual efficiency of transfection and is not influenced significantly by the expression of each reporter plasmid, as it is demonstrated by the large portion of pGL3b= NKE24. The normalized values of reporter activity (Ratio F/R) demonstrate that in 67% of the samples the *nkx 2.5* promoter succeded to drive the expression of the firefly luciferase reporter. The increase in firefly luciferase reporter activity from *nkx 2.5* pro::*luc* (NKE24) was about 25-fold (mean value) with a standard deviation of 27.7.

2.4.3.2 The influence of desmin expression on *nkx 2.5* promoter-driven luciferase reporter

Although desmin is already expressed by C2C12 myoblast, it was included in the transfection series, to see if this ectopic expression leads to surprising effects.

Firefly luciferase (∑3)	Renilla luciferase (∑3)	Ratio F/R (∑3)
1 NKE24< NKE24+Des	2 NKE24< NKE24+Des	0 NKE24< NKE24+Des
1 NKE24= NKE24+Des	0 NKE24= NKE24+Des	1 NKE24= NKE24+Des
1 NKE24> NKE24+Des	1 NKE24> NKE24+Des	2 NKE24> NKE24+Des

Table 2.4.3.2: The results of the luciferase activities from nkx 2.5 promoter-driven reporter (NKE24) compared to luciferase reporter activity from the same vector in co-expression with desmin. NKE24: nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity. Des: ectopic expression of desmin from pBK-RSVcDesmin.*

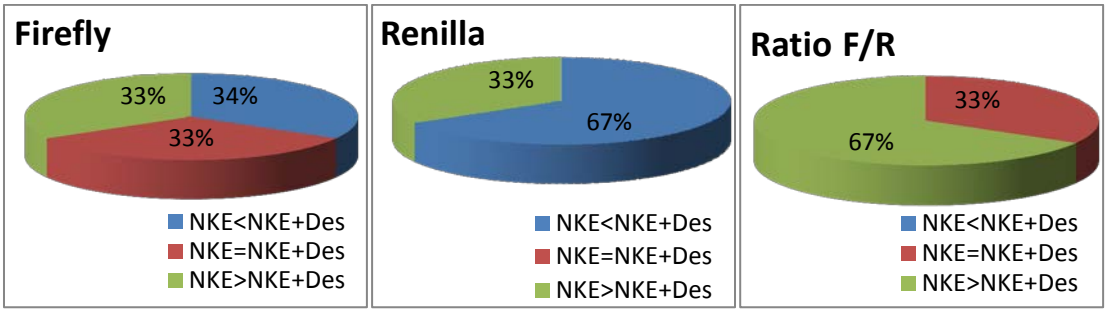


Figure 2.4.3.2*: Show circular diagrams of the results of nkx 2.5 promoter-driven reporter activities, compared to the co-expression with desmin.*

Indeed the ectopic expression of desmin from pBK-RSVcDesmin led to a surprising result. While the raw data from the firefly luciferase reporter does not show any effect of desmin co-expression on the reporter, the normalized reporter signals (Ratio F/R) indicate that desmin might inhibit the luciferase reporter downstream of the *nkx 2.5* promoter. The luciferase reporter activity is decreased when desmin is co-expressed. The mean value of decrease is 9-fold with a standard deviation of 12. Despite the great standard deviation, the observed 9-fold decrease in reporter activity upon co-expression of desmin is compared to the other results a relatively clear and strong effect. The degree of reliability of the apparent repression of the luciferase reporter by desmin will be demonstrated by desmin knock down at mRNA level by pshRNA-GFP. The knock down of desmin then should result in increased luciferase activity.

2.4.3.3 The influence of a short hairpin RNA negative control on the luciferase reporters

To test if the short hairpin RNA-GFP negative control serves as an adequate negative control, the pshRNA-GFP negative control plasmid was co-transfected with NKE24 into C2C12

myoblasts. The luciferase reporter activity was assayed in the Dual Luciferase Assay System (Promega) 48 hours after transfection.

Firefly luciferase (5)	Renilla luciferase (5)	Ratio F/R (5)
1 NKE<NKE+Kdneg	1 NKE<NKE+Kdneg	1 NKE<NKE+Kdneg
1 NKE=NKE+Kdneg	1 NKE=NKE+Kdneg	4 NKE=NKE+Kdneg
3 NKE>NKE+Kdneg	3 NKE>NKE+Kdneg	0 NKE>NKE+Kdneg

Table 2.4.3.3: The compared luciferase reporter activities from NKE24 expression alone and in combination with a negative control shRNA (Kdneg). NKE24: nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity.*

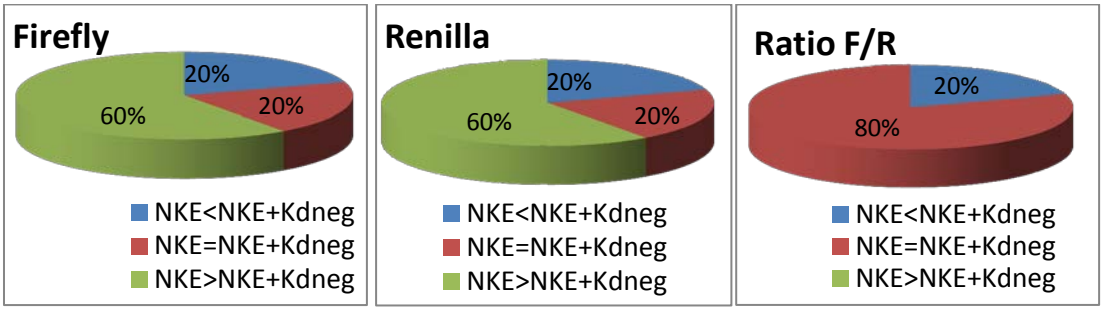


Figure 2.4.3.3*: The results from comparison of the luciferase reporter activities of NKE24 alone and in combination with pshRNA-GFP negative control, shown as circular diagrams.*

In this experiment the pshRNA-GFP negative control plasmid was shown to leave the luciferase reporters uninfluenced. Therefore, it is proven to be a satisfying negative control for subsequent knock down of desmin at mRNA level by pshRNA-GFP clones 1-4.

2.4.3.4 The influence of short hairpin RNA negative control and desmin and luciferase reporters

The independence of the luciferase reporters from pshRNA-GFP negative control was also tested under co-expression of desmin in C2C12 myoblasts. Unfortunately, this test was only done in duplicates, which is too little to show significant results. Anyway, the data is shown for completeness of the reporter assay data.

Firefly luciferase (2)	Renilla luciferase (2)	Ratio F/R (2)
1 N+Des<N+Des+Kdneg	0 N+Des<N+Des+Kdneg	1 N+Des<N+Des+Kdneg
0 N+Des=N+Des+Kdneg	1 N+Des=N+Des+Kdneg	1 N+Des=N+Des+Kdneg
1 N+Des>N+Des+Kdneg	1 N+Des>N+Des+Kdneg	0 N+Des>N+Des+Kdneg

Table 2.4.3.4: Shows the compared luciferase reporter activities from NKE24, which is co-expressed with desmin from pBK-RSVcDesmin, without or in combination with a negative control shRNA (Kdneg). NKE24: nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity. Des: ectopic expression of desmin from pBK-RSVcDesmin.*

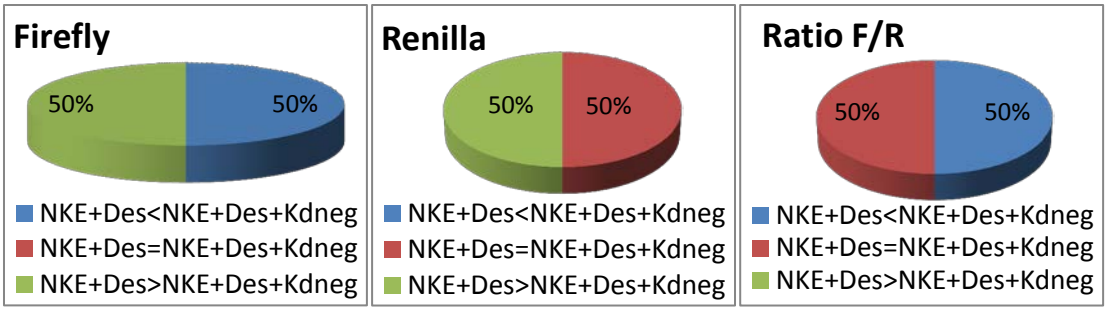


***Figure 2.4.3.4**: Depicts the results shown above in circular diagrams.*

This set up was only repeated twice, so the results are not significant.

2.4.3.5 The influence of desmin knock down on the luciferase reporters

A) Short hairpin RNA clone 1

Firefly luciferase (3)	Renilla luciferase (3)	Ratio F/R (3)
1 NKE24< NKE24+Kd1	1 NKE24< NKE24+Kd1	1 NKE24< NKE24+Kd1
0 NKE24= NKE24+Kd1	0 NKE24= NKE24+Kd1	2 NKE24= NKE24+Kd1
2 NKE24> NKE24+Kd1	2 NKE24> NKE24+Kd1	0 NKE24> NKE24+Kd1

Table 2.4.3.5A: Shows the compared activities of the luciferase reporters from NKE24 and phRL-TK alone or in combination with the shRNA clone 1 (Kd1). NKE24: nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity.*

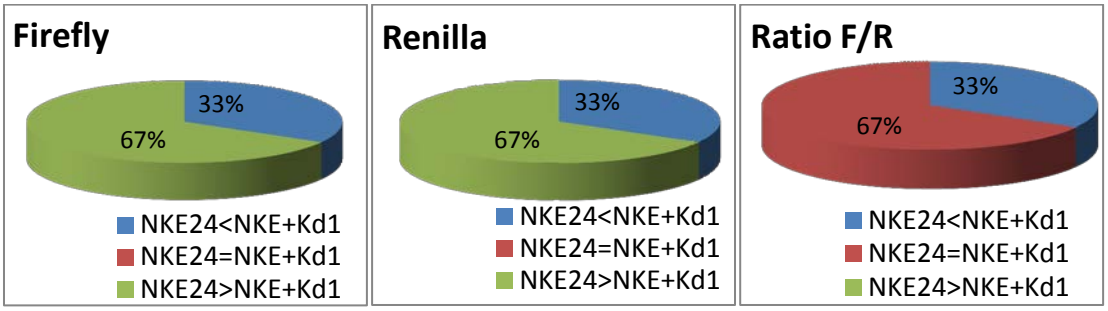


***Figure 2.4.3.5A**: Depicts the relative difference in luciferase activities under the influence of the shRNA clone 1.*

Unfortunately, the presented set up was only done in triplicate. If the great deviation from previous experiments is taken into account, a triplicate of one experiment might be to little to be statistically relevant. Despite this, the normalized results from this reporter assays show that the knock down of desmin at mRNA level does not change the reporter activity. Therefore, this knock down could not proove the theory that desmin is acting as a repressor on the *nkx 2.5* promoter. The 33% of the results, which show that the knock down resulted in increased reporter activity is below the limit of significance.

In later semi-quantitative real time PCR with cDNA from transfected C2C12 myoblasts, it is demonstrated that the knock down of desmin mRNA by pshRNA-GFP is incomplete at the

time of RNA extraction 48 hours after the transfection, as it is shown in figure 2.4.7AB. Concommitantly, the knock down is incomplete when the Dual Luciferase Assay is performed 48 hours after transfection. The results from the semi-quantitative real time PCR, which unfortunately was done after the reporter assays, indicates that in C2C12 myoblasts, which naturally express desmin, the timing and or the degree of the knock down is insufficient to abolish desmin mRNA from the targeted cells. In parallel the effect of desmin knock down is not fully developed at the time when the reporter assay is performed. Most probably, resident desmin subunits are still acting on the system, while the newly transcribed desmin mRNA is decayed more or less incomplete. Unfortunately, it was missed to assess the degree of desmin mRNA knock down in extensive real time PCR experiments.

If the results from semi-quantitative real time PCR, which are shown and described in chapter 2.4.7, are taken into account, the 33% of results, which show increased reporter activity upon desmin mRNA knock down, maybe the best result, which is possible under conditions of incomplete knock down.

However, the only conclusion, which can be drawn from the results of this reporter assay, is that desmin does not act as an activator on the *nkx 2.5* promoter in C2C12 myoblasts.

B) Short hairpin RNA clone 2

Firefly luciferase (3)	Renilla luciferase (3)	Ratio F/R (3)
0 NKE24< NKE24+Kd2	0 NKE24< NKE24+Kd2	1 NKE24< NKE24+Kd2
0 NKE24= NKE24+Kd2	0 NKE24= NKE24+Kd2	1 NKE24= NKE24+Kd2
3 NKE24> NKE24+Kd2	3 NKE24> NKE24+Kd2	1 NKE24> NKE24+Kd2

Table 2.4.3.5B: The compared activities of the luciferase reporters under the influence of the short hairpin RNA clone 2(Kd2). NKE24: nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity.*

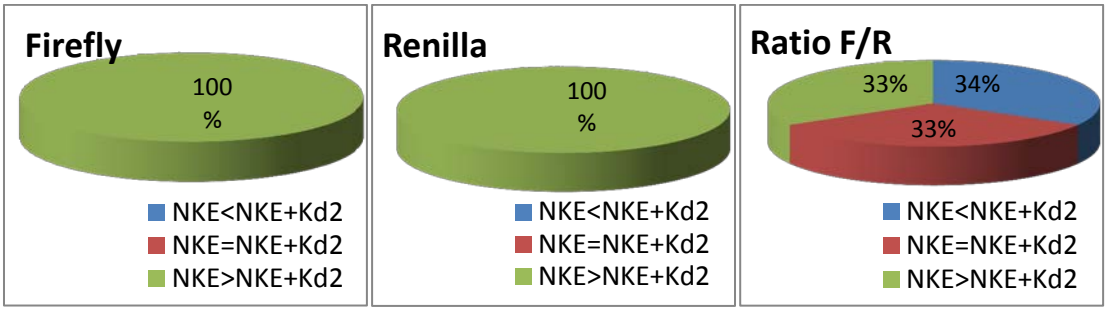


Figure 2.4.3.5B*: The relative difference in luciferase activities under the influence of pshRNA-GFP clone 2.*

At the level of the firefly luciferase, the knock down of desmin at mRNA level seems to reduce the reporter activity. The renilla luciferase reflects that the transfection efficiency was lower when pshRNA-GFP clone 2 was co-transfected, so the apparent reduction of the firefly

luciferase activity might be a consequence of lower transfection efficiency. However, the normalized result shows that the knock down of desmin at mRNA level by pshRNA-GFP clone 2 does not influence the luciferase reporter activity in C2C12, which is reflected by a 1:1:1 distribution of smaller:equal: greater reporter activity, as shown in figure 2.4.3.5B.

C) Short hairpin RNA clone 3

Firefly luciferase (3)	Renilla luciferase (3)	Ratio F/R (3)
0 NKE24< NKE24+Kd3	0 NKE24< NKE24+Kd3	1 NKE24< NKE24+Kd3
0 NKE24= NKE24+Kd3	1 NKE24= NKE24+Kd3	2 NKE24= NKE24+Kd3
3 NKE24> NKE24+Kd3	2 NKE24> NKE24+Kd3	0 NKE24> NKE24+Kd3

Table 2.4.3.5C: The compared activities of the luciferase reporters from NKE24 and phRL-TK alone or in combination with shRNA clone 3 (Kd3). NKE24: nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity.*

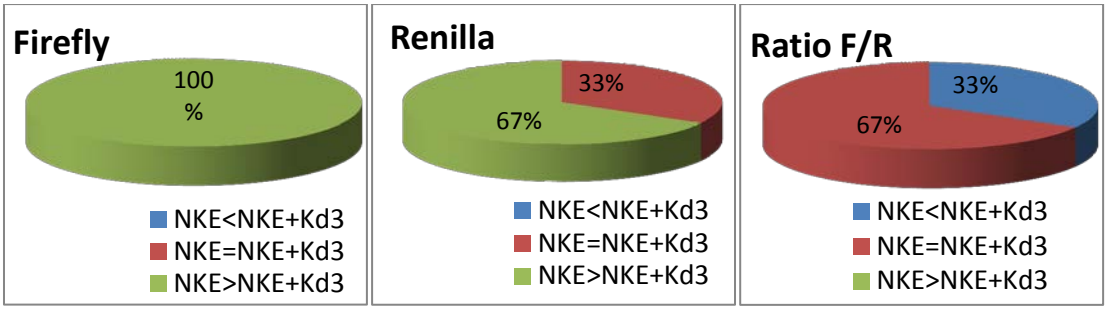


Figure 2.4.3.5C*: The relative difference in luciferase activities under the influence of the shRNA clone 3.*

As it was already before observed from other transfections, the efficiency-or at least the renilla luciferase activity, which reflects the transfection efficiency, is lower in the presence of pshRNA-GFP (clone 3). However, the normalized reporter activity shows that the knock down of desmin at mRNA level does not influence the luciferase reporter activity significantly, because 67% (two samples!) show equal reporter activity from NKE24 alone and in combination with pshRNA-GFP clone 3. As it is demonstrated by results, which were shown previously, a triplicate of one experiment seems to be too little to be statistically relevant. In addition to that, as it is also mentioned in chapter 2.4.3.5A, the knock down of desmin mRNA in C2C12 by the pshRNA-GFP plasmids was shown to be incomplete in semi-quantitative real time PCR (chapter 2.4.7). In consequence, the effect of the knock down is not reflected unambiguously in the data. Due to the incompleteness of the knock down, we have to assume that already slight tendencies, which deviate from statistical distribution of 1:1:1 or from 100% equality in reporter signal, are the most significant results, which we can expect from incomplete knock down. Similarily to the knock down of desmin by pshRNA-

GFP clone 1, which is described in chapter 2.4.3.5A, the results from knock down by pshRNA-GFP clone 3 show 67% of equal results from NKE24 (*nkx 2.5* pro::*luc*) alone and in combination with pshRNA-GFP clone 3 (NKE24= NKE24+Kd3), whereas 33% of the results show that the luciferase reporter activity decreased upon the knock down of desmin at mRNA level. If the incompleteness of the knock down is taken into account, the 33% of the results, which reflect a decrease in reporter activity upon desmin knock down, demonstrate that desmin might act inhibitory on the *nkx 2.5* promoter.

D) Short hairpin RNA clone 4

C2C12 myoblasts, which naturally express desmin, were transfected with the *nkx 2.5* pro::*luc* reporter alone or combined with a desmin targeting shRNA (Kd4).

Firefly luciferase (9)	**Renilla luciferase (9)**	**Ratio F/R (∑9)**
2 NKE24< NKE24+Kd4	2 NKE24< NKE24+Kd4	2 NKE24< NKE24+Kd4
1 NKE24= NKE24+Kd4	2 NKE24= NKE24+Kd4	5 NKE24= NKE24+Kd4
6 NKE24> NKE24+Kd4	5 NKE24> NKE24+Kd4	2 NKE24> NKE24+Kd4

Table 2.4.3.5D: Shows the compared activities of the luciferase reporters from NKE24 and phRL-TK alone or in combination with shRNA clone 4 (Kd4). NKE24: nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity.*

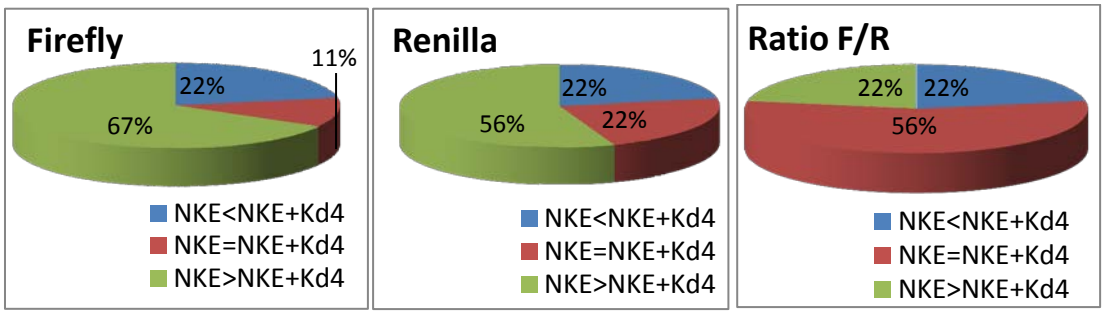


Figure 2.4.3.5D*: Depicts the relative difference in luciferase activities under the influence of the shRNA clone 4.*

The results from co-expression of pshRNA-GFP clone4 show that the knock down of desmin does not influence the luciferase reporter significantly.

Firefly luciferase (3)	**Renilla luciferase (3)**	**Ratio F/R (3)**
0 N+Des< N+Des+Kd4	0 N+Des< N+Des+Kd4	2 N+Des< N+Des+Kd4
0 N+Des= N+Des+Kd4	0 N+Des= N+Des+Kd4	1 N+Des= N+Des+Kd4
3 N+Des> N+Des+Kd4	3 N+Des> N+Des+Kd4	0 N+Des> N+Des+Kd4

Table 2.4.3.5D: The comparison of the luciferase reporters under ectopic desmin expression from pBK-RSVcDesmin alone or in combination with short hairpin RNA clone 4(Kd4). N(KE24): nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity. Des: ectopic expression of desmin from pBK-RSVcDesmin.*

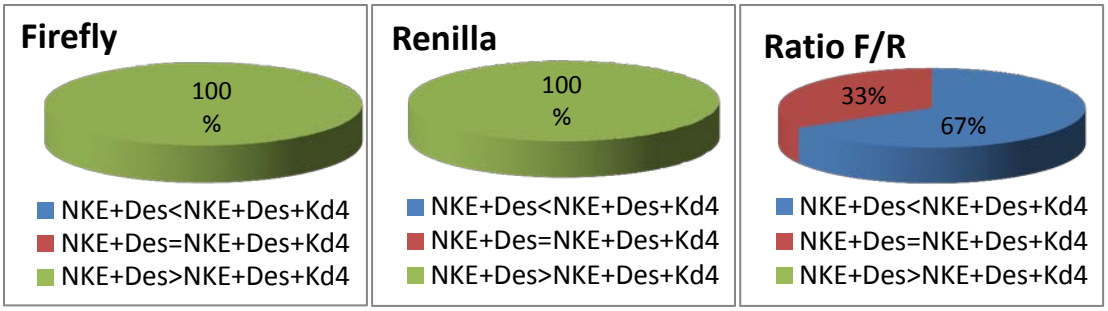


***Figure 2.4.3.5D**: Depicts the relative difference in luciferase activities under the influence of desmin and the shRNA clone 4.*

When desmin is co-expressed with the *nkx 2.5* pro::*luc* (NKE24) reporter, the simultaneous knock down of desmin by pshRNA-GFP clone 4-expression, the luciferase activity increases in 67% (two samples) upon the knock down. The mean value of increase is 5.76-fold with a standard deviation of 3.4. The observed increase in luciferase reporter activity from *nkx 2.5* pro::*luc* (NKE24) upon the knock down of desmin, suggests that desmin acts inhibitory on the *nkx 2.5* promoter.

On one hand, this interpretation of the result should be handled with care, because in the manual of the Dual Luciferase Reporter Assay (DLR-Promega) it is documented that „it is important to realize that *trans* effects between promoters on co-transfected plasmids can potentially affect reporter gene expression“ *(Farr and Roman, 1992; DLR-manual III.B "Important considerations in co-transfection experiments")*. The expression of GFP from pshRNA-GFP is controlled by a CMV (CytoMegaloVirus) promoter. This type of promoter is very strong and might over rule the normal mechanisms of transgene expression. To exclude the chance of promoter interference, another study is needed, in which the CMV promoter is transfected on a separate plasmid, without the short hairpin RNA sequence.

On the other hand, such a *"trans"* effect was not observed from any other transfection of the shRNA-GFP clones, which carry all the same CMV-promoter to drive GFP expression. Therefore, it is more likely that the revealed increase in reporter activity upon desmin knock down and simultaneous ectopic expression of desmin suggests an inhibitory role of the intermediate filament protein at the *nkx 2.5* promoter.

2.4.4 Luciferase assays of transfected primary cardiomyocytes

Primary cardiomyocytes (pCMC) were prepared from newborn BalbC and Black6 mice and used for transfection one day after isolation without further subculture. From fluorescence microscopy of transfected pCMC we know that only a very small subset of cardiac cells is susceptible to transfection. The cells were screened for transfection efficiency 24 to 48 hours after the transfection with pshRNA-GFP. Transfected cells appeared green in fluorescence

microscopy. The Dual Luciferase Assays were performed 48 hours after transfection with the same cells.

2.4.4.1 The *nkx 2.5* promoter drives luciferase expression in primary cardiomyocytes

Firefly luciferase (∑ 11)	Renilla luciferase (∑ 12)	Ratio F/R (∑ 11)
11 pGL3b< NKE24	10 pGL3b< NKE24	9 pGL3b< NKE24
0 pGL3b= NKE24	1 pGL3b= NKE24	1 pGL3b= NKE24
0 pGL3b> NKE24	1 pGL3b> NKE24	1 pGL3b> NKE24

Table 2.4.4.1: The results of the reporter activities of the promoter-less pGL3b vector and the nkx 2.5 promoter-driven luciferase expression (NKE24), in comparison.
NKE24: nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity.*

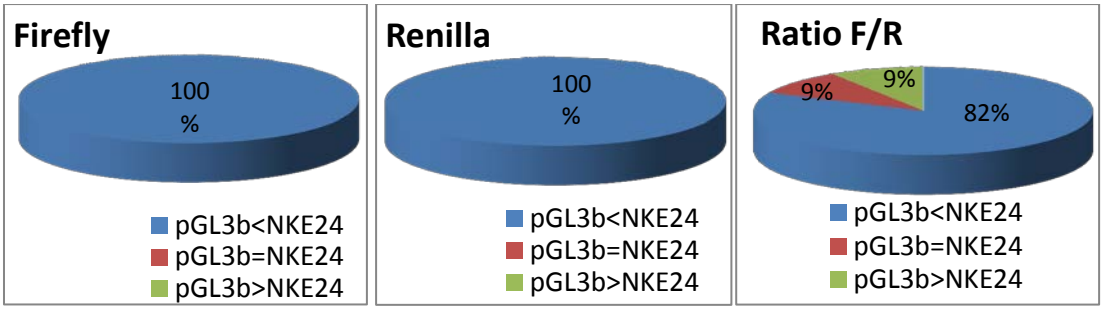


Figure 2.4.4.1*: The results of the luciferase reporter activities in circular diagrams.*

Again it was successfully proven that the *nkx 2.5* promoter is able to promote the firefly luciferase reporter expression. Interestingly, from the renilla luciferase activity we see that the transfection efficiency was disproportionally greater from NKE24 transfections. However, based on the normalized reporter values (ratio F/R) we found in 82% of the primary cardiomyocytes increased luciferase reporter expression after *nkx 2.5* pro::*luc* (NKE24) transfection. The mean value of increase in reporter activity upon NKE24 transfection was12-fold with a standard deviation of 9.5. Once a peak of a 29-fold activation was observed, compared to the promoter-less pGL3b. The lowest observed activation was 1.8-fold. This large deviation in the results led to the great standard deviation, which overshadows the reliability of the results.

2.4.4.2 The expression of pshRNA negative control might elicit side effects in transfected cardiomyocytes

A) pGL3b and pshRNA-GFP negative control

Primary cardiomyocytes were isolated from newborn mice and transfected with the promoter-less pGL3b alone or in combination with the short hairpin RNA negative control plasmid

(Kdneg). This experiment was included to show that the random scrambled sequence of the pshRNA-GFP negative control plasmids does not influence the luciferase reporters, which could not be demonstrated unambiguously.

Firefly luciferase (4)	Renilla luciferase (4)	Ratio F/R (4)
1 pGL3b< pGL3b+Kdneg	2 pGL3b< pGL3b+Kdneg	1 pGL3b< pGL3b+Kdneg
3 pGL3b= pGL3b+Kdneg	2 pGL3b= pGL3b+Kdneg	1 pGL3b= pGL3b+Kdneg
0 pGL3b> pGL3b+Kdneg	0 pGL3b> pGL3b+Kdneg	2 pGL3b> pGL3b+Kdneg

Table 2.4.4.2: Compared results of reporter assays from transfected primary cardiomyocytes (pCMC). pGL3b: promoter-less firefly luciferase reporter, Kdneg: a negative control shRNA (pshRNA-GFP).

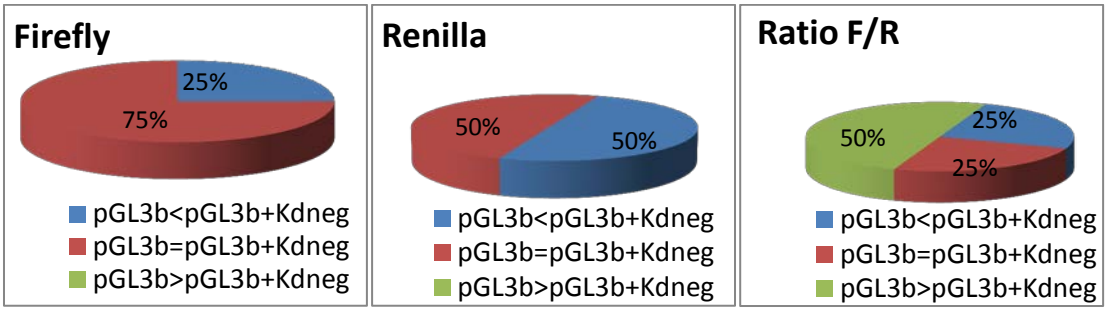


***Figure 2.4.4.2**: Shows the results of the luciferase reporter activities in circular diagrams.*

As we expected, the expression of the pshRNA-GFP negative control does not significantly influence the firefly luciferase activity. Despite the insignificance, in 50% of the results we found that the reporter activity was 3.7-fold greater without the addition of pshRNA-GFP negative control (Kdneg) with a standard deviation of 2.8. This result is somewhat similar to the results from the desmin targeting short hairpin clones in C2C12 myoblasts. Although, the result is below the limit of significance, it might indicate that the short hairpin RNA negative control found some off-target in the pCMC and subsequently leads to reduced reporter activity after co-expression.

B) NKE24 and pshRNA-GFP negative control

Primary cardiomyocytes (pCMC) were isolated from newborn mice, as described in chapter 4.1.7. The pCMC were subjected to transfection with the *nkx 2.5* pro::*luc* reporter plasmid (NKE24) alone or in combination with a short hairpin RNA-GFP negative control plasmid (Kdneg), which expresses a random scrambled hairpin RNA sequence. The luciferase reporter activities were assayed in a Dual Luciferase Assay (Promega) 48 hours after transfection. The reporter activities from NKE24 transfected cells were compared to the reporter activities from cells transfected with NKE24 and pshRNA-GFP negative control. As the different transfections in general showed great deviation in the reporter activities, the results were

summarized as “smaller” (NKE24< NKE24+Kdneg), “equal” (NKE24= NKE24+Kdneg), and “greater” (NKE24> NKE24+Kdneg) and listed in the table below.

Firefly luciferase (11)	**Renilla luciferase (11)**	**Ratio F/R (11)**
0 NKE<NKE+Kdneg	5 NKE<NKE+Kdneg	0 NKE<NKE+Kdneg
5 NKE=NKE+Kdneg	3 NKE=NKE+Kdneg	3 NKE=NKE+Kdneg
6 NKE>NKE+Kdneg	3 NKE>NKE+Kdneg	8 NKE>NKE+Kdneg

Table 2.4.4.3: Shows the compared luciferase reporter activities from NKE24 expression alone or in combination with a negative control shRNA (Kdneg). NKE24: nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity.*

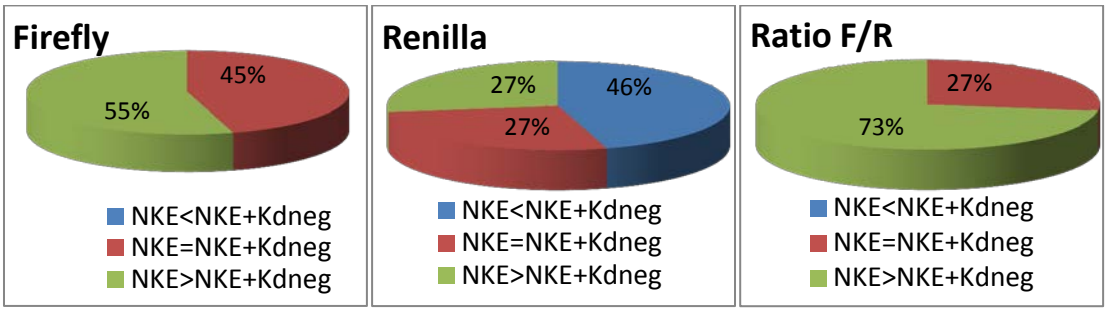


Figure 2.4.4.3*: Depicts the results, which are shown above as circular diagrams.*

The transfections of NKE24 and NKE24 in combination with the pshRNA-GFP negative control (Kdneg) are supposed to show balanced fractions of the three optional results (< smaller, = equal, or > greater). Although from firefly luciferase a great portion of the results shows that NKE24 and NKE24+Kdneg (pshRNA-GFP negative control) give equal reporter activity, the normalized results (ratio F/R) demonstrate that the combination with pshRNA-GFP negative control reduced the luciferase activity in 73%. The decrease in reporter activity is 2.5-fold (mean value) with a standard deviation of 0.5. The reduced firefly activity from NKE24+Kdneg does not seem to be a consequence of lower transfection efficiency, because the data from renilla luciferase show balanced results with a slight tendency to a greater efficiency of transfection from the samples with pshRNA-GFP negative control. The greater transfection efficiency in the samples, which were transfected with *nkx 2.5* pro::*luc* (NKE24) and pshRNA-GFP negative control (Kdneg), should result in the production of more firefly luciferase reporter, unless there is an unknown factor, which interferes with one of the components.

In the Nature Cell Biology editorial “Wither RNAi?” *(2003; Vol.5 (6): 489-490)* the use of a scrambled siRNA/shRNA is considered to be of “little benefit”. In addition to that, a comparison of the effects of siRNA/shRNA targeting a non-endogenous gene, such as lac Z or GFP, and a shRNA, which targets the gene of interest, is stated to be more useful as a negative control, than a scrambled sequence.

The results from co-expression of the pshRNA-GFP negative control implicate that the expression of the scrambled sequence of the short hairpin elicits an off-target effect in pCMCs. Therefore, the sequence of the short hairpin *(as shown in 2.4.1.1A)*, which is transcribed from pshRNA-GFP negative control, was subjected to a BLAST search of the mouse genome and nucleotide database *(NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi)*. The search revealed six mRNA sequences of the mouse, which could be targeted by the negative control short hairpin RNA sequence due to sequence similarity. The following mRNA sequences were identified as potential targets:

Name	**Function**
Bone Marrow kinase in the X-chromosome (Bmx) non-receptor tyrosine kinase (Bmx/Etk)	TNF signalling and cell cycle entry *(Al-Lamki et al.2009)*
leucine rich repeat protein 1 neuronal (Lrrn1)	Myotome specific expression *(Haines et al.2005);* cell affinity and compartment segregation in brain development *(Tossell et al.2011)*
exonuclease 3'-5' domain containing 2 (Exd2)	Not available on NCBI database search
ubiquitin specific peptidase 34 (Usp34)	Regulation of axin stability and Wnt/β-catenin signalling *(Lui et al.2011)*
natural cytotoxicity triggering receptor 1 (Ncr1/NKp46)	Tumor immunoediting *(Elboim et al.2010)*
anthrax toxin receptor 1 (Antxr1/Tem8)	Tumor endothelial marker *(Carson-Walter et al.2001)*

The potential consequences of RNAi targeting of these mRNAs by the pshRNA-GFP negativ control in transfected pCMC are unknown. As the potential targeting of the listed mRNAs was not verified by real time PCR, the mechanism behind the decrease in reporter activity upon co-expression of pshRNA-GFP negative control remains elusive.

2.4.4.3 The influence of the knock down of desmin at RNA level on the luciferase reporters

To elucidate desmins potential to interact with the *nkx 2.5* promoter in fully differentiated primary cardiomyocytes, the *nkx 2.5* pro::*luc* reporter plasmid (NKE24) was transfected alone and in combination with a short hairpin RNA-expressing plasmid, which serves to target desmin at mRNA level. If desmin does not only bind to the *nkx 2.5* promoter, but also interferes with its activation, the knock down is expected to result in changed reporter activity after transfection into pCMC, which were isolated from newborn mice.

A) Short hairpin RNA clone 4

Firefly luciferase (11)	**Renilla luciferase (11)**	**Ratio F/R (11)**
0 NKE24< NKE24+Kd4	5 NKE24< NKE24+Kd4	0 NKE24< NKE24+Kd4
7 NKE24= NKE24+Kd4	4 NKE24= NKE24+Kd4	5 NKE24= NKE24+Kd4

4 NKE24> NKE24+Kd4	2 NKE24> NKE24+Kd4	6 NKE24> NKE24+Kd4

Table 2.4.4.3A: Shows the compared activities of the luciferase reporters after transfection with nkx 2.5 *pro::*luc *(NKE24) alone or in combination with the short hairpin RNA clone 4 (Kd4). Renilla luciferase from phRL-TK internal control vector. Ratio F/R: normalized reporter activity, which corrects deviation from difference in transfection efficiency.*

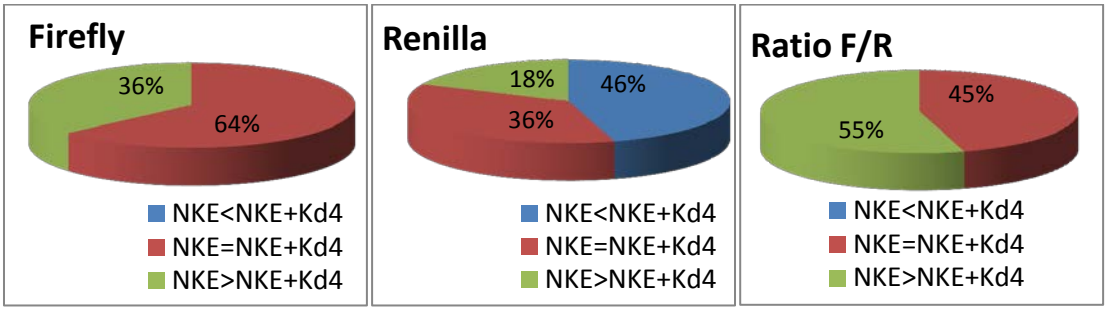


Figure 2.4.4.3A*: The relative difference in luciferase activities upon the co-expression of the desmin targeting shRNA clone 4 (Kd4).*

The normalized values of luciferase reporter activity reflect a 55%: 45% ratio of NKE24>NKE24+Kd4 : NKE24=NKE24+Kd4. So a little less than the half of the the results reflect unchanged reporter activity upon the co-expression of pshRNA-GFP clone 4 (Kd4). The other half (55%) shows a decrease in reporter activity when pshRNA-GFP clone 4 is co-expressed. The mean value of decrease is about 4-fold with a standard deviation of 2.85. Despite the fact that 55% is not a convincingly significant result, the data suggest that desmin might act as activator on the *nkx 2.5* promoter in pCMC. This result is in contrast to the results from the reporter assays with 10T ½ fibroblasts as well as with C2C12 myoblasts, in which the simultaneous co-expression and knock down of desmin revealed increased reporter activity, and therefore suggests that desmin acts inhibitory on the *nkx 2.5* promoter.

B) Short hairpin RNA clone 1 and clone 4 combined

To enhance the effect of desmin knock down on the *nkx 2.5* pro::*luc* reporter, pCMC were transfected with two different pshRNA_GFP clones (Kd1+4).

Firefly luciferase (3)	**Renilla luciferase (3)**	**Ratio F/R (3)**
2 N< N+Kd1+Kd4	2 N< N+Kd1+Kd4	0 N< N+Kd1+Kd4
0 N= N+Kd1+Kd4	1 N= N+Kd1+Kd4	0 N= N+Kd1+Kd4
1 N> N+Kd1+Kd4	0 N> N+Kd1+Kd4	3 N> N+Kd1+Kd4

Table 2.4.4.3B: Shows the compared activities of the luciferase reporters from pCMC, which were transfected with NKE24 alone or in combination with the shRNA clones 1+4. N(KE24): nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity.*

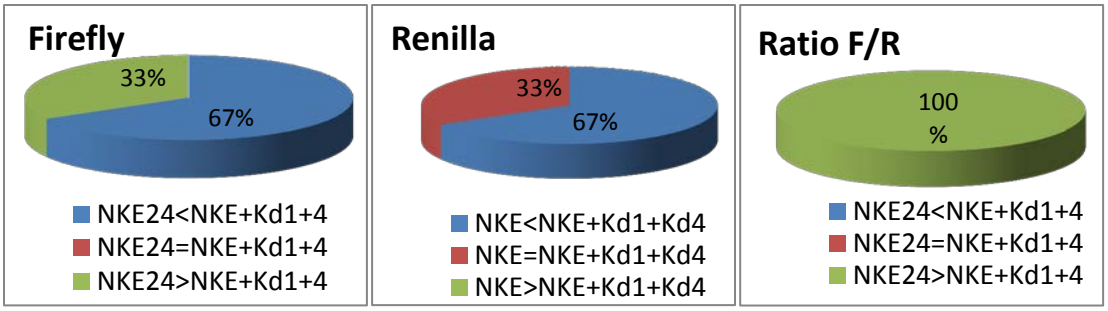


***Figure 2.4.4.3B**: Depicts the relative difference in luciferase activities upon co-expression of two different shRNA clones.*

The data from the reporter assay of primary cardiomyocytes after the transfection with NKE24 or NKE24 in combination with pshRNA-GFP clones 1+4 shows at the level of the firefly luciferase that in 67% of the samples the reporter activity increased upon the co-expression of the pshRNA-GFP clones 1+4. This increase in reporter activity after the knock down of desmin might be interpreted in the way that desmin acts as inhibitor on the *nkx 2.5* promoter. This interpretation is contradicted by the results from the normalized values of reporter activity (ratio F/R). The ratio F/R shows 100% of NKE24> NKE24+Kd1+Kd4. The mean value of decrease was 1.76-fold with a standard deviation of 0.08. The decrease in reporter activity allows for the suspicion that desmin might act as a very weak activator at the *nkx 2.5* promoter or alternative as a "permissive factor", which binds to the *nkx 2.5* promoter to substitute another molecule. Once more the results from the reporter assays with transfected pCMC contradict the findings from the same study in 10T ½ fibroblasts and in C2C12 myoblasts. Upon simultaneous ectopic expression of desmin and knock down of desmin at mRNA level, the reporter activity from *nkx 2.5* pro::*luc* (NKE24) increased and therefore desmin seems to be rather inhibitory to the *nkx 2.5* promoter in 10T ½ fibroblasts and C2C12 myoblasts.

2.4.5 Summarized results of luciferase assays from transfected fibroblasts and myoblasts

The transfections of 10T ½ fibroblasts and C2C12 myoblasts revealed similar results from Dual Luciferase Assays. Therefore, in this chapter the data from 10T ½ fibroblasts and C2C12 myoblasts are summarized with respect to the reporter plasmids, which were used in transfection. This was done to achieve more significant results and greater sample quantitiy, especially from the transfections, which were only done in duplicates in the different cell types. The results from transfected primary cardiomyocytes are not included, because they revealed results, which are contradictory to the results from fibroblasts and myoblasts.

2.4.5.1 The *nkx 2.5* promoter drives reporter expression

The luciferase reporter activities from 10T ½ fibroblasts and C2C12 myoblasts, which are shown in chapters 2.4.2 and 2.4.3, are summarized in the following table.

Firefly luciferase (∑25)	**Renilla luciferase (∑ 25)**	**Ratio F/R (∑ 25)**
20 pGL3b< NKE24	9 pGL3b< NKE24	19 pGL3b< NKE24
3 pGL3b= NKE24	8 pGL3b= NKE24	4 pGL3b= NKE24
2 pGL3b> NKE24	8 pGL3b> NKE24	2 pGL3b> NKE24

Table 2.4.5.1: The compared reporter activities of the promoter-less pGL3b and the nkx 2.5 *pro::*luc *reporter (NKE24). Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity.*

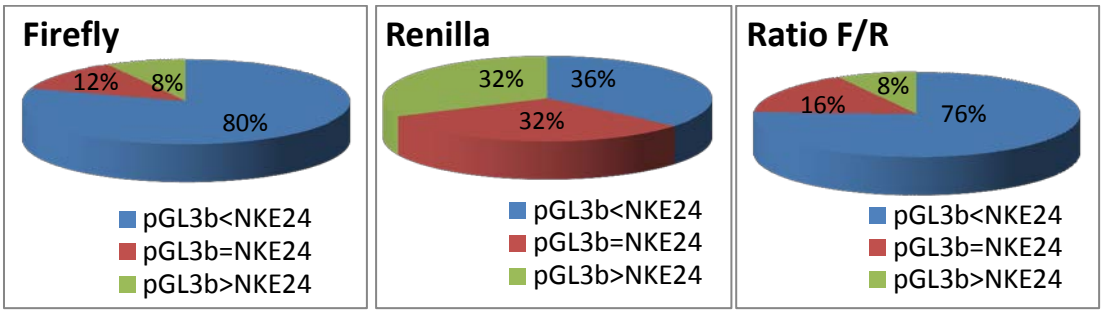


Figure 2.4.5.1*: Shows the results of the luciferase reporter activities in circular diagrams.*

The summarized results of transfected 10T ½ fibroblasts and C2C12 myoblasts confirm that the *nkx 2.5* promoter upstream of the *firefly luciferase* gene (NKE24) drives the expression of the firefly luciferase reporter. The mean value of the summarized increase in reporter activity is 29-fold with a standard deviation of 82.9. The great standard deviation implicates that the summary of the results from the two different cell types might be inappropriate.

2.4.5.2 The influence of ectopic desmin expression

A) on luciferase activity from promoter-less pGL3b (negative control)

Firefly luciferase (13)	**Renilla luciferase (13)**	**Ratio F/R (13)**
0 pGL3b< pGL3b+Des	3 pGL3b< pGL3b+Des	5 pGL3b< pGL3b+Des
11 pGL3b= pGL3b+Des	6 pGL3b= pGL3b+Des	3 pGL3b= pGL3b+Des
2 pGL3b> pGL3b+Des	4 pGL3b> pGL3b+Des	5 pGL3b> pGL3b+Des

Table 2.4.5.2A: Shows the results of the luciferase reporter activities from the promoter-less pGL3b vector upon desmin co-expression. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity. Des: ectopic expression of desmin from pBK-RSVcDesmin.

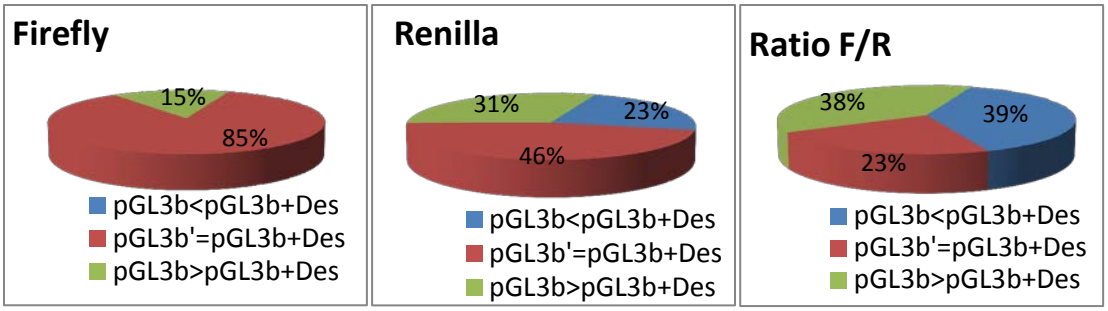


***Figure 2.4.5.2A**: Shows the results of the luciferase reporter activities in circular diagrams.*

As expected, the co-expression of desmin with the promoter-less pGL3b firefly luciferase reporter plasmid does not change the luciferase reporter activities significantly.

B) The influence of ectopic desmin expression on luciferase reporter downstream of the nkx 2.5 promoter

To assess the influence of desmin on the *nkx 2.5* promoter the NKE24 reporter plasmid was transfected into 10T ½ fibroblasts and C2C12 alone or in combination with the desmin expression vector pBK-RSV-cDesmin, as it is described in chapter 2.4.2 and 2.4.3. The results, which are shown here, reflect a summary of the previously shown results, in order to increase the sample quantity and statistical relevance.

Firefly luciferase (∑18)	**Renilla luciferase (∑18)**	**Ratio F/R (∑18)**
2 NKE24< NKE24+Des	2 NKE24< NKE24+Des	2 NKE24< NKE24+Des
6 NKE24= NKE24+Des	6 NKE24= NKE24+Des	11 NKE24= NKE24+Des
10 NKE24> NKE24+Des	10 NKE24> NKE24+Des	5 NKE24> NKE24+Des

Table 2.4.5.2B: Shows the results of the activities from nkx 2.5 promoter-driven luciferase reporter (NKE24) compared to NKE24 in combination with desmin. NKE24: nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity. Des: ectopic expression of desmin from pBK-RSVcDesmin.*

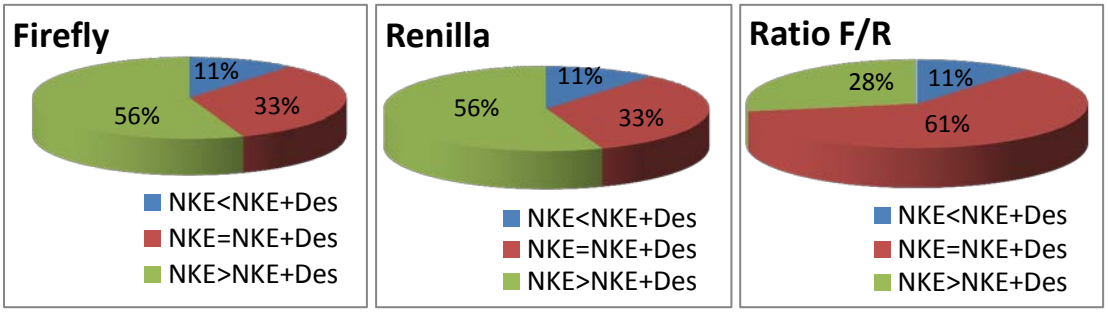


***Figure 2.4.5.2B**: Show circular diagrams of the results of nkx 2.5 promoter-driven reporter activities, compared to the co-expression with desmin.*

The ectopic expression of desmin from pBK-RSV-cDesmin does not lead to a significant change in the reporter activity from the *nkx 2.5* pro:luc (NKE24) after transfection in 10T ½

fibroblasts and C2C12 myoblasts. The majority (61%) of the samples (N18) revealed that the luciferase reporter activity is not influenced by the ectopic expression of desmin.

C) The influence of ectopic desmin expression on the MCE reporter plasmid

Firefly luciferase (4)	Renilla luciferase (8)	Ration F/R (4)
0 MCE<MCE+Des	1 MCE<MCE+Des	0 MCE<MCE+Des
1 MCE=MCE+Des	0 MCE=MCE+Des	4 MCE=MCE+Des
3 MCE>MCE+Des	7 MCE>MCE+Des	0 MCE>MCE+Des

Table 2.4.5.2C: Comparison of luciferase reporter activities. MCE: minimal cardiac enhancer sequence upstream of nkx 2.5 *promoter and* firefly luciferase *gene. Des: ectopic expression of desmin from pBK-RSV-cDesmin.*

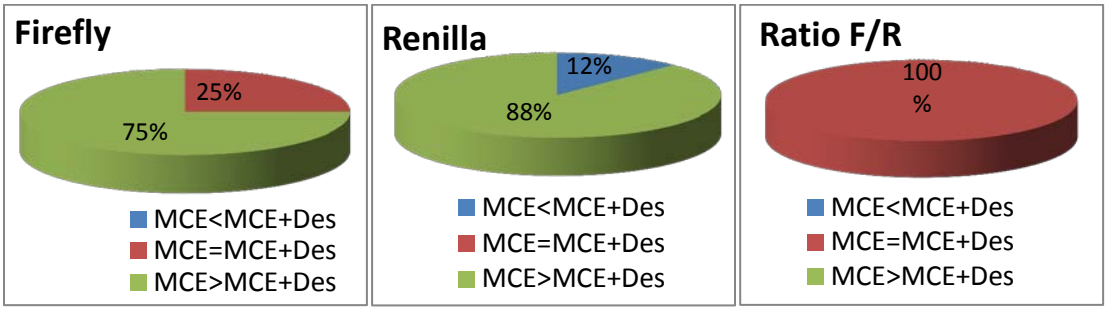


***Figure 2.4.5.2C**: Shows circular diagrams of the results shown above.*

The expression of desmin does not significantly change the luciferase reporter activity. Unfortunately, the sample quantity is too small to be statistically significant.

2.4.5.3 Cloned minimal cardiac enhancer fails to increase reporter activity

Firefly luciferase (∑5)	Renilla luciferase (∑5)	Ratio F/R (∑5)
1 NKE24 < MCE	0 NKE24 < MCE	1 NKE24 < MCE
3 NKE24 = MCE	3 NKE24 = MCE	3 NKE24 = MCE
1 NKE24 > MCE	2 NKE24 > MCE	1 NKE24 > MCE

Table 2.4.5.3: Shows a comparison of the results from NKE24 and MCE transfections. NKE24: nkx 2.5 *pro::*luc *reporter. MCE:* mce*::*nkx2.5 *pro::*luc*Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity.*

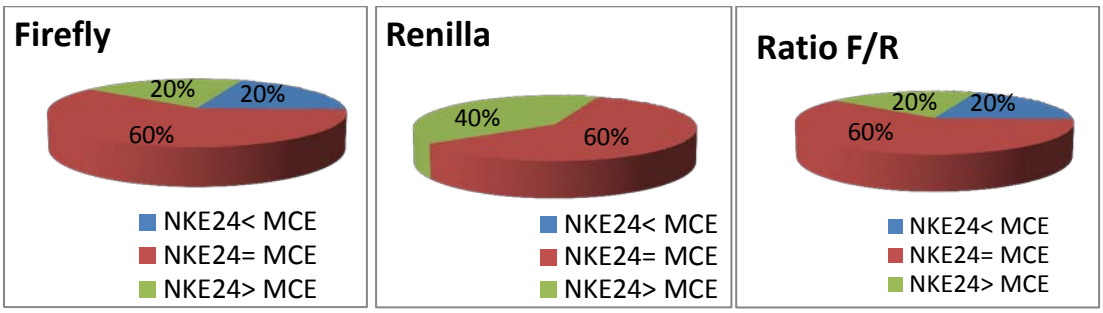


***Figure 2.4.5.3**: Shows the compared emergence of increased (<), unchanged (=), and decreased (>) reporter activity from NKE24 and MCE reporter plasmids.*

The expression of the luciferase reporter failed to increase from the cloned sequence of the minimal cardiac enhancer, compared to NKE24 (*nkx 2.5* pro::luc reporter). The cloned sequence of the minimal cardiac enhancer from the MCE plasmid was not sufficient to increase the reporter activity. This might be due to several reasons. The first is that the minimal cardiac enhancer (*Lien et al. 1999*) might fail to induce the *nkx 2.5* promoter. The second reason may be that the cloned sequence of the MCE contains additional base pairs compared to the published version, as it is shown in figure 2.2.5C. Unfortunately it was impossible to find out if these base pairs derive from a polymorphic region or if they derive from mistakes in the PCR, because the respective sequence of the C3H mouse genome is not available in the NCBI database. The GATA-binding sites within the MCE sequence, which were shown to be vital for the proper function of the minimal cardiac enhancer *(Lien et al.1999),* were not affected in the cloned sequence.The third reason for the failure of the minimal cardiac enhancer sequence to increase the luciferase reporter activity might be that the interaction of the enhancer sequence with a promoter needs alignment of the two regulatory elements. The arrangement of the minimal cardiac enhancer (MCE) within the context of a plasmid might be too tight to enable the alignment of the enhancer element and the promoter. For another enhancer element (μ) a need for precise alignment of regulatory sites was reported to be vital for enhancer activation *(Nikolajczyk et al.1996).* Similarily, precise spatial arrangement might be crucial to the activation of the minimal cardiac enhancer. In the study from Lien and colleagues transgenic mice were used as a model to explore the boundaries and the function of the minimal cardiac enhancer. When transgenic mice are generated by homologous recombination the sequence is incorporated site specific and therefore, the sequence remains in the context of the genome. In contrast, when reporter studies are done with plasmids in transfected cells, the lack of the proper spatial arrangement of the sequences, which are the subject of the study, might lead to complete loss of function of the respective element. The underlying mechanisms of activation of enhancer elements in consequence of precise spatial arrangement range from protein binding and complex formation to DNA bending (flexure) and to altered DNA conformation.

2.4.5.4 The influence of a negative control shRNA on the luciferase reporters

10T ½ fibroblasts and C2C12 myoblasts were transfected with the *nkx 2.5* pro::*luc* reporter (NKE24) and the internal control phRL-TK (*renilla* luciferase), as usual. Additionally, a negative control shRNA plasmid (pshRNA-GFP) was co-transfected to demonstrate that any observed effect from the later knock down of desmin at mRNA level by shRNA is specific.

The use of a scrambled negative control shRNA is discussed in more detail in chapter 2.4.4.2. The negative control short hairpin RNA-GFP from pshRNA-GFP was co-transfected to demonstrate that it leaves the activities of the firefly and the renilla luciferase unchanged.

Firefly luciferase (∑8)	**Renilla luciferase (∑8)**	**Ratio F/R (∑8)**
2 NKE<NKE+Kdneg	2 NKE<NKE+Kdneg	2 NKE<NKE+Kdneg
2 NKE=NKE+Kdneg	3 NKE=NKE+Kdneg	5 NKE=NKE+Kdneg
4 NKE>NKE+Kdneg	3 NKE>NKE+Kdneg	1 NKE>NKE+Kdneg

Table 2.4.5.4: The comparison of luciferase reporter activities from NKE24 expression alone or in combination with a negative control shRNA (Kdneg). NKE24: nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity.*

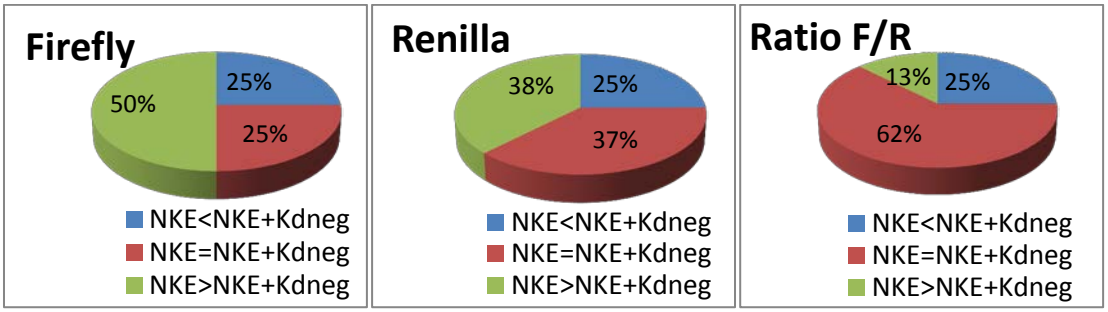


Figure 2.4.5.4*: Depicts the results, which are shown above as circular diagrams.*

Here, it is shown that the negative control shRNA-GFP does not interfere with the reporter activity of neither firefly luciferase from NKE24, nor renilla luciferase from phRL-TK. The result clearly demonstrates the absence of any off-target effect of the random scrambled RNA sequence, which is expressed from the negative control pshRNA-GFP.

2.4.5.5 The influence of ectopic desmin expression and negative control shRNA

Firefly luciferase (6)	**Renilla luciferase (6)**	**Ratio F/R (6)**
2 N+Des<N+Des+Kdneg	0 N+Des<N+Des+Kdneg	1 N+Des<N+Des+Kdneg
3 N+Des=N+Des+Kdneg	4 N+Des=N+Des+Kdneg	5 N+Des=N+Des+Kdneg
1 N+Des>N+Des+Kdneg	2 N+Des>N+Des+Kdneg	0 N+Des>N+Des+Kdneg

Table 2.4.5.5: The compared luciferase reporter activities from nkx 2.5 *pro::*luc *(N) and simultaneous ectopic desmin expression, alone or combined with shRNA negative control (Kdneg).*

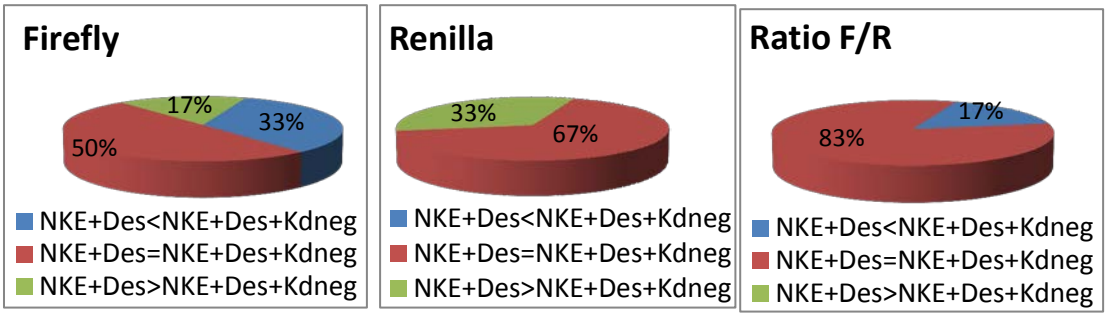


***Figure 2.4.5.5**: The compared luciferase reporter activities in circular diagrams. NKE:* nkx 2.5 *pro::*luc, *Des: ectopic desmin expression from pBK-RSVcDesmin, Kdneg: negative control shRNA-GFP.*

In 10T ½ fibroblasts and C2C12 myoblasts the negative control shRNA does not influence the luciferase reporter.

2.4.5.6 The influence of desmin knock down on the luciferase reporters

In this part of luciferase assay results, the effect of desmin knock down at mRNA level by pshRNA-GFP clones in 10T ½ fibroblasts and C2C12 myoblasts is discussed. The results derive from the transfections of 10T ½ fibroblasts and C2C12 myoblasts, which are discussed in chapters 2.4.2 and 2.4.3. The following data represents a summary of luciferase reporter assay results of these two cell types.

A) Short hairpin RNA clone 1

Firefly luciferase (5)	Renilla luciferase (10)	Ratio F/R (5)
1 NKE24< NKE24+Kd1	1 NKE24< NKE24+Kd1	1 NKE24< NKE24+Kd1
2 NKE24= NKE24+Kd1	2 NKE24= NKE24+Kd1	4 NKE24= NKE24+Kd1
2 NKE24> NKE24+Kd1	2 NKE24> NKE24+Kd1	0 NKE24> NKE24+Kd1

Table 2.4.5.6A: Shows the compared activities of the luciferase reporters from NKE24 and phRL-TK alone or combined with a desmin targeting shRNA (Kd1). NKE24: nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity.*

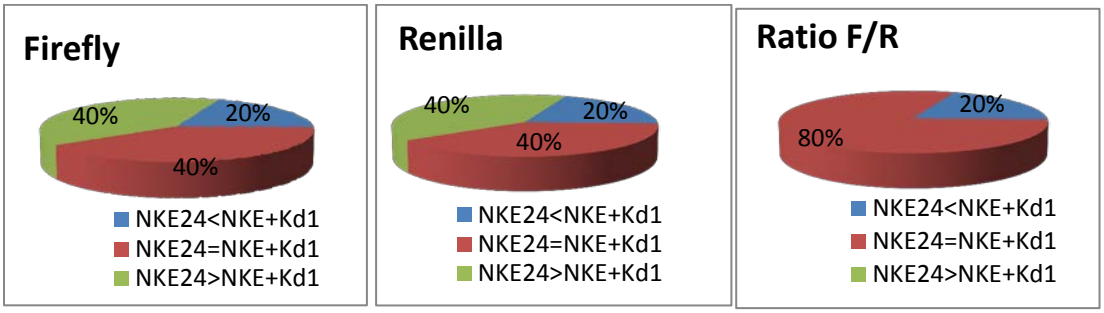


***Figure 2.4.5.6A**: Depicts the relative difference in luciferase activities under the influence of the shRNA clone 1.*

The summarized results of transfections of 10T ½ fibroblasts and C2C12 myoblasts demonstrate that the knock down of desmin at mRNA level by pshRNA-GFP clone 1 (siRNA mechanism) failed to change the luciferase reporter activity signficantly.

B) Short hairpin RNA clone 3

Firefly luciferase (5)	Renilla luciferase (5)	Ratio F/R (5)
0 NKE24< NKE24+Kd3	0 NKE24< NKE24+Kd3	1 NKE24< NKE24+Kd3
2 NKE24= NKE24+Kd3	3 NKE24= NKE24+Kd3	4 NKE24= NKE24+Kd3
3 NKE24> NKE24+Kd3	2 NKE24> NKE24+Kd3	0 NKE24> NKE24+Kd3

Table 2.4.5.6B: Shows the compared activities of the luciferase reporters under the influence of the shRNA clone 3(Kd3). NKE24: nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity.*

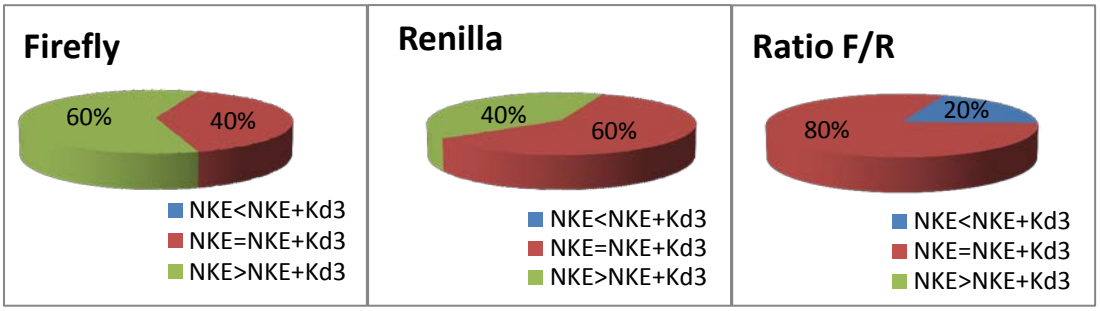


***Figure 2.4.5.6B**: Depicts the relative difference in luciferase activities under the influence of desmin and the shRNA clone 3.*

The results from the co-expression of pshRNA-GFP clone 3 resemble the data from pshRNA clone 1. The knock down of desmin left the reporter activity unchanged.

C) Short hairpin RNA clone 4

Firefly luciferase (∑13)	Renilla luciferase (∑13)	Ratio F/R (∑13)
2 NKE24< NKE24+Kd4	2 NKE24< NKE24+Kd4	2 NKE24< NKE24+Kd4
1 NKE24= NKE24+Kd4	2 NKE24= NKE24+Kd4	9 NKE24= NKE24+Kd4
10 NKE24> NKE24+Kd4	9 NKE24> NKE24+Kd4	2 NKE24> NKE24+Kd4

Table 2.4.5.6C: Shows the compared activities of the luciferase reporters alone or in combination with the short hairpin RNA clone 4(Kd4). NKE24: nkx 2.5 *pro::*luc *reporter. Renilla luciferase: from phRL-TK internal control vector, reflects the transfection efficiency. Ratio F/R: normalized reporter activity.*

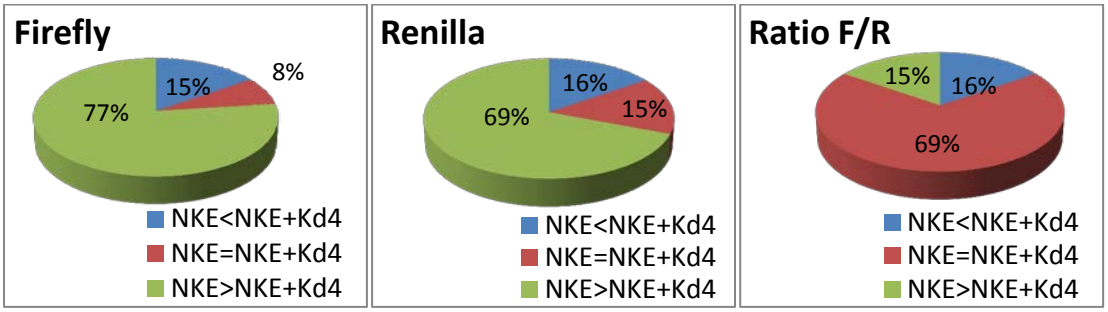


***Figure 2.4.5.6C**: Depicts the relative difference in luciferase activities under the influence of the shRNA clone 4.*

The knock down of desmin at mRNA level by a siRNA mechanism (pshRNA-GFP clone 4) does not change the luciferase reporter activity significantly. The result, which is shown here, resembles the result from the co-expression of a negative control pshRNA-GFP. The normalized reporter activities demonstrate that the knock down of desmin at mRNA level does not influence the reporter activity under the control of the *nkx 2.5* promoter. This

indicates that desmin does not act on the *nkx 2.5* promoter, although the intermediate filament protein binds to this regulatory element, as it was found in ChIP experiments.

D) *Short hairpin RNA clone 4 and desmin*

Firefly luciferase (∑9)	Renilla luciferase (∑9)	Ratio F/R (∑9)
3 N+Des< N+Des+Kd4	2 N+Des< N+Des+Kd4	5 N+Des< N+Des+Kd4
3 N+Des= N+Des+Kd4	3 N+Des= N+Des+Kd4	4 N+Des= N+Des+Kd4
3 N+Des> N+Des+Kd4	4 N+Des> N+Des+Kd4	0 N+Des> N+Des+Kd4

Table 2.4.5.6D: Compared luciferase reporter activities are summarized as smaller (<), equal (=), or greater (>). N (NKE24): nkx 2.5 *pro::*luc *reporter. Kd4: desmin targeting pshRNA-GFP clone 4.*

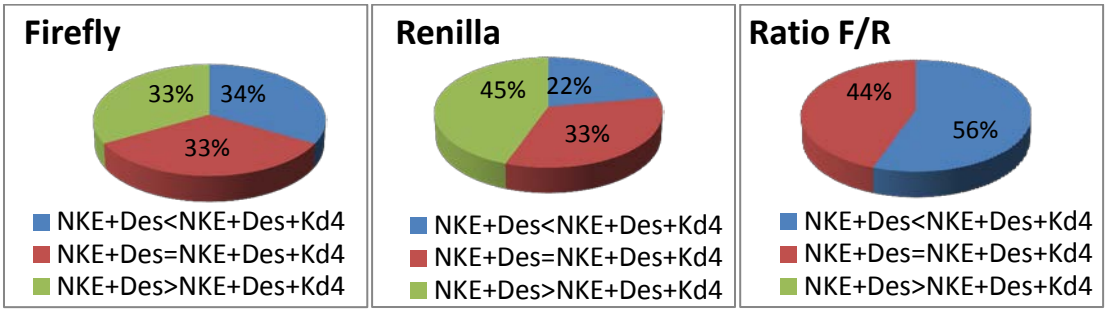


Figure 2.4.5.6D*: The compared reporter activities depicted in circular diagrams. NKE24:* nkx 2.5 *pro::luc reporter, Des: pBK-RSV-cDesmin expression vector, Kd4: desmin targeting pshRNA-GFP clone4.*

When desmin is expressed ectopically from pBK-RSVcDesmin and knocked down at mRNA level by shRNA (pshRNA-GFP) simultaneously, the luciferase reporter activity increases, compared to the ectopic expression of desmin alone. The mean value of reporter increase upon desmin knock down was 3.5-fold with a standard deviation of 2.7. (peak increase: 8-fold). The increase in reporter activity from desmin knock down was only observed when desmin is expressed ectopically.

2.4.6 Results of the luciferase assays

To elucidate why desmin binds to the *nkx 2.5* promoter and the minimal cardiac enhancer sequence, two different types of reporter plasmids, which carry the respective regulatory elements in *cis*, were engaged. A third plasmid, which carried the MCE sequence in *trans* without reporter gene, was included.

First, the NKE24 vector was proven to increase the firefly luciferase reporter activity via the *nkx 2.5* promoter. In all three transfected cell types the expression of NKE24 (*nkx 2.5* pro::*luc*) resulted in increased reporter activity compared to the promoter-less pGL3b.

Second, the minimal cardiac enhancer sequence *(Lien et al.1999)* was cloned upstream to the *nkx 2.5* pro::*luc* reporter, to demonstrate that desmin not only binds to the *nkx 2.5*

promoter and the minimal cardiac enhancer, but also interferes with reporter activity downstream of these well characterized regulatory elements. Unfortunately, the cloned MCE sequence was found to be unable to significantly increase the reporter expression from the firefly reporter vector. There may be several reasons for the failure of reporter enhancement. The first reason might be that the cloned sequence contains additional base pairs compared to the published sequence (*Lien et al.1999*). It is unclear if these bases came from a polymorphism within this region or from an error in the PCR. The possibility of a polymorphism arises from the fact that C3H mouse gDNA was used for cloning. Lien and colleagues were apparently exploring B6C3 gDNA. The second reason may be that the distance of the MCE sequence and the *nkx 2.5* promoter is too short. Therefore, the MCEcis vector was cloned.

The MCE-cis vector contains the MCE sequence at a maximum possible distance to the *nkx 2.5* pro::luc reporter. The reporter assays with MCE-cis revealed that the cloned sequence of the minimal cardiac enhancer fails to increase the luciferase reporter activity. Probably the results would have been different if the vector was aligned before transfection. The third reason for the failure of the MCE-cis vector to enhance the reporter activity of downstream *firefly luciferase* gene might be found in the fact that the genomic context is missing in the plasmid. In literature a theory is stated that the enhancer needs to align with the promoter elements. The precise spatial alignment of an enhancer was shown to be critical to the proper function of the enhancer *(Nikolajczyk et al.1996).* The concert of transcription factor (TF) binding needs a certain and apparently invariable arrangement of binding sites for proper complex formation. The TF-complex formation might further lead to DNA flexure and subsequent alteration of DNA conformation *(Nikolajczyk et al.1996).* Therefore, the lack of the genomic context within a reporter plasmid may be responsible for the failure of the MCE-cis to enhance the reporter activity. In the study of the minimal cardiac enhancer, Lien and colleagues used transgenic mice to explore the function and the boundaries of this regulatory element. Within transgenic mice, the MCE sequence remains in a genomic context, because homologous recombination is needed to establish a certain genotype *in-vivo*. Therefore, the ability of the MCE sequence to function in a reporter plasmid needs to be studied in more detail.

Third, to eliminate the chance that changed reporter activities result from a possible *trans* effect *(Farr et al.1992)* of the minimal cardiac enhancer, we tried to clone the MCE-trans vector. Unfortunately, the efforts to clone the minimal cardiac enhancer sequence into a

pBluescriptKS-backbone without luciferase reporter or any other additional regulating element remained unsuccessful.

The major task of this thesis was to elucidate desmins ability to interact with the *nkx 2.5* promoter. Therefore, desmin was ectopically expressed after transfection of 10T ½ fibroblasts and C2C12 myoblasts. An *nkx 2.5* pro::*luc* reporter (NKE24) was co-transfected to assess the influence of desmin on the *nkx 2.5* promoter. Subsequent Dual Luciferase Assays (Promega) revealed that ectopic desmin expression does not significantly change the activity of the luciferase reporter.

It remains elusive why desmin binds to the *nkx 2.5* promoter, as it was found in ChIP experiments (*chapter 2.1*). Concomitantly with the failure of desmin to change the luciferase reporter activity, the knock down of desmin at mRNA level from the expression of the shRNA-GFP plasmids failed to significantly change the reporter activity.

In contrast the simultaneous ectopic expression and knock down of desmin at mRNA level leads to increased reporter activity in the Dual Luciferase Assays (Promega) in 10T ½ fibroblasts and C2C12 myoblasts. In the discussion of the reporter assay results from 10T ½ fibroblasts after desmin targeting at mRNA level (2.4.2.7C), it is shown that the shRNA does not influence the reporter activity, as it is demonstrated by 100% equality in reporter activity with and without desmin knock down. This is in line with the fact that 10T ½ fibroblasts do not express desmin naturally. In contrast, when desmin is ectopically expressed during simultaneous knock down in 10T ½ fibroblasts the results reflect equal activity and increased reporter activity in a 50:50 ratio. Although not significant, the mean value of increase reporter activity in 10T ½ was 2-fold with a standard deviation of 0.43.

In C2C12 myoblasts, which naturally express desmin, the knock down of desmin lead to 56% of equal reporter activity. In contrast, when desmin is ectopically expressed and simultaneously knocked down in C2C12 myoblasts, the reporter activity increased in 67% of the samples. The mean value of incease in reporter activity in C2C12 was 5.76-fold with a standard deviation of 3.4. In summary, the results from 10T ½ fibroblasts and C2C12 myoblasts, therefore suggest an inhibitory action of ectopically expressed desmin on the *nkx 2.5* promoter. In contrast the co-transfection of pBK-RSVcDesmin and the luciferase reporter vector into 10T ½ fibroblasts revealed that desmin fails to change reporter activity significantly. As we did not expect to find such great difference in the results from natural and ectopical desmin expression, it was omitted to increase sample quantities of C2C12 myoblasts with ectopically expressed desmin levels. We rather expected to find changed reporter activity upon the knock down of naturally expressed desmin. In addition to that, we expected to see

changed luciferase reporter activity from transfected 10T ½ fibroblasts, which were forced to ectopic desmin expression, compared to *nkx 2.5* pro::*luc* reporter (NKE24) controls. The fact that only ectopic desmin expression showed significant results in reporter assays was rather surprising and makes conclusive interpretation difficult.

The reporter assays with transfected primary cardiomyocytes (pCMC) revealed a result, which is the opposite from what was observed in 10T ½ fibroblasts and C2C12 myoblasts. As fully differentiated pCMC naturally express relatively large amounts of desmin, these cells were never subjected to ectopic desmin expression. In pCMC reporter assays we relied on the effect of shRNA, which targets desmin at mRNA level, and therefore was expected to reveal significant results. The knock down of desmin in pCMC, which were transfected with the *nkx 2.5* pro::*luc* reporter (NKE24) revealed in 55% of the samples a mean 4-fold decrease in luciferase reporter activity with a standard deviation of 2.85. So in pCMC the results from knock down of desmin at mRNA level suggest that desmin might act as a very weak and potentially conditional activator on the *nkx 2.5* promoter. The intermediate filament protein desmin might rather be a "permissive" factor on the *nkx 2.5* promoter in pCMC and in contrast a "non-permissive" factor in 10T ½ fibroblasts and C2C12 myoblasts, than a transcription factor (TF), which actively interferes with the activity of the *nkx 2.5* promoter. It might serve as a binding site for TFs, which subsequently induce activation or repression of the *nkx 2.5* promoter. As the levels of desmin expression are relatively low in 10T ½ fibroblasts and C2C12 myoblasts compared to pCMC, the proposed role for desmin as a co-factor in the regulation of heart relevant gene expression would fit to the process of intermediate filament assembly. The subunits of the intermediate filament type III-protein desmin bind to the *nkx 2.5* promoter and the minimal cardiac enhancer, as it was demonstrated in ChIP experiments, at the early onset of expression and act "non-permissive" on these genomic regulatory elements, until the onset of intermediate filament assembly. With the progress of intermediate filament assembly, desmin subunits are sequestered from the nucleus to fullfill their role in the maintenance of the structural integrity of muscle cells, due to greater affinity of intermediate filament protein subunits to each other than to DNA. The sequestering of desmin from the nucleus to cytoplasmic IF assembly is demonstrated by the disappearance of desmin binding to the *nkx 2.5* promoter and the minimal cardiac enhancer at day 9 of development in $des^{+/+}$ EBs and at day 8 in $des^{+/+/ect}$ EBs in ChIP experiments.

2.4.7 Semi-quantitative Real Time PCR

To demonstrate the effects of desmin targeting at mRNA level by shRNA (pshRNA) on the expression levels of desmin, transfected cells were harvested for subsequent RNA isolation 48 hours after transfection. The RNA was isolated with the QIAGEN RNeasy kit. The genomic DNA was digested with DNaseI. Thereafter, the samples were tested for residual gDNA in a PCR with primers, which are specific for the housekeeping gene *glycerinealdehyde-3-phosphat-dehydrogenase (GAPDH).* After the samples were proven to be free of gDNA, the residual mRNA was reversely transcribed with Superscript II reverse transcriptase from Invitrogen and oligo-dT primers. The cDNA was used to set up another GAPDH-PCR to equilibrate the cDNA concentration in the samples.

Thereafter, a PCR with primers, which are specific for *desmin*, was run under the following conditions:

Temperature	Duration	Cycles
95°C	5 min	
95°C	1 min	x 22
52°C	30 sec	x 22
72°C	45 sec	x 22
72°C	1 min 30 sec	
4°C	Infinitely	

Figure 2.4.7.1*: Semi-quantitative real-time PCR. Samples from transfected 10T ½ fibroblasts and C2C12 myoblasts. Vectors were used as indicated in the picture.*
NKE24: nkx 2.5 *pro::*luc *reporter, Kd4: shRNA plasmid clone4 (pshRNA),*
GAPDH: housekeeping gene for glycerinealdehyde-3-phosphat-dehydrogenase serves to equilibrate [cDNA]; pGL3b: promoter-less firefly luciferase reporter plasmid. Desmin (Des): pBK-RSVcDesmin, Kdneg: negative control shRNA. RNA was isolated 48 hours post transfection.

The figure 2.4.7.1 demonstrates the absence of desmin mRNA in 10T ½ fibroblasts and successful expression of desmin in 10T ½ after transfection with pBK-RSVcDesmin. The levels of ectopically expressed desmin in 10T ½ fibroblasts are not affected by the negative control shRNA, whereas the targeting of desmin by shRNA (Kd4) resulted in complete knock down of ectopically expressed desmin in 10T ½ fibroblasts. The semi-quantative RT PCR with C2C12 cDNA demonstrates that desmin is expressed naturally in untransfected C2C12 myoblasts. In addition to that, it demonstrates that the knock down of desmin at mRNA level by pshRNA-GFP (Kd4) is incomplete in C2C12 myoblasts (NKE24+Kd4). Moreover, this semi-quantitative RT PCR failed to detect changes in the levels of desmin expression upon ectopic expression. Either the levels of naturally expressed desmin are already saturated, so that ectopic expression of desmin fails to further increase the expression level, or the semi-quantitative real time PCR is simply too insensitive to monitor changes in the levels of desmin expression. In the cDNA of transfected C2C12 myoblast there is no evident difference in the levels of natural desmin expression compared to the ectopic expression of desmin from pBK-RSVcDesmin and compared to the knock down of desmin mRNA. The results from the semi-quantitative real time PCR indicate that the timing of desmin knock down in C2C12 myoblasts might be inappropriate. In consequence, this indicates that the reliability of the results from Dual Luciferase Assays with the C2C12 myoblasts should be questioned. Due to the fact that the knock down of desmin was obviously incomplete we can only expect effects in the reporter assay, which slightly miss the furrow of significance. Furthermore, the semi-quantitative RT PCR demonstrates that the expression of additional desmin could not elevate the levels of desmin expression in C2C12 myoblasts. Neither the knock down of desmin at mRNA level nor its rescue could be verified in the present RT PCR. As a consequence for this thesis the whole chapter on Dual Luciferase Assays was interpreted according to this, in the way that increase or decrease in luciferase reporter activity was indicated even if reporter activity was changed only insignificantly. To finally elucidate desmins ability to interfere with regulatory DNA elements during development, the knock down of desmin and its rescue by the co-expression of desmin from pBK-RSVcDesmin should be verified in a more sensitive approach like a Light Cycler Real Time PCR (Roche) before reporter activity may be assayed.

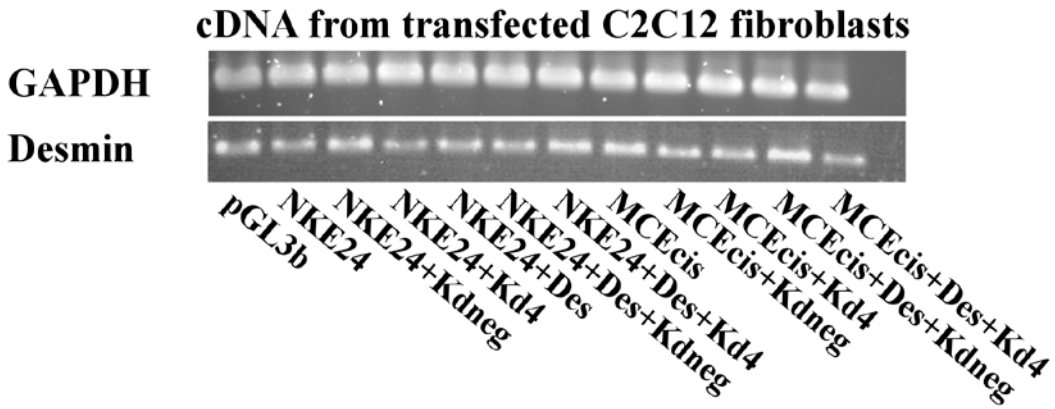


Figure 2.4.7.2*: Semi-quantitative real time PCR. RNA was isolated from C2C12 myoblasts 48 hours after transfection. Vectors were used as indicated. pGL3b: promoter-less firefly luciferase reporter plasmid. NKE24:* nkx 2.5 *pro::*luc *reporter plasmid, Desmin (Des): from the pBK-RSV-cDesmin vector. Kdneg: negative control shRNA, Kd4: desmin targeting shRNA. MCE-cis:* mce::nkx 2.5 *pro::*luc *reporter.*

In Figure 2.4.7.2 the results of a different semi-quantitative real time PCR (RT PCR) is depicted. It shows the natural expression level of desmin in C2C12 myoblasts in the first three samples (pGL3b, NKE24, and NKE24+Kdneg). The fourth lane shows the knock down of desmin at mRNA level by pshRNA-GFP clone 4. Here the knock down became more apparent than in figure 2.4.7.1. The level of desmin mRNA appears slightly reduced when compared to the first three lanes. The ectopic expression of desmin from the pBK-RSVcDesmin is visible as a weak increase in signal intensity in lane 5 (NKE24+Des). Unfortunately, this increase is not remarkable when compared to the natural levels of desmin expression (e.g lane 3: NKE24+Kdneg). Moreover, the “knock down and rescue” sample in lane 7 (NKE24+Des+Kd4) shows greater level of desmin expression than in the samples, in which desmin was expressed ectopically (e.g. lanes 5: NKE24+Des and 6: NKE24+Des+Kdneg). This also demonstrates that the timing of desmin expression, its knock down and rescue in the transfection and reporter assay study might be inappropriate to reveal more significant results.

2.4.8 Fluorescence microscopy reveals levels of transfection efficiency

The shRNA plasmids, which code for a desmin-targeting short hairpin RNA or a negative control shRNA, co-express a green fluorescent protein (hMGFP). The expression of hMGFP enables tracking of transfected cells in fluorescence microscopy, and therefore to visualize the transfection efficiency.

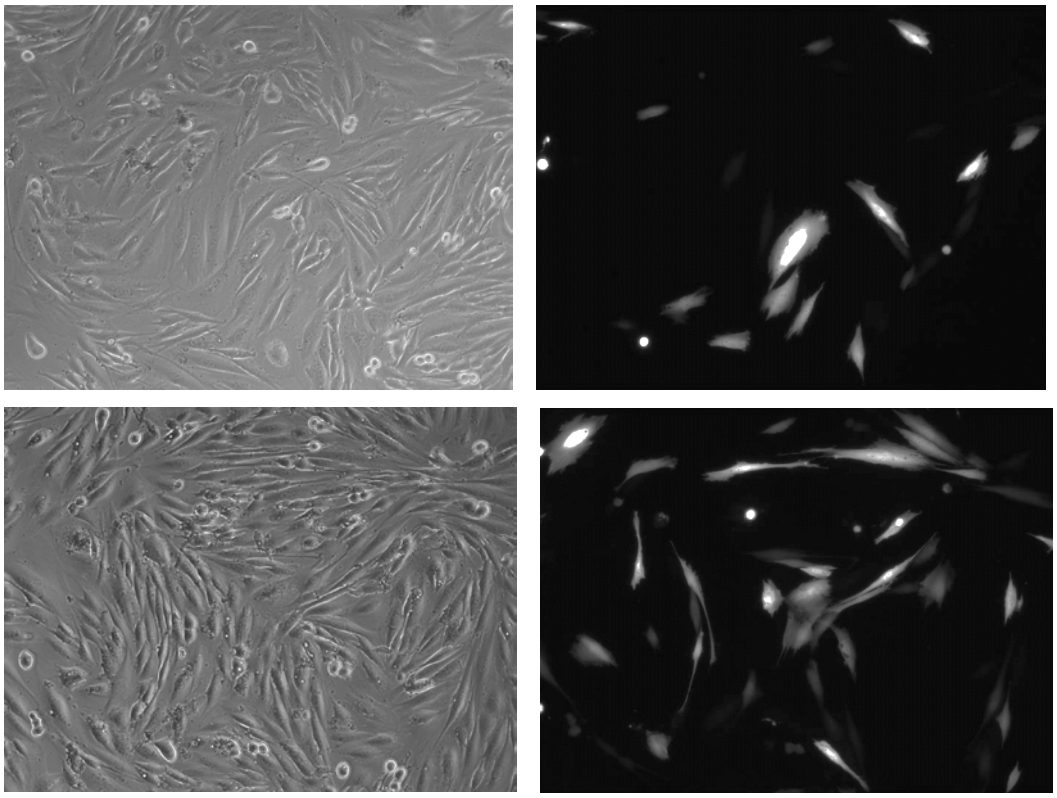


Figure 2.4.8.1: *Shows C2C12 myoblasts after the transfection with pshRNA-GFP clone 4, which targets murine desmin mRNA. Left: viable C2C12 cells (20x) in phase contrast. Right: fluorescence image of the same cells(20x). GFP-fluorescence represents successfully transfected (pshRNA-GFP) cells. Transfection efficiency ranged from ~10-15%.*

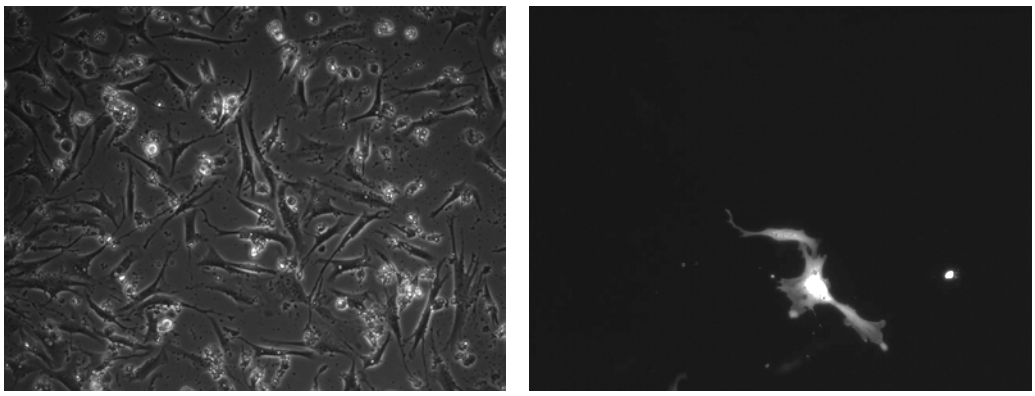
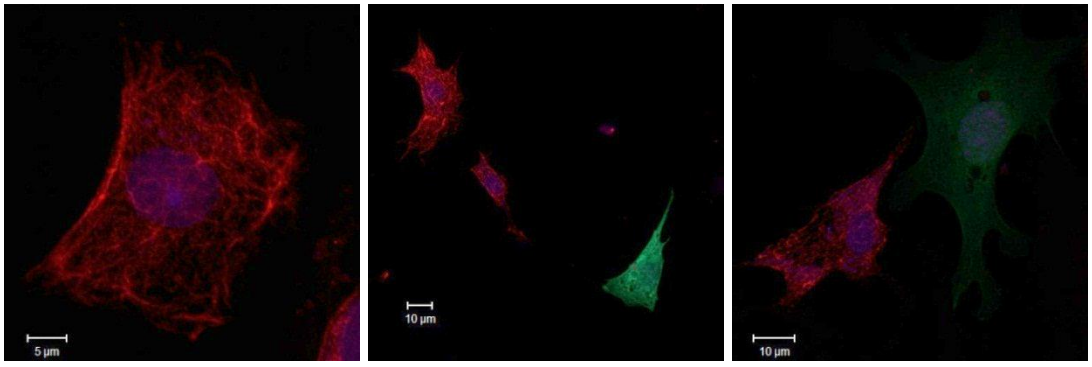


Figure 2.4.8.2: *Transfected primary cardiomyocytes (pCMC) in phase contrast microscopy (left) and in fluorescence microscopy (right). The fluorescence results from the transfection of pshRNA-GFP clone 4, which targets the murine desmin mRNA. Transfection efficiency of pCMC: ~5%*

2.4.9 Primary cardiocytes represent a heterogenous population

To assess the consequences of desmin knock down, transfected pCMC were subjected to immunofluorescence staining. Therefore, primary cardiomyocytes were transfected with plasmids, which express shRNA variants (pshRNA). The pshRNA-GFP clone 4 serves to specifically knock down the mRNA of the *desmin* gene in target cells, by mechanisms of RNA interference (RNAi). The negative control shRNA was included to explore co-

localization of desmin-staining in successfully transfected cells (GFP^+). Similarily to the Dual Luciferase Assays and semi-quantitative RT PCR, transfected cells were fixed two days after transfection. The fixed cells were stained with desmin-specific mouse monoclonal antibodies, and a secondary Texas red-conjugated antibody. This enables to visualize the desmin protein in the red channel of the Zeiss confocal laser scanning microscope, while the GF protein is visualized in the green channel.



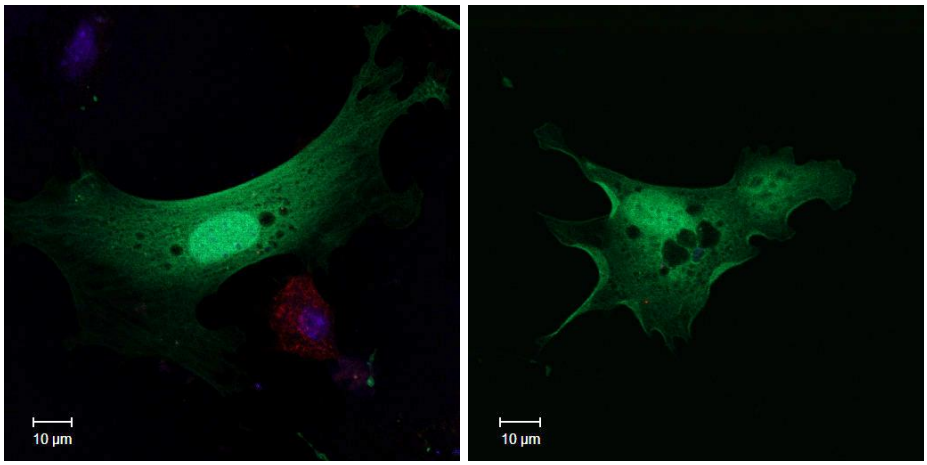
A) *B)* *C)*

***Figure 2.4.9.1**: Immunofluorescence microscopy of pCMC with antiDesmin antibody and detection of GFP.*

***A) untransfected pCMC,** Red: desmin monoclonal mouse AB. DAPI (blue): nucleus.*
***B) transfected pCMCs** (pshRNA-GFP clone 4). Green: GFP^+ from successful transfection with desmin targeting shRNA-GFP. Red: desmin monoclonal mous AB. Blue: DAPI stainig of the nuclei.*
***C) transfected pCMCs.** Green: GFP^+ (pshRNA-GFP clone 4), red: desmin specific monoclonal antibody, DAPI (blue): nuclei.*

Figure 2.4.9.1A shows a fixed primary cardiomyocyte, positively stained for desmin (red). The intermediate filament type III protein is mostly located to the cytoplasm and more condensed around the nucleus (blue). Figure 2.4.9.1B shows fixed pCMCs, which are either successfully transfected (GFP^+) or desmin positive (red). It remains unclear if the introduction of desmin targeting shRNA leads to RNAi-mediated protein decay, or if in advance only desmin negative pCMCs are susceptible to transfection. To examine the effect of desmin knock down at mRNA level on the levels of pre-existing protein (e.g. RNAi-mediated protein decay), a series of western blot studies need to be done.

However, the immunofluorescence staining of transfected pCMC demonstrates that primary cells represent a very heterogenous population of cells, in which only some are susceptible to genetic manipulation.



A) ***B)***

Figure 2.4.9.2*: Transfected pCMCs.*
Green: GFP positive, Red: desmin positive immunofluorescence staining, Blue (DAPI): nuclei
A) *transfected with desmin targeting pshRNA-GFP clone 4.*
B) *transfected with a negative control pshRNA-GFP.*

Figure 2.4.9.2AB demonstrates the observed heterogeneity of pCMC with respect to the susceptibility of individual cells to transfection. Interestingly, in virtually all the samples of fixed pCMC after transfection, we only found large cardiac fibroblasts to be successfully transfected (GFP positive). The desmin positive cells (red) were usually smaller. Two exceptions were found in all examined samples. The exceptions are shown in figure 2.4.9.1B and 2.4.9.3 on the left. In both pictures des^+ and GFP^+ neighbouring cells appear in similar size and shape.

It seems that not all of the cell types, which are present in a cardiac tissue, are susceptible to transfection. This observation is in line with the very low transfection efficiency we obtained from the primary cardiomyocytes (~5%).

Unfortunately, the transfections of the negative control shRNA failed to reveal a single des^+/GFP^+ cell, although the negative control shRNA (pshRNA-GFP) codes for a scrambled short hairpin RNA sequence, which does not target desmin mRNA. In Dual Luciferase Assays (Promega) of transfected pCMCs we found evidence that the negative control shRNA elicits off-target effects. In subsequent search of the NCBI nucleotide database six potential targets were identified. Still it remains elusive if the GFP expression over rules the desmin staining or interferes with its structure in a way that masks the epitopes for desmin-specific antibody binding or if desmin is simply not expressed uniformely in all cell types of the cardiac tissue. However, it is remarkable that even after the transfection with the negative control, the cells were either GFP positive, or desmin positive.

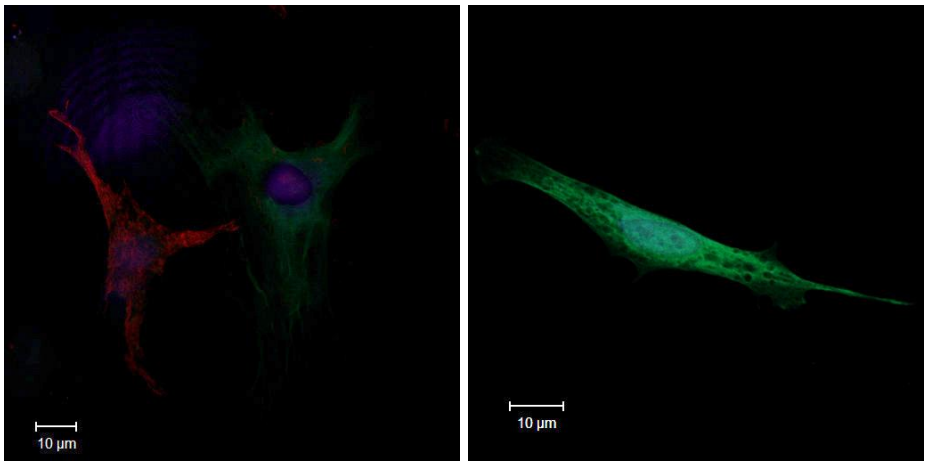


Figure 2.4.9.3*: transfected Primary cardiocytes (pshRNA-GFP clone4). Red: desmin-specific staining from monoclonal mouse antibodies and secondary texas red (TR) antibody. Green: GFP$^+$, DAPI (blue): nuclei.*

Unfortunately, in none of the specimens a co-localization of GFP (successful transfection) and desmin-texas red staining was found. This leads to the suspicion that it is only a special subset of primary cardiocytes, which is susceptible to transfection. More precisely, the cardiac muscle cells may be too specialized and too sensitive to be susceptible to transfection and therefore, to produce a short hairpin RNA. In a cell type of that importantance in the organism there might be at least one mechanism to suppress any attack from foreign DNA or RNA. In addition to that, it is very unlikely that the short hairpin RNAs are able to act on pre-existing desmin protein, or even on large quantities of desmin mRNA, as the semi-quantitative Real Time PCR of the transfected samples revealed (*chapter 2.4.7*). So in summary, the primary cardiomyocytes represent a model, which is not ideal for transfection and subsequent reporter assays, due to their variable susceptibility to transfection.

3. Material

3.1. Chemicals for molecular biology

Acetic acid Merck, D
Acrylamide BioRad, USA
Agarose, Biozyme LE Biozyme, D
Ampicillin Sigma, USA
BCIP=5'Brom-4'Chlor-3'Indoylphosphat Diagnostic Chemicals Limited, D
Bovine Serum Albumin Roth, D
ß-Mercaptoethanol Loba Feinchemie, A
Bis N,N'-Methylenbisacrylamide BioRad, USA
Brompheolblue Sigma, USA
Coomassie Brilliant Blue R250 Merck, D
DAPI Sigma, USA
Dabco Sigma, USA
dNTPs MBI Fermentas, Lithuania
Dimethylsulfoxid (DMSO) Acros, B
Dimethylformamid Fluka, CH
Dithiothreitol (DTT) Acros, B
Ethanol Merck, D
Ethidiumbromide Fluka, CH
EDTA Acros, B
EGTA Acros, B
Formaldehyde Merck, D
Glycerin Merck, D
Hydrochloric acid (HCl) Acros, B
Kanamycin Sigma; USA
Lumiglo® cell signaling, D
Methanol Merck, D
MgCl2 MBI Fermentas, Lithuania
Mineraloil Sigma, USA
Mowiol 2-88 Hoechst, D
Nitrotetrazolium Blue Chloride (NBT) Fluka, CH
NEB Buffers New England Biolabs, USA
PCR-buffer without MgCl2 MBI Fermentas, Lithuania
Phenylmethansulfonylfluoride (PMSF) Flucka, CH
Ponceau-S Sigma, USA
Protease inhibitor complete mini Roche, CH
Reverse Transcriptase Buffer Invitrogen, USA
Saponin Roth, D
SDS BioRad, USA
Sodiumchloride Salinen Austria, A
Sodiumhydrogencarbonate LifeTechnologies, USA
Tris Base LifeTechnologies, USA

T4-Ligase Buffer Promega, USA

3.2. Chemicals for tissue culture

ß-Mercaptoethanol Loba, A
Ampothericin B Invitrogen, USA
D-Glucose Acros, B
DMEM powder LifeTechnologies;USA
DMSO (Dimethylsulfoxide) Sigma, USA
Fetal Bovine Serum (FBS) HyClone, USA
Gibco, USA
Sigma, USA
Gelatine Difco, USA
L-(+)-Glutamin Acros, B
Mitomycin C Acros, B
Natriumhydrongencarbonate LifeTechnologies, USA
Penicillin G Kaliumsalz Merck, D
Streptomycin Sigma, USA

3.3 Enzymes

CIP (Calf Intestine Phosphatase) NewEnglandBiolabs, USA
Collagenase Gibco, USA
DNAse I, RNase free Roche, D
Pancreatin Worthington, USA
Pfu-Polymerase Fermentas, Lithuania
Restriction Enzymes NewEnglandBiolabs, USA
RNaseOUT® LifeTechnologies, USA
Superscript®II RNase Reverse Transcriptase LifeTechnologies, USA
Taq DNA Polymerase MBI Fermentas, Lithuania
Trypsin LifeTechnologies, USA
T4 DNA Ligase Promega, USA

3.4 Antibodies

Anti-Desmin (mouse) monoclonal (epitope approx: AA 325-372), Sigma (ProdNr.D1033)
Anti-Histone H3 (rabbit), Abcam (ProdNr.: ab1791)
Anti-cMyc (mouse), from the group of E.Ogris
Anti-Vimentin (mouse), Abcam
donkey anti-rabbit TexasRed-conjugated
primary pre-immune serum from rabbits, from the group of C.Seiser
goat anti-mouse FITC-conjugated

3.5 PCR primers

Primer name	Sequence		
nkx2-5 promoter 1 fwd	5'-CTCTGTTTGCTTTCTCGCCA-3'	52°C	245 bp
nkx2-5 promoter 1 rev	5'-ATTGGAGACAGGCAGCGTTA-3'	52°C	
nkx2-5 promoter 2 fwd	5'-GGCGTTGTTGAAGATAAAGC-3'	48°C	275 bp
nkx2-5 promoter 2 rev	5'-GCCTTTTAAAGACTTGGTGC-3'	48°C	
min.card.enh 1 fwd	5'-GAGATAAGATGACATACCAGA-3'		349 bp
min.card.enh 1 rev	5'-ATTAGTGTGAACACAACACT-3'	57,8°C	
min.card.enh 2 fwd	5'-TTATCTTCCCCGGAGGAAAT-3'	51,9°C	327 bp
min.card.enh 2 rev	5'-GCAGACACTCAAGCCCTTAA-3'		
MCE1107 2 fwd	5´-CGACAGGAAACTCGGAGCTA-3´	60°C	121 bp
MCE1107 2 rev	5´-CTCTGCTGTGTGGCCTTGTA-3´	60°C	
nkx2.5ProSG fwd	5´-TAAAGAAACCCCCACAGGTCA-3´	55°C	166 bp
nkx2.5ProSG rev	5´-TGTCAAGAAAAAGGAAGAAAACTC-3´	53°C	
nkx2-5 distal 1 fwd	5´-AGATTCCCAGCAGTCCGTA-3´	54,5°C	~340 bp
nkx2-5 distal 1 rev	5´-CAAGAGTCACTGCCCGAA-3´	54,5°C	
nkx2-5 distal 2 fwd	5´-GACATTGTGCATCTTAGCA-3´	54,5°C	~440 bp
nkx2-5 distal 2 rev	5´-TTCCACACTGCTGACCTG-3´	54,5°C	
nkx2-5 proximal 1 fwd	5´-GTCCCAGAACAACTGTCT-3´	54,5°C	~270 bp
nkx2-5 proximal 1 rev	5´-CCAGCCAGACACACTCAA-3´	54,5°C	
nkx2-5 proximal 2 fwd	5´-CTCACTCTGTCCTGTTTCCT-3´	54,5°C	~400 bp
nkx2-5 proximal 2 rev	5´-GAGTCCTGTTAAGTGAATCGG-3´	54,5°C	
GAPDH fwd	5´-CGTCTTCACCACCATGGAGA-3´	55°C	~300 bp
GAPDH rev	5´-CGGCCATCACGCCACAGTTT-3´	55°C	
Dra III fwd	5'-TAATCACGTAGTGTCTGGGTCCTAATGC-3'	63°C	510 bp
Not I rev	5'-TATTCGCCGGCCGCTCAAGGTGCACATGA-3'	63°C	
MCEtrans fwd	5'-GATAGGGTTGAGTGTTGTTCCAGTTT-3'	66°C	1066 bp
MCEtrans rev	5'-ATTACCGCCTTTGAGTGAGCTGATA-3'	66°C	
Sal I fwd	5'-TAATGTCGACTCTGGGTCCTAATGC-3'	56,5°C	~ 510 bp
Sal I rev	5'-TATTGTCGACTCAAGGTGCACATGA-3'	56,5°C	
MCEcis fwd	5'-TATGTTTCAGGTTCAGGGGGAGGTG-3'	57°C	~ 681 bp
MCEcis rev	5'-TAAGTGCGGCGACGATAGTCATGC-3'	57°C	
Sal I fwd and MCEcis rev			~ 597 bp

3.6 Plasmids

Vector name	Origin	Specification
pGL3b	Promega	Basic FF (w/o promoter), Amp^R (Luc.Assay)
pUC18	ATCC	*LacZ*, Amp^R (Transfection/DNA balance)

NKE24	Based on pGL3b (Promega), cloned and kindly provided by K.Yutzey from Cincinnati, USA.	*nkx 2.5* promoter-*FF; Amp*^{R} (Luc.Assay)
MCE-NKE1	Based on NKE24, cloned by C.Fuchs (2007) MCE (*Lien et al.; 1999)*	MCE- *nkx 2.5* promoter-*FF; Amp*^{R} (Luc.Assay)
MCE(3) or (i)	Based on NKE24, cloned by S.Gawlas in cooperation with C.Fuchs in 2007.	MCE- *nkx 2.5* promoter-*FF ; Amp*^{R} (Luc.Assay)
MCEcis	Based on NKE24, MCE cloned at max. possible distance by S.Gawlas in 2008	*nkx2.5* promoter-*FF*….-MCE (at max.distance) *Amp*^{R} (Luc.Assay)
MCEtrans	Based on pBluescriptKS from Stratagene, cloned by S.Gawlas in 2008	MCE only without promoter or *FF* *Amp*^{R}(transfection/Luc.Assay)
pBK-RSV-cDes	Backbone from Stratagene, *(Sonja Puz diploma thesis 1999) (Hofner et al.2007).*	RSV promoter-cDNA of *desmin*; *Kan*^{R} (expression/transfection/Luc.Assay)
phRL-TK	Promega	HSV-TK promoter- *RL; Amp*^{R}; (Luc.Assay)
pMG3-GFP-Vim	group of Prof.G.Wiche, backbone pEGFP-C1(Clontech)	Expression of *vimentin-GFP* (CMV promoter) (transfection/Luc.Assay)
pshRNA	SA Biosciences, (hMGFP backbone)	U1 promoter-short hairpin RNA targeting *desmin* mRNA; CMV promoter-*GFP; Amp*^{R}; (transfection/Luc.Assay)

Abbreviations: *FF* (firefly *luciferase* gene), *RL* (renilla reniformis *luciferase*), *Amp*^{R} (ampicillin resistance gene), *LacZ* (*β-Galactosidase* gene for blue-white screening), MCE (minimal cardiac enhancer; *Lien et al. 1999*), RSV (Rous-Sarcoma Virus promoter, relatively strong promoter), HSV-TK (Herpes simplex virus-thymidine kinase promoter, provides low to moderate levels of (renilla luciferase expression), CMV (CytoMegaloVirus promoter, a very strong promoter), *GFP* (gene for green fluorescent protein), *Kan*^{R} (Kanamycin resistance gene)

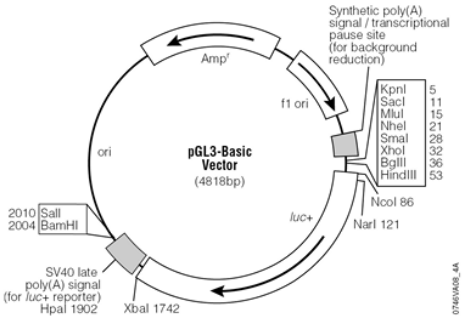


Figure 3.6.1: Shows a map of pGL3b (Promega).

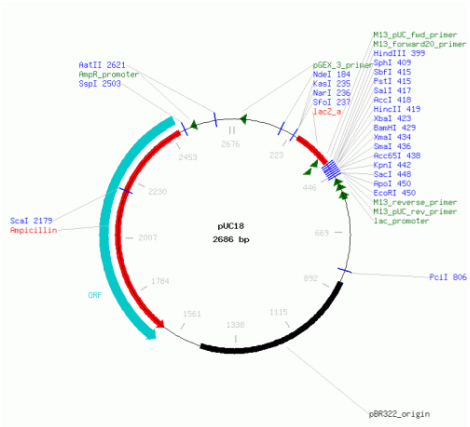


Figure 3.6.2: Shows a map of pUC18.

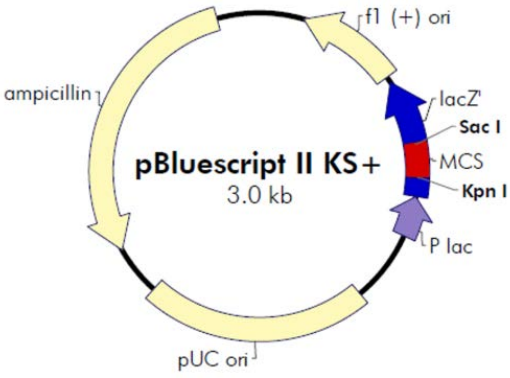


Figure 3.6.3.: Shows a map of pBluescriptKS (Stratagene).

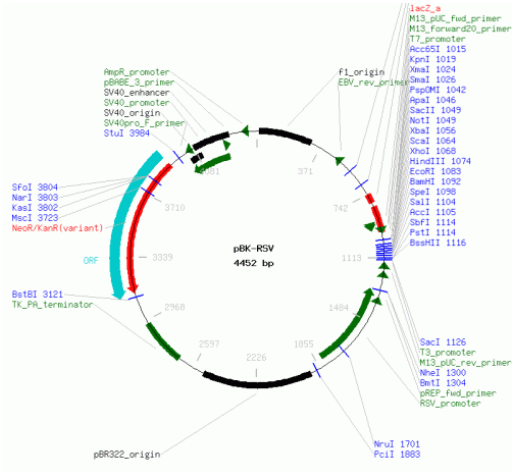


Figure 3.6.4: Shows a map of pBK-RSV (Stratagene).

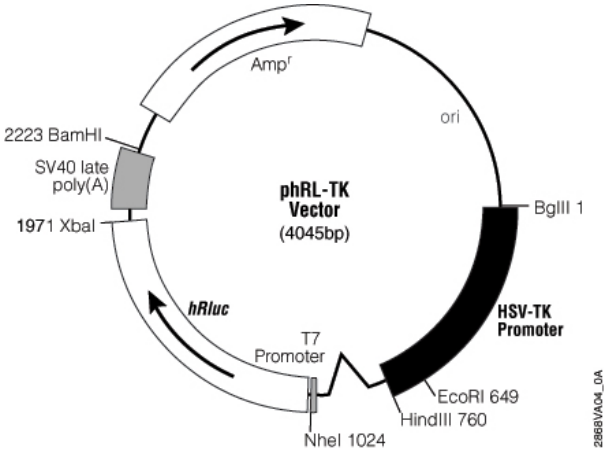


Figure 3.6.5: Shows a map of phRL-TK (Promega).

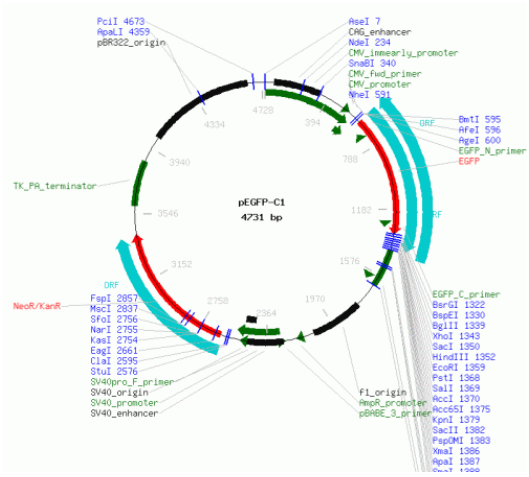


Figure 3.6.6: Shows a map of pEGFP-C1 (Clontech).

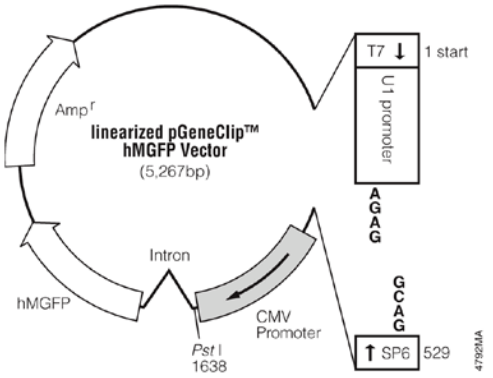


Figure 3.6.7: Shows a map of the pshRNA (SA Biosciences).

3.7 Kits

RNeasy® Mini Kit Qiagen, D
QIAGEN Endotoxin free Maxi Plasmid Prep Qiagen, D
QIAquick Gel extraction Kit Qiagen, D
Wizard® *Plus* SV Minipreps DNA Purification System Promega, USA
PureYield™ Plasmid Midiprep System Promega, USA
Dual Luciferase® Reporter System Promega, USA

3.8 Bacterial strains

E.coli TOP10
E.coli XL-1

3.9 Material for bacterial culture

3.9.1 LB medium

For one liter of LB-medium for bacterial culture, 10g Sodium Chloride, 10g Tryptone, and 5g yeast extract are mixed in a beaker and dissolved in one liter of distilled water. The pH is adjusted to 7 and aliquoted to 500ml glass bottles. Then the LB-medium is sterilized by autoclaving at 120°C at 1,4bar steam pressure for at least 20 minutes.

3.9.2 LB-agar plates

To produce LB-agar plates, 7.5g agarose (1.5%) is added to a 500ml bottle of LB-medium, before autoclaving. One 500ml bottle contains enough volume to produce twenty 10cm plates. After autoclaving, the LB-agar medium is either directly used to pour the plates, or it may be stored at 4°C until use. In case the LB-agar is not directly used, the agarose must be melted in a microwave at 180 watt for 30 minutes.
Thereafter or after autoclaving, the LB-medium must be allowed to cool down to about 50°C before antibiotics may be added (e.g. 0.08g Ampicillin). To ensure proper distribution, gently shake the media. The LB-agar is poured into 10cm plates for bacterial culture under (semi-) sterile conditions. The agar needs about one hour at room temperature to solidify. Thereafter, the plates are stored at 4°C in the cold room. Before use, the plates have to be dried and heated to room temperature under (semi-) sterile conditions.

3.10 Material for cell culture

Glass bottles

Used empty glass bottles are filled with a dash of hypochlorite and tap water. To destroy all potentially contaminating agents, the bottles are left with the hypochlorite for 10 minutes. Thereafter, the bottles are rinsed until no chlorine smell is left.

The bottles are rinsed, filled with milli pore water and are left for 24hrs. On the next day the bottles are dried and autoclaved at 121°C and 1bar steam pressure for at least 20 minutes.

Glass pipettes

After use, the pipettes are stored in quivers, containing hypochlorite and tap water, to prevent them from running dry, while they are still dirty. A dirty pipette, which dried out would be almost impossible to clean, when detergent must not be used.

The glass pipettes are rinsed in tap water in a special quiver for at least two hours. Overnight they are incubated in milli pore water. On next day they are dried at 80°C, plugged. To sterilize the glass pipettes, they are baked at 180°C for 8hrs (dry sterilization). These pipettes and bottles are exclusively used for cell culture. They must not be mixed up with normal detergent treated bottles and pipettes.

In addition to that, single use plastic ware, like 10cm dishes, cell lifters and pipette tips are needed.

3.11 Cell lines

All cell lines are from murine origin.

Embryonic Stem Cells	*Des* $^{+/+}$ (AB2.2)	wild type ESC isolated by Allan Bradley from 129Sv mice *(Soriano et al;1991)*
	Des$^{+/+}$*des*$^{\mathbf{ect}}$ (DC6)	Constitutively overexpressing *desmin* Produced by Sonja Putz in 1999 (diploma thesis)
	Des$^{-/-}$	*desmin* knocked out by Georg Weitzer *(Weitzer et al.;1995)*
	Desmin$^{\mathbf{\Delta 1\text{-}48/\Delta 1\text{-}48}}$ (1B44)	N-terminally truncated version of desmin is ectopically expressed *(Höllrigl et al.;2002)*
primary	Cardiomyocytes	Isolated from newborn mice (BalbC and B6)
Fibroblasts	10T 1/2	Murine embryonic fibroblast line (do not express desmin)
	C2C12	Murine myoblasts (muscle pre-cursor cells), express desmin
	SNL 76/7	Transgenic mouse embryonic fibroblast STO feeder cell line *(McMahon and Bradley;1990)*

4. Methods

4.1 Tissue culture

In general, working should be absolutely sterile. To maintain murine embryonic stem cells (ESC), the glassware (pipettes and bottles) must be completely free of washing detergents and the cells need to be fed every 24 hours. Whenever water is included in a TC protocol, it is sterile (autoclaved > 20minutes at 121°C and 1bar steam pressure) water from a MilliPore Synergy® Ultrapure Water System. The water from this system is deionized (optimal 18.2 MegaOhm) and free of any (bacterial) toxin.

4.1.1 Media and solutions for tissue culture

Water

sterile (autoclaved > 20minutes at 121°C and 1bar steam pressure) water from a MilliPore Synergy® Ultrapure Water System.

10x Phosphate Buffered Saline

NaCl	80 g
KCl	2 g
Na_2HPO_4 . 7 H_2O	10.72 g
KH_2PO_4	2 g

The ingredients are dissolved in 800ml MilliPore water, pH is adjusted to 7.2 using saturated Na_2HPO_4. Afterwards, the solution is filled to 1 liter (MilliPore) and sterile filtered (Nalgene Filter , Nalgene membrane; 0.22µm pore size)

100x Glutamine-Penicilline-Streptomycine

NaCl	4.25 g
Penicilline	1.5 g
Streptomycine	2.5 g
L-(+)-Glutamine	14.6 g

Ingredients are dissolved in 500ml MilliPore water.

Sterile filtered, aliquoted in 50ml Falcon tubes, and stored at -20°C for longer periods. The currently used GPS stock is stored at 4°C after thawing.

β-Mercaptoethanol

1x PBS	200 ml
β -Mercaptoethanol	144 µl

In ESC culture, β-ME is used to disarm free radicals in the nutrition media. Free radicals may be generated during exposure to oxygen, CO_2, or just during the cellular metabolism. Before use, the solution is sterile filtered, aliquoted and stored at -20°C until use.

Trypsin/EDTA

NaCl	3.5 g
D-Glucose	0.5 g
Na_2HPO_4. $7H_2O$	0.09 g
KCl	0.185 g
KH_2PO_4	0.12 g
EDTA	0.2 g
Trypsin (Gibco)	1.25 g
TrisBase	1.5 g

The ingredients are dissolved in 450ml MilliPore water, pH is adjusted to 7.6 (with concentrated HCl), and the solution is filled to a final volume of 500ml. Before use, it is sterile filtered, aliquoted and stored at -20°C.

1% Gelatine Solution

To prepare this 10x stock solution, 10 g gelatine (Difco) are dissolved in 1l MilliPore water and sterile filtrated or autoclaved.

A 0.1 % gelatine solution is used to cover plates with collagen matrix to facilitate attachment of ESC, “feeder cells” or embryoid bodies (EBs).

Dulbeco´s Modified Eagle Medium

To prepare 5 l of DMEM medium, half a can of powdered high glucose DMEM (Gibco) and 18.5 g $NaHCO_3$ are dissolved in 4.5 l milli pore water. After pH-adjustment to pH 7.4, the solution is expanded with MilliPore water to a final volume of 5 liters and is sterile filtered, through a Nalgene filter membrane with 0.22 µm pore size, into cell culture bottles. From each sterile filtered bottle of DMEM a small aliquote is incubated at 37°C over night, to see if sterilization was successful.

Cell culture Media

Name	**Composition**	**Purpose**
M10Gi	10% FCS (Gibco), 1% GPS, DMEM	Fibroblast culture
M15Hi	15% FCS (High Clone), 1% GPS, 1% β-ME, DMEM	ESC culture
M15Si	15% FCS (Sigma), 1% GPS,	EB culture

	1% β-ME, DMEM	
M4Si	4% FCS (Sigma), 1% GPS, DMEM	Cardiomyocyte culture
2x Freezing Medium	20% FCS according to cell type, 20% DMSO, 60% DMEM	Freezing of all cell types

2x Freezing Medium

The freezing medium contains dimethylsulfoxide (DMSO), which is an amphophilic substance that prevents the formation of (big) ice crystals during a freezing procedure. If DMSO is omitted, the ice crystals can grow during the freezing procedure and will harm the membranes and the organelles of the cells. The freezing medium always should be prepared freshly, as DMSO is sensitive to light and oxygen.
Before cells are frozen, freshly prepared 2x FM is added 1:1 to the cells in culture medium; e.g.: 1.2ml 2x FM + 1.2ml cells suspended in the appropiate medium (M10Gi, M15Hi)

4.1.2 Collagen coating of tissue culture plates

The tissue culture (TC) plates, which are used to plate feeder cells, to grow EBs, to grow primary cardiomyocytes must be freshly covered with 0.1% collagen (gelatin). To cover the plates, they are filled with the gelatin solution until the bottom is covered. Then the plates are incubated at room temperature (RT) for two hours. Thereafter, the remaining liquid is sucked off with a Pasteur pipette, which is connected to a vacuum pump.

4.1.3 Culture of fibroblasts

In general for the culture of fibroblasts, TC-plates do not need to be additionally collagen-coated. The fibroblasts usually manage to adhere to TC plates within few hours. As growth medium, M10Gi is used and cells can be split up to 1:8 before they reach confluency. The fibroblast cell lines in culture were 10T ½ (an embryonal mouse fibroblast cell line), C2C12 myoblasts, and SNL 76/7 (fibroblasts which ectopically express Leukemia Inhibitory Factor-LIF).
For long term storage, the cell lines are kept in liquid nitrogen (Cp: 77K / < -196°C). Therefore, the cells have to be thawed. To remove the cell-containing vials from the liquid nitrogen, a protective shield must be worn. Once the cells are out of the liquid nitrogen, the thawing procedure should be done quickly.

Before the cells are thawed, a beaker, which contains hand-warm water, is prepared. In addition, a 1000µl Gilson pipette, a pipette man, a sterile 12ml Falcon tube, a 10cm TC plate and M10Gi will be needed.

The vial, which contains the cells, is thawed in hand warm water. When only a small ice core is left, the vial is rinsed with 70% EtOH, dried and flamed. The cell suspension is transferred to the sterile 12 ml Falcon and 10 ml M10Gi are added slowly and drop-wise to carefully reduce the DMSO concentration. This is necessary to avoid osmotic shock, which would be the consequence when nutrition media is added too suddenly to the cells in DSMO-containing media. The falcon tube, which contains the cell suspension is flamed, tightly closed, and centrifuged at room temperature (RT) and 1000 rpm for 7 minutes. The supernating medium, which contains DMSO, is sucked off and the cells are resuspended in 4ml M10Gi. The cell suspension is then transferred to a 10cm TC plate. To catch all cells, the falcon tube is washed with another 4ml M10Gi. Alternatively the pellet of cells may be resuspended in 8ml M10Gi and cells are then directly plated on a 10cm TC plate. The fibroblasts are incubated at 37°C and 5% CO_2.

To maintain cells in exponential growth phase it is important to split them before complete confluency is reached. To split cells, medium is sucked off the plate, the cells are washed once with ~ 5ml 1xPBS. Then the PBS is removed, the cells are covered with 1ml trypsin /10cm plate. The cells detach during incubation at 37°C for 5-10 minutes. The detachment of the cells is controlled in a light microscope (using fourfold magnification). If the cells are completely detached, trypsin is inhibited by adding of at least 2 parts of serum-containing medium (e.g 2ml medium). The fibroblasts can be split up to 1:8 (e.g. 1ml out of 8ml cell suspension is resuspended in 7ml fresh M10Gi). To achieve an equal distribution of the cells on the plate, it is rocked back and forth, left and right. Rotary movements should be avoided, as this would lead to the concentration of the cells in the middle of the plate. After plating, the incubation of the cells is continued at 37°C and 5% CO_2.

To protect the cells from senescence, they are frozen as soon as they are no more needed in continuous culture. To guarantee that the cells are not harmed during the freezing procedure, they are protected by 20% DMSO in the freezing medium. The DMSO is an amphophilic substance, which has the ability to prevent the formation of big ice crystals. To freeze viable cells, the culture medium of a slightly sub-confluent culture vessel is sucked off. Then, the cells are washed once with 1x PBS. To detach the cells, 0.7 ml trypsin is equally applied onto a confluent cell layer and is incubated at 37°C and 5% CO_2 for 10 minutes. The detached cells are resuspended in 2ml M10Gi and are transferred to a sterile 12 ml Falcon

tube. Analogical to the thawing procedure, it is important to slowly increase DMSO concentration in the cell suspension. If the DMSO -concentration is raised too quickly the cells would shrink. Therefore 2.7 ml 2x Freezing medium are added slowly and drop-wise, while pivoting the tube. Then 1.8ml of this suspension is aliquoted into cryo-tubes. The cryo-tubes have to be flamed, to decrease the risk of contamination, before they are closed tightly. The tightly closed vials are put into a polystyrene box, which is hermetically sealed. The box is put to a -80°C freezer for at least 24hrs (up to several weeks). For long term storage the cells should be kept in liqu.N.

4.1.4 Production of feeder-cells

The “feeders” are needed to maintain ESC in an undifferentiated state.
As feeder cells we use mitotically inhibited SNL76/7 cells, which secrete LIF (leukemia inhibitory factor) into culture medium. The ESC are plated on these feeder cells.

One confluent 10cm plate of SNL76/6 fibroblasts is sufficient for at least two 24-well plates. The culture medium of SNL76/6 cells is sucked off, but 4ml. Then 80µl mitomycine C are added drop-wise and incubated 3-4 hrs at 37°C to achieve complete mitotic arrest. The experimentator should wear gloves, when mitomycin C is handled, because it is very toxic to cells. The mitomycin C disables mitotic division of any cell type. In the meantime, 24-well plates are covered with collagen for two hours. After four hours the medium of the SNL fibroblasts, which contains the mitomycin C, is sucked off. Then cells are washed twice with 1x PBS and 1 ml trypsin is added to achieve detachment of the cells. The plate is then, as usual, incubated at 37°C for 10 minutes. The mitotically inhibited fibroblasts are resuspended in ~ 5ml M10Gi and transferred to a 50ml falcon tube. To be sure of having transferred all cells, the original cell culture plate is washed with an additional volume of 5ml media and the suspension is added to the cells in the falcon tube. As even traces of mitomycine C would be harmful to the ESC, the toxin has to be removed completely. Therefore the suspension of mitotically arrested SNL (“feeders”) is centrifuged at 1000 rpm for 7 minutes, and the supernatant (SNT) is sucked off. The pellet of feeder cells is resuspended in 20ml M10Gi and the cells are counted in Thoma-chamber (number of cells in all squares x 10.000 = number/ml).

The concentration of the cells in the suspension is adjusted/ or rather diluted to 1.5-1.75 x 10^5 cells per ml. Thereafter the cell suspension is plated onto the collagen covered TC-plates: for a 6-well plate 2ml cell suspension are needed; for a 24-well plate: 1ml; for a 48-well plate: 0.5 ml; for a 96-well plate 0.2 ml. Before the ESC are plated on top, the feeders

must be incubated at 37°C over night, to ensure attachment to the plate. The feeders can be kept for two weeks, but they should be fed at least once a week.

4.1.5 Maintenance of embryonic stem cells (ESC)

To maintain ESC in an undifferented state, they need to be grown on a layer of "feeder cells". In addition to that, the ESC must be fed every 24hrs (1.5 ml M15 Hi) for two reasons. First, they have a high turnover rate and need a lot of nutrients. Second, it is necessary to exchange the nutrition medium every 24hrs, to avoid accumulation of signaling factors from neighboring cells. Likewise important is that ESC must be split as soon as they reach confluency, otherwise they would begin to differentiate.

The ESC are kept in liquid nitrogen until they are needed. To thaw the ESC, one follows the general procedure as is was described above for the fibroblast. The only difference is, that the "feeder-wells" must be pre-fed with ~1ml M15 Hi two hours before the ESC are thawed. The pre-feeding is critical to maintain the ESC in an undifferentiated state, because the nutrition medium will be enriched with LIF in the meantime. The thawing procedure itself is the same as with fibroblasts: The vial, which contains the ESC, is thawed in hand-warm water until only a small ice core is left. Then the ESC suspension, which contains DMSO, is transferred to a sterile 12ml falcon tube. To reduce the DMSO-concentration, 10ml M15Hi are added slowly and drop-wise. Then the ESC suspension is centrifuged at 1000 rpm for 7 minutes. After the centrifugation, the cells are resuspended in 1ml M15Hi taken from the pre-fed feeder well. To avoid pre-mature differentiation, the ESC are carefully resuspended in the LIF-enriched medium. Equally important to the presence of LIF, is that ESC are seeded as single cells, because any kind of aggregation would lead to the onset of a differentiation process. The ESC are finally seeded into a 24-well for normal maintenance of the cells. The plates are incubated as usual at 37°C and 5% CO_2.

To keep the ESC in an undifferentiated state and in the exponential growth phase, it is absolutely necessary to split them, before they reach complete confluency. When the ESC have to be split, the ESC and the feeder wells, on which ESC will be plated after splitting, must be pre-fed two hours before (1.5ml M15Hi/24-well). This ensures that the medium is enriched with LIF. After the two hours of pre-feeding, the old medium is sucked off the ESC, then the ESC are washed **TWICE** with 1xPBS. To detach and separate the cells from each other, they are treated with 200µl trypsin and are incubated at 37°C and 5 % CO_2 for 20 minutes. The detachment is controlled in a light microscope. When the cells are detached, 1ml M15Hi from a pre-fed feeder well is used to inactivate the trypsin. To plate the ESC as single

cells they have to be resuspended thoroughly and to be split 1:3 for maintenance (e.g. transfer 400µl per 24-well).

To ensure the transfer of all cells, the old well can be washed with another 1ml fresh M15Hi and is distributed to the new well(s) in equal amounts. Thorough resuspension is crucial to prevent ESC from differentiation. The ESC only may be kept in an undifferentiated state if they are seeded as single cells. For full attachment of ESC on top of the "feeder layer" it is necessary to incubate the plate at least over night at 37°C and 5% CO_2.

4.1.6 Generation of embryoid bodies

The embryoid body (EB) model of *in-vitro* ESC differentiation enables the generation of all cell types, which are obtained from the three germ layers.

The day before the generation of the EBs, the ESCs should be split 1:2, to obtain them in optimal condition. Prepare autoclaved Millipore water and an automatic pipette.
On day 0, the ESCs are pre-fed two to four hours with M15Si. Later, to get rid of the feeder cells, they will be pre-adsorbed to a collagen-coated 6-well. Therefore, a 6-well plate is coated with collagen and left for two hours. After 2-4 hours, the medium is sucked off the pre-fed cells. Then the cells are washed twice with 1x PBS. To detach ESCs from each other and the feeders, they are incubated with 200µl trypsin per 24-well at 37°C for 20 minutes. In the meantime the collagen is sucked off the plate for pre-adsorption. The detached cells are then resuspended in 800µl M15Si. The feeders are pre-absorbed on the coated 6-well plate. To ensure that all cells are transferred it is recommended to wash after with 1ml M15Si. The feeder cells are pre-adsorbed for 1hr on the collagen-coated 6-well. The attachment of the fibroblasts is monitored in a light microscope. The pre-adsorption is fine when huge fibroblasts stick to the bottom of the plate and the smaller and round ESC are still in suspension. The cell suspension is transferred to a sterile 50ml Falcon. Before the cells are counted in a Thoma-chamber, the cell suspension is diluted in M15Si to a volume of 20ml. The cell density is adjusted to $4x10^4$ (±2000) cells per ml. To calculate the amount of the cell suspension, which is necessary for the experiment, we assume that about 100 EBs fit to one 10cm plate. Each EB is made up by a drop of 20µl of the [$4x10^4$ cells/ml] suspension. Therefore, about 2ml ESC suspension will be needed per plate. To prevent the EBs from drying out, during the first 4 days of incubation as hanging drops, they must be provided with a humid environment. The droplets of 20µl cell suspension are set in the lid of petri-dishes, which are filled with sterile, autoclaved, Millipore water. An automatic pipette is used to drop about 100 times 20 µl of the cell suspension into lid of the petri-dishes. Each drop contains about 800 cells. The formation of EBs is happening during a continuous incubation at 37°C

and 5% CO_2 for 4.5 days. This allows for the attachment of the ESC to each other in a snow-ball like manner. In the meantime, some of the ESCs develop to cells of the primitive endoderm, the visceral and the parietal endoderm. At day 4.5 the EBs are gently rinsed to collagen-covered TC-plates using at least 6.5 ml M15Si. Then, to enable the attachment of the EBs to the bottom of the TC-plates, the plates are incubated for at least 24 hrs.

Feeding protocol for EBs

The nutrition medium is not changed completely, because it is enriched ("conditioned") with EB-derived factors, which influence the further differentiation.

Day 0-4.5	800 ESC in 20µl
Day 4.5	Rinse with ~6.5 ml M15Si
Days 7 and 10	3ml conditioned medium+8ml fresh M15Si
Days 13,16,19, and 22	4ml cond.med.+ 10 ml fresh M15Si
Days 25, 28 and 31	5 ml cond.med. + 12 ml fresh M15Si

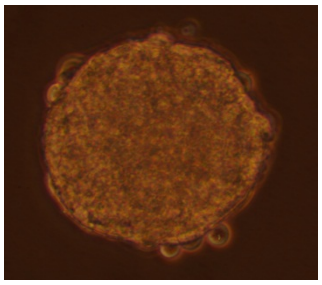


***Fig.4.1.6A**: day 4.5 WT EB (20x)*

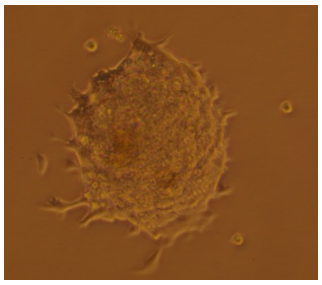


***Fig.4.1.6B**: day 5 WT EB (10x)*

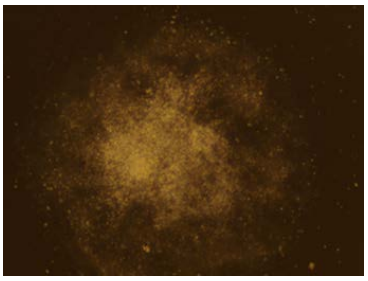


***Fig.4.1.6C**: day 7 WT EB (4x)*

Note the bubbles of visceral endoderm; the cells at the borders of EBs belong to parietal endoderm, which is essential for a process that mimics implantation.

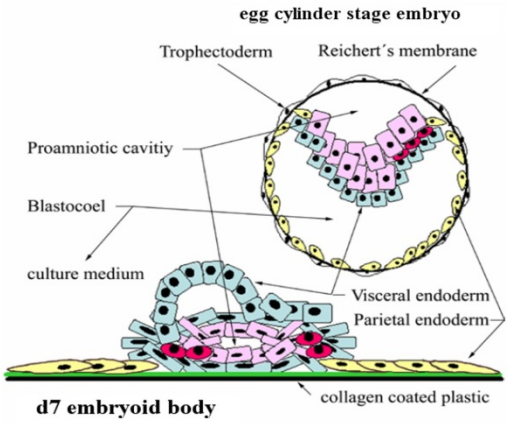


***Fig.4.1.6D**: Comparison of an early egg cylinder stage embryo with an embryoid body. Note the lack of a real trophectoderm in the embryoid body model. (cross sections)*

4.1.7 Isolation of primary cardiomyocytes from neonatal mice

The hearts of neonatal mice (BalbC and Black6) are prepared and placed in 1x PBS. Then the hearts are cut into very small pieces with sterile scissors. To clean the tissue, the pieces are spun at 1000rpm for two minutes. The supernatant is removed and 1ml of a

pancreatin/collagenase mixture is added to digest the connective tissue. The digestion is incubated shaking at 37°C for 15 minutes. Thereafter, the cells are spun at 1000rpm for two minutes, the supernatant is removed, and another 1ml of fresh Pancreatin/Collagenase mixture is added. This second digestion at 37°C shaking is continued until a homogenous appearance is achieved (5-10 minutes). To get rid of the cell debris, the cells are spun at 1000rpm for two minutes. The supernatant is removed and the cardiac cells are resuspended in M4Si culture medium.

To get rid of the cardiac fibroblasts, the cell suspension is pre-adsorbed on collagen coated tissue culture plates for two to three hours. Finally the supernatant, which contains the enriched cardiomyocyte population, is plated onto new collagen coated tissue culture plates. The cardiomyocytes are cultured at 37°C and 5% CO_2. The culture medium has to be renewed after 24 hours to remove the remaining cell debris. The media has to be changed every three to four days.

Pancreatin/Collagenase enzyme mix

0.4 ml Pancreatin (Gibco)
5 mg Collagenase (Worthington)
9.6 ml 1x PBS

4.1.8 Transient transfection of eukaryotic cells

4.1.8a Calcium-phosphate Transfection

Material: Exponentially growing eukaryotic cells (e.g.10 T ½ fibroblasts)
Complete medium (M10Gi)
Purified Plasmid DNA (10 µg per 6 well)
2,5 M $CaCl_2$ (30µl)
2x HEPES-buffered saline
1x PBS (Phosphate-buffered saline)

2,5M $CaCl_2$: 183.7 g $CaCl_2 \cdot 2H_2O$ is dissolved in 500ml Millipore water, is sterile filtered through a nitrocellulose filter, and is stored at -20°C in 10ml aliquots.

2x HEPES-buffered saline(HeBS) solution :
16.4g NaCl [0.28M final]
11.9g HEPES (**2-4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfonic acid)**
0.21g Na_2HPO_4 [1.5mM final]

The ingredients are dissolved in 800ml Millipore water. The pH is adjusted to 7.05 (- 7.12 max.) with 5N NaOH. The solution is sterilized by filtration. The precipitation potential is tested by mixing 0.5 ml 2x HeBS and 0.5 ml $CaCl_2$. The two solutions are combined and vortexted. A fine precipitate should form. The precipitate appears cloudy and white in an eppendorf tube or opalescent under the light microscope. If the precipitation failed, the pH has

to be checked **(optimal range pH 7.05-7.12)**. The pH is absolutely critical for the transfection efficiency and the viability of the cells.

At the day of the transfection, the optimal cell density for a 6 well is $5x10^5$. Therefore, exponentially growing cells (e.g.10T ½) are plated to 6 wells in a density of approximately $2.5x10^5$ on the day before transfection. In the next morning, the cells are pre-fed with 2ml M10Gi. Then, the transfection reagents are heated to room temperature to guarantee for the proper formation of precipitate. For the transfection of each 6 well, 10 µg of transfection grade plasmid DNA is mixed in sterile Millipore water to a final volume of 256.5µl. To keep the amount of transfected DNA steady in all the different experiments, it is necessary to divide the total DNA concentration of 10µg, if there is more than one plasmid to be transfected. Then, 13.5µl 2M $CaCl_2$ is added to reach a total volume of 270µl. In parallel, the same volume of 2xHeBS (270µl) per 6 well is aliquoted to sterile 12ml falcon tubes or 2 ml Eppendorf tubes. The mixture of plasmid DNA and $CaCl_2$ is resuspended and added dropwise to one HeBS-aliquote, while it is vortexed simultaneously. The simultaneous vortexing prevents formation of complexes that would be too large for the uptake by the cells. The vortexing therefore increases the efficiency of the transfection. The complete transfection mix is allowed to sit at room temperature for at least 30 minutes. Thereafter, the transfection solutions are vortexed again and are added to the cells slowly and drop-wise. To evenly distribute precipitate all over the cell layer, the medium is carefully resuspended. The plates are incubated at 37°C and 5% CO_2 over night.

An additional step of a DMSO-shock may be useful to enhance the efficiency of the transfection, but this also may kill sensitive cells. The DSMO-shock is performed after the incubation of the cells with the transfection reagents (this could be from 2hrs after the transfection to the next morning). Therefore, 10% DMSO in PBS is prepared freshly (2ml per 6well). The transfection medium is removed from cells the shock solution (10% DMSO in PBS) is added to the cells and incubated at RT for up to 2.5 minutes. The DMSO is removed, the cells are washed twice with 1x PBS and are fed with regular growth medium (M10Gi in case of fibroblasts).

Due to the great variations in the transfection efficiency that arise from the Calcium-Phosphate method, an alternative transfection reagent was also used.

4.1.8b Lipofectamine 2000 (Invitrogen) Transfection

When Lipofectamine 2000 is used as a transfection reagent, one has to follow the principle of keeping the DNA amount constant in all the different experiments. Actually, this is important independently from the method and the transfection reagent.

In “lipofection”, less DNA is needed, compared to the calcium phosphate method of transfection. Therefore, only 4µg DNA per 6 well are diluted in DMEM without serum or antibiotics to a final volume of 250µl. Then, 12µl of lipofectamine 2000 are diluted in DMEM without serum or antibiotics to a total volume of 250µl. Then the diluted DNA and the lipofectamine in DMEM are combined. This mixture is incubated at room temperature for 30 minutes. After 30 minutes, the lipofection mix is added to the pre-fed cells. Then the lipofected cells are incubated at 37°C and 5% CO_2 for four hours. Thereafter, the cells are washed with 1xPBS and fed with regular growth medium.

4.1.9 Immunofluorescence staining

The immunofluorescence staining is a method to localize proteins or any other antigen. It relies on proper fixation of the cells to retain cellular distribution of the antigen and to preserve the morphology of the cell. After fixation, the cells are exposed to a primary antibody, which is directed against the protein of interest. To ensure for the accessibility of the epitopes of the antigen, the cells are also exposed to permeabilizing reagents. The primary antibody is then bound by a species specific secondary antibody, which binds its Fc-portion. The secondary antibody is conjugated to a fluorophore, which is excited by a certain wavelength of laser light. This enables the visualization of defined structures within a cell. First, the cells have to be fixed. The choice of the fixation reagent depends on the cell type and the antibodies, which will be used. One fixation reagent is ice-cold (-20°C) 96% ethanol. To fix cells with 96% EtOH, media is sucked off the culture plate and the cells are washed twice with ice-cold 1x PBS. The ice-cold PBS should not be directly applied onto the cells, but rather led down the wall of the culture plate. After the washing, the cells are fixed with an appropriate volume (2ml for a 6 well) of ice-cold 96% EtOH. The EtOH must be added slowly, because alcohol leads to de-hydration of cellular structures. Then the fixed cells are incubated at –20°C for 20 minutes. Thereafter, the cells are slowly re-hydrated by adding sterile distilled water to the 96% EtOH to receive 70% EtOH (800µl for a 6 well). If the water is not added slowly, the cells would burst due to osmotic forces. Then the culture plate is sealed with parafilm and is incubated at –20°C for 10 minutes. Afterwards, the culture plate with the fixed cells and the 70% EtOH may be kept at 4°C for a few weeks if it is sealed tightly. The fixation with EtOH is said to be potentially harmful to fine cellular structures, because the alcohol dehydrates the cells. On one hand, this dehydration may destroy the epitope, which should be bound by the specific antibody. On the other hand, EtOH fixation at the same time serves for permebilization of the cell, and therefore might increase the

accessibility of the epitope. In general, when the fixation reagent is chosen, one should follow the advice of the manufacturer of the antibody.
Another fixation reagent is 2% formaldehyde in PBS. To fix cells with 2% formaldehyde in PBS, the medium is aspirated and replaced by formaldehyde fixative (2ml per 6 well). The fixative is incubated at room temperature for 10 minutes. Then the cells are washed twice with 1xPBs. The culture plate is sealed with parafilm and may be stored at 4°C over night. Here to permeabilize the cells, a solution of 0.1% saponin in 1xPBS is used (140µl 10% saponin in 14ml 1xPBS). Saponin is used as a mild permeabilizing reagent. The saponin solution is incubated shaking at room temperature for 20 minutes. Thereafter, the cells are washed 1x TBS/ 0.1% Triton x-100. If EtOH fixation was used, the saponin permeabilization and the TBS washing steps are not compulsory. To block unspecific binding, the cells are incubated at room temperature with 1xPBS and 10% fetal calf serum (FCS) for 20 minutes. In the meantime, the primary antibodies are diluted according to the manufacturer's protocol usually in 1xPBS (here in PBS/ 0.1% saponin/10% FCS; e.g. antiDesmin 1:40, antiHistone H3 1:500). To reduce background staining, it is recommended to centrifuge the mixtures at full speed in a table top centrifuge for five minutes, to bring down aggregates of antibody. Then a 60µl drop of this antibody containing solution is placed in the lid of the culture plate. With forceps the coverslips are lifted from the plate and excess liquid is blotted on a Kim-wipe, by touching it with the edge of the cover slip. The cover slips are inverted and laid onto the drop with the cells facing the antibody solution. The culture plate is emptied and rinsed with tap water and distilled water, and then turned into a humid chamber, by filling the wells with humid paper towels (if available, a humid chamber is used). Then the coverslips are incubated with the primary antibody at room temperature in the humid chamber for about 1.5 hours. Before the incubation with the secondary antibodies, the coverslips are inverted again and placed back to their original wells, where they are washed three times with a 0.1% Triton x solution in 1x TBS. Then the species-specific secondary antibodies are diluted (e.g. 1:200; usually 1:100- 1:500 in 0.1% saponin/10% FCS in PBS) and centrifuged at full speed at room temperature for 5 minutes to pellet aggregated antibodies. The secondary antibodies are incubated at room temperature in the dark in a humid chamber for about one hour. To counter-stain the nuclei, a DAPI staining is included. Therefore, DAPI (Hoechst) is diluted between 1:2500 and 1:5000 in 1x PBS. After the incubation with the secondary antibody, the coverslips are washed three times with PBS (EtOH fixed) or twice with 0.1% Triton x in TBS and a third time in regular PBS for 10 minutes each. To the EtOH fixed cover-slips the DAPI counter-stain may be included in the first washing step with PBS. To the formaldehyde fixed

cover slips, the DAPI is added to the third washing step with PBS. The coverslips are incubated with DAPI at room temperature in the dark for five minutes. Then the samples are washed three times with 1x PBS at room temperature for 5 minutes. Finally the stained cover slips are fixed to microscope slides. Therefore, the mounting medium Mowiol is pre-heated to 65°C and 40µl are laid to a clean microscope slide. The coverslips are laid on the slides, with the cell-side facing the mouting medium. The Mowiol is dried at room temperature over night. On the next day the edges of the cover slips are sealed with nail polish.

10x Tris Buffered Saline (TBS)

500 mM Tris. HCl (pH 7.4)
1500 mM NaCl

10% Saponin in 1x PBS

dissolve 1g Saponin in 10ml PBS, aliquote to Eppies (500µl) and store at -20°C.

2% Formaldehyde in PBS

2ml 37% Formaldehyde are diluted in 35ml 1x PBS.

100 ml 0.1% Triton X in TBS

100 µl Trition X-100 in 100ml 1x TBS

Mowiol 2-88

6 g Glycerol
2.6 g Mowiol 2-88 (Hoechst)
5 % DABCO
6 ml dH_2O

The ingredients are mixed and are stired for two hours until all components are dissolved. Then 12 ml 0.2 M Tris/HCl pH 8.5 are added. The solution is heated to 50°C for 10 minutes. Then the solution is centrifuged at room temperature and 5000 rpm for 15 minutes. The solution is aliquoted and stored at -20°C.

4.1.10 The use of a Zeiss confocal laser scanning microscope

The system is turned on at a main switch. The LSM 510 meta program is started in expert mode. The lasers, which are needed to excite the fluorophores, are turned on. To excite DAPI staining a wavelength of 405nm (UV) is needed. To excite FITC labelled samples, an argon 2 laser is needed. This laser is most sensitive and has to be set on standby to pre-heat, before it is turned on. To excite Texas Red stained samples, a wavelength of 561nm is needed. Thereafter, the microscope has to be configured for its scanning method. There is the option to choose single or multitracking. The use of the multitracking mode is of advantage, because

it scans the signals from the different channels one after the other. This minimizes the chance of detecting wrong signals from “bleeding” of other fluorophores. Then the tracks have to be defined individually. The exciting laser is chosen, then HFT and mirrors are chosen. Finally the filters have to be chosen: A “long pass” filter allows wavelengths greater than 420nm to pass, whereas all wavelengths shorter than this are deflected. A “band pass” filter allows a defined wavelength to pass and deflects others. Therefore, it is important to know, which wavelength is emitted by the used fluorophore. An example for the set up to detect FITC would be to set the HFT to 488nm, a BP filter to 505-550. A setting for TR detection: laser 561nm on, detector 530nm. To adjust the channels, the size of the pinhole is set to 1, which is the setting to see a confocal picture. It is not recommended to change the detector gain or the amplifier gain, because both work by photo-multipliers, which would also increase all background noise. It is better to start with the detector set to 750, then if necessary to increase the laser power.

To turn off the laser microscope, the 561nm laser must be turned off in the “makro”, because there is a software bug. If the next microscope user is coming not before one hour, the argon laser is switched to the standby mode. If the system may be shut down, all lasers are turned off in the laser menu. Now, the argon laser shows “cooling”. It is most important to wait until it says “connected” and not to turn off the system before the laser is cooled. After that, the LSM-meta program may be closed (exit), the computer may be shut down and the main switch is turned off.

4.2 Molecular biology methods

4.2.1 mRNA isolation (Quiagen RNeasy®)

The mRNA is isolated from eukaryotic cells, which are washed twice with 1x PBS. Then the cells are overlaid with 1xPBS and scraped off the culture surface by either using a cell scraper or a gilson pipette tip. The loosened cells are resuspended and transferred to 1,5ml eppies. The suspension is centrifuged at full speed and 4°C for 5 minutes. Thereafter, the supernatant is sucked off, and the pellets are resuspended in 600µl buffer RLT and 6µl β-mercaptoethanol. Then, the suspension is transferred to the QIA-shredder column and is centrifuged at full speed and RT for 2 minutes. The flow-through is collected and mixed with the same volume of 70% RNase-free ethanol. This suspension is put on an RNA-binding column in two steps, each 600µl. The column is centrifuged at full speed and RT for 15 seconds and the flow-through is discarded. The column, which has bound the RNA, is washed twice with buffer RW1. The flow-through is discarded. Then, the column is washed twice

with 500µl of buffer RPE and centrifuged at full speed and RT for 15 seconds. After the second wash the column with bound RNA is centrifuged again at full speed and RT for 15 seconds to remove all residual washing liquid. Finally, the column is added to a new eppi, and the RNA is eluted with 40µl RNase-free water by centrifugation at full speed and RT for one minute. To remove possible DNA-contamination, 3,75µl DNase I buffer and 3,75µl DNase I (Fermentas) is added and incubated at RT (or 37°C) for 30 minutes. To inactivate the DNase, the RNA suspension is incubated with 3,75µl EDTA at 65°C for 10 minutes. Thereafter, the RNA is stored at -80°C until reverse transcription. Before reverse transcription, the RNA has to be checked for contamination with gDNA. Therefore, a PCR with primers, which are specific for a eukaryotic housekeeping gene(e.g.: GAPDH; glyceraldehyde-3-phosphate dehydrogenase) is performed. The sequences of primers are listed in chapter 3.5.
The following table shows the conditions for a GAPDH PCR:

Temperature	Time	Cycles
94°C	1 minute	
94°C	45 seconds	30 (29 repetitions)
55°C	45 seconds	30 (29 repetitions)
72°C	1 minute	30 (29 repetitions)
72°C	4 minutes	
4°C	Infinitely	

The results of the GAPDH-PCR is analysed on a 1,5% agarose gel. A negative control and a positive control should be included, to be sure that there is no gDNA contamination in the RNA samples. If no gDNA contamination was found, the mRNA is reverse transcribed into cDNA.

4.2.2 Reverse transcription of mRNA

The master mix for each reverse transcription reaction is set up as the following:

10 µl	5x RT buffer
5 µl	0.1 M DTT
1.5µl	RNA out
2 µl	10mM dNTPs

1µl of oligo dTTT-primer is added to each mRNA-solution and is incubated at 70°C for 10 minutes. Thereafter the mixture is cooled on ice for 3 minutes. After the cooling the mRNA mixture is centrifuged at full speed and RT for 15 seconds to collect condensed liquids. Then, 18,5µl of the RT-PCR mix is added to each mRNA solution. The RT-mixes are incubated at 42°C for 2 minutes then 1µl of reverse transcriptase enzyme is added. To reverse transcribe the mRNA, the mixes are incubated at 42°C for 50 minutes, followed by incubation at 70°C for 15 minutes. The reverse transcribed mRNA, which is now cDNA, is cooled on ice for 5

minutes and finally centrifuged at full speed and RT for 2 minutes. The cDNA is stored at –80°C until use, or –20°C. For semi-quantitative RT-PCR it is necessary to balance the levels of total cDNA. Therefore, a PCR is set up with primers, which are specific for a house keeping gene, like GAPDH. The result of this PCR is used to balance the concentration of the template cDNAs.

4.2.3 Polymerase chain reaction

The PCR is a standard laboratory method to selectively amplify target DNA. It is rapid and versatile, but one of the limitations of a PCR is that some information about the target sequence needs to be known. This information is needed to design the primers or amplimers. The primers are specific for the target sequence and usually consist of 15 to 25 nucleotides. They serve as the priming unit, which enables the DNA polymerase in presence of deoxyribonucleotide triphosphate units (dNTPs) to elongate the template DNA. The template DNA is melted at 94°C, so that the separated DNA strands are available for the binding of the complementary primers (annealing). Thereafter, the PCR reaction mix is heated to 72°C, which is the working temperature of the commonly used DNA polymerase of *Thermus aquaticus* a thermophile bacterium. The Taq-DNA polymerase then adds the dNTPs according to the sequence on the complementary DNA strand, the target DNA is extended and amplified. After 15 to 30 rounds of heating and cooling of the PCR mix, the reaction is stopped by cooling to 4°C, the target DNA was selectively amplified. The product of a PCR is usually analysed in an agarose gel electrophoresis.
The following table shows the conditions of a typical PCR:

Temperature	Time	Cycles
94°C (DNA melting)	1 minute	
94°C (melting)	45 seconds	14 to 29 repetitions
55°C (primer annealing)	45 seconds	14 to 29 repetitions
72°C (extension by pol)	1 minute	14 to 29 repetitions
72°C (final extension)	4 minutes	
4°C (cooling)	Infinitely	

4.2.4 Analysis of PCR products in agarose gel electrophoresis

To prepare an analytic 1% agarose gel (7cm x 12cm) for electrophoresis, 1g agarose (electrophoresis grade) is heated in 100ml 1xTBE buffer in a microwave oven at full power for about two minutes.
For a preparative gel it is better to use 1xTAE buffer.

Table 4.2.4: shows the range of efficient DNA separation on agarose gel.

Agarose % [w/v]	Range of efficient separation of linear DNA
0.3%	5- 60 kb
0.6%	1- 20 kb
0.7%	0.8- 10 kb
0.9%	0.5- 7 kb
1.2%	0.4- 6 kb
1.5%	0.2- 3 kb
2%	0.1- 2 kb

4.2.5 DNA isolation

To isolate gDNA from (embryonic stem) cells, the cells are washed twice with room temperature 1xPBS. Then the cells are detached from the culture plate by applying 200µl of trypsin (EDTA) at 37°C for 20 minutes. In the meantime, the lysis buffer is prepared as the following: 10mM Tris pH 7,4 ; 10mM EDTA ; 300mM NaCl ; 0.4% SDS ; 200ng /ml proteinase K. The lysis buffer is added in 10 times more volume than the volume of the trypsin, directly to the trypsin treated cells. The cells are briefly resuspended in lysis buffer and then transferred to a 2ml eppi. The lysis proceeds at 55°C over night. On the next day the lysate is cleared from protein by phenol-chloroform-isopropanol extraction. Therefore, 500µl PCI is added and the suspension is vortexed to mix. Then the suspension is centrifuged in a table top centrifuge at full speed and 4°C for 10 minutes. The supernatant is transferred to a new Eppendorf tube and PCI extraction is repeated. After the second transfer of the supernatant (the aqueous phase), it is divided into two and precipitated with room temperature isopropanol. To achieve successful precipitation it is necessary to use at least twice the volume of the supernatant. The precipitate is spun down in a centrifuge at full speed at 4°C for 30 minutes. The supernatant is carefully discarded and the DNA pellet is washed with 70% ethanol. After the centrifugation, the supernatant again is carefully discarded and the pellet is allowed to air-dry. The DNA pellet is allowed to dissolve in 100µl TE buffer over night at 4°C.

4.2.6 Chromatin Immuno Precipitation

The ChIP is a technique to confirm physical interaction of a protein of interest (POI) and a genomic DNA (gDNA) region of interest (ROI). It is based on cross-linking of residual proteins to gDNA, and purification in the presence of antibodies, which are specific for the POI. The analysis of the ChIP results is achieved by PCRs with primers, which are specific for the region of interest.

ChIP Stocks Solutions

Chemicals	Amount	pH adjustment	Solvent	Volume
1M Tris pH 8,1	12.1 g	HCl	ddH2O	100ml
1M Tris pH 6,5	12.1 g	HCl	ddH2O	100ml
1M Tris pH 7,5	12.1 g	HCl	ddH20	100ml
1M HEPES pH 7.9	23.8 g	2M NaOH	ddH2O	100ml
0.5M EGTA pH 7.5	19.0 g	2M NaOH	ddH2O	100ml
0.5M EDTA pH 7.5	18.6 g	2M NaOH	ddH2O	100ml
3M NaOAc pH 5.2	24.6 g	HCl	ddH2O	100ml
10% TritonX-100	20 ml		ddH2O	200ml
10% NP-40	20 ml		ddH2O	200ml
10% SDS	20 ml		ddH20	200ml
1M LiCl	16.96 g		ddH2O	400ml
1M Tris pH 8	48.4 g		ddH2O	400ml
4M NaCl	23.36 g		ddH2O	400ml
10% NaDoc	10 ml		ddH2O	100ml

ChIP Buffers

Wash-Buffer I *(400ml)*	***Lysis Buffer*** *(desired vol.)*
10ml 10% Triton X-100	10% SDS
8ml 0.5M EDTA pH 7.5	10mM EDTA pH 7.5
400µl 0.5M EGTA pH 7.5	50mM Tris pH 8
4ml 1M HEPES pH 7.9	Proteinase Inhibitor (Roche, Complete) 1 tablet dissolved in sterile Millipore water = 50x stock
ddH20 ad **400ml**	0.1mM **P**henyl**M**ethan**S**ulfonyl**F**lourid (MW 174.19g/mol ; solved in Isopropanol; 10µl from 1000x stock)
Wash-Buffer II *(400ml)*	***Hi-Salt Buffer*** *(400ml)*
20ml 4M NaCl	50ml 4M NaCl
800µl 0.5M EDTA pH 7.5	20ml 1M Tris pH 8
400ml 0.5M EGTA pH 7.5	4ml 10% SDS
4ml 1 M HEPES pH 7.9	40ml 10% NP-40
ddH2O ad 400ml	ddH_2O ad 400ml
Dilution Buffer *(400ml)*	***LiCl Buffer*** *(400ml)*
400µl 10% SDS	100ml 1M LiCl
44ml 10% Triton X-100	20ml 1M Tris pH 8
960µl 0.5M EDTA pH 7.5	20ml 10% NaDoc
6.68ml 1M Tris pH 8.1	40ml 10% NP-40
16.7ml 4M NaCl	ddH2O ad 400ml
ddH2O ad 400ml	***Elution Buffer*** *(5ml)*
RIPA Buffer *(400ml)*	1ml 10% SDS
15ml 4M NaCl	0.5ml 1M NaHCO3
20ml 1M Tris pH 8.1	50µl 1M DTT
4ml 10% SDS	***TE Buffer*** *(400ml)*
20ml 10% NaDoc	4ml 1M Tris pH 8
40ml 10% NP-40	800µl 0.5M EDTA
ddH2O ad 400ml	ddH2O ad 400ml

Preparation of Protein-A-coupled Sepharose beads

Before use, the protein-A sepharose beads have to be prepared, washed and blocked. About 0.1g protein-A sepharose beads, which equals to three times the tip of a small spatula, is put into a 2ml eppi. Then 1ml TE buffer is added and the beads are allowed to soak at room temperature for one hour. Then the beads are centrifuged in a table top centrifuge at 1000 rpm and at room temperature for 3 minutes. The supernatant is taken off cautiously and the beads are washed three times with 1ml TE buffer in the same way as described. To avoid unspecific binding of DNA or protein to the beads, they have to be blocked. For 100µl beads, a blocking solution is prepared as the following: combine 2µl herring or salmon sperm [10mg/ml TE], with 10µl BSA [10mg/ml], with 5µl of a 2% sodium azide solution, and with 84µl TE buffer. The protein-A sepharose beads are overlaid with one volume of blocking solution and blocked shaking at 4°C for two hours. Thereafter, the beads are washed and overlaid with the same volume of TE buffer.

ChIP procedure

Day 1

To crosslink residual protein to chromatin, 54µl 37% formaldehyde per 2ml culture medium (270µl/10ml) are added into culture wells and incubated in a fume hood at RT for 20 minutes. To sequester the formaldehyde, 100µl 2.5 M glycine is added and incubated shaking at RT for 5 minutes. The culture medium containing formaldehyde and glycine is aspirated and the cell layers are washed twice with ice-cold 1xPBS. The cells are harvested in 2ml 1xPBS. Therefore, a 1000µl gilson pipette is used to scratch and to resuspend the fixed, harvested cells in PBS. The yield is transfered to a sterile 12 ml falcon tube and is centrifuged 1200rpm at 4°C in a table top centrifuge for 5 minutes. To avoid loss of POI through degradation, 200µl of a solution of 50x Roche Complete Protease Inhibitor (PI) in sterile Millipore water and 10µl of a 1000x PMSF in isopropanol solution are added to 10ml WASH I buffer in the mean time. The supernatant (SN) is sucked off, the pelleted cells are washed with WASH I buffer by resuspending them in 1ml Wash buffer I and adding of another 4ml. Then the cells are incubated on ice for 10 minutes. The same procedure is repeated with WASH II buffer: The suspension is centrifuged, SN is aspired, the pellet is resuspended in WASH II +PI+ PMSF and incubated on ice for 10 minutes. Thereafter the suspension is centrifuged 1200 rpm at 4°C for 5 minutes. The supernating wash II is sucked off, the pellet is resuspended in 500µl lysis buffer (+PI+PMSF) and incubated at 4°C over night.

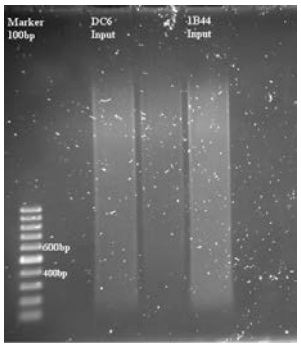
Day 2

5ml Elution Buffer are prepared according to the formula listed above. To fragment chromatin (genomic DNA of the cells), a sonicator is used. The lysate is sonicated 5 times at 90% dot cycle, 45% output for 15 seconds with a cooling step on ice between each cycle for 30 seconds. To clear the suspension from unwanted membrane proteins, the sonicated lysate is centrifuged at full speed (14krpm) in an Eppendorf centrifuge at 4°C for 10min. The cleared SN is transfered to a new Eppendorf tube. To estimate the concentration of protein in the sample, the optical density at a wavelength of 280nm is measured in a NanoDrop photometer. Therefore, the samples are diluted 1:50 in lysis buffer before measurement of OD_{280} (protein concentration) in the NanoDrop photometer. First of all a so called "input" sample is prepared. The input sample serves to check if sonication was sufficient. In addition to that, the input sample serves as internal control: In following PCRs, the DNA content of all other samples will be compared to the DNA content in the input sample. Sufficient sonication is essential for a successful ChIP. As "sufficient sonication" is considered a mean length of sonicated gDNA fragments of 400-600 bp. Otherwise, in later PCR analysis false positive signals may occur, if chromatin fragments are too long. The ChIP is based on several purification steps of DNA fragments in the presence of IgG antibodies, which are specific for the protein of interest. On the other hand the antibodies bind to protein A coupled sepharose beads. ChIP functions because specific antibodies keep DNA fragments with bound protein down with the beads, whereas other DNA fragments and proteins remain free in the suspension. So if DNA fragments are too long, the protein of interest (POI) may be bound to a gDNA sequence far more upstream or downstream, which would give false positive signals in the following PCR, even if primers, specific for a certain DNA sequence are used.
However, for a measured OD_{280} of 0.1 we take 100µl of sonicated material to set up the input sample. (max.possible volume is 140µl)
To reverse crosslinking of protein and gDNA in the input sample, the sample volume is extended to a final volume of 400µl with elution buffer. Thereafter, 20µl 4M NaCl are added and the sample is incubated at 65°C 300rpm shaking O/N.

Day 3

Protein in the sample is degraded by proteinase K:
8µl 0.5 M EDTA, 16µl 1M Tris pH 6.5 and 2µl proteinase K solution [20mg/ml] are added and incubated at 55°C 500rpm shaking for one hour. Thereafter, DNA in the sample is purified by phenol-chloroform-isopropanol (PCI 25:24:1) extraction. Phenol is always handled in a fume hood and gloves should be worn. 600µl PCI are added to input samples. The tubes are vortexed to mix and centrifuged at full speed (14krpm) at 4°C for ten minutes. The centrifugation is needed to separate the aqueous phase, which contains DNA, from the phenol phase, which contains degraded protein. The aqueous phase is transfered to a new eppi and is mixed with 800µl –20°C cold 96% Ethanol (EtOH p.a.), 12µl 3M NaOAc, and 1µl glycogen [20mg/ml dH_2O]. To precipitate the DNA, this solution is incubated at –20°C for 30 minutes. Following precipitation DNA is pelleted in a centrifuge at full speed at 4°C for 10 minutes. The SN is discarded and the DNA pellet is washed with 500µl 70% EtOH. As the DNA pellet is more loosely attached after the 70% EtOH washing, an additional centrifugation step at full speed and 4°C is needed for 5 minutes. The SN is discarded, pellets are air dried and allowed to dissolve in 200µl sterile dH_2O. While DNA dissolves in dH_2O, RNA is digested with 1µl RNase A at 37°C and 1200rpm shaking for 30 minutes.
Before proceeding to sample preparation in presence of antibodies specific for protein of interest, the length of sonicated chromatin fragments in the input samples have to be checked on a 0.8% agarose gel.

Fig.4.2.3: Analysis of sonicated chromatin on a 0.8% agarose gel



The setup of an immuno-precipitation: for each immuno-precipitation one 100µl aliquote of the sonicated sample is needed. (if the OD_{280} is 0.1→ 100µl). Then 900µl dilution buffer (+200µl PI+10µl PMSF/10ml) are added. Thereafter, to pre-clear the sonicated samples, 20µl protein A bead suspension is added and incubated vertically shaking at 4°C for one hour. To separate the sonicated sample from the beads, which should be loaded unspecifically with protein, the vial is centrifuged at 1200rpm at 4°C for 5 minutes. Then the SN is transfered to a

new eppi and antibodies specific for the protein of interest (POI) are added [4µg per IP]. The antibodies are incubated at 4°C in a vertical shaker over night.

Day 4

The complexes of antibodies bound to POI are harvested with 30µl protein A beads shaking at 4°C in a vertical shaker for one hour. The underlying principle is that sepharose beads are conjugated to *staphylococcus aureus* protein A, which binds the Fc-portion of IgG molecules. After harvesting, the IPs are centrifuged at 1200rpm at 4°C for 5minutes. Then the SNT is removed. The protein A-sepharose beads have POI bound via IgG and their Fc-potion. To get rid of unspecific bindings, the beads are washed with 900µl RIPA-buffer and HI salt buffer. All the following washing steps consist of vertical shaking at 4°C for ten minutes and centrifugation at 1200 rpm and 4°C for five minutes, to collect the beads at the bottom. The same washing procedure repeated with 900µl LiCl buffer to get rid of crosslinked RNA. Finally the beads were washed twice with 900µl TE-buffer to neutralize and wash away salts. Finally to elute DNA-POI-antibody complexes from the beads, the beads are vigorously shaked at room temperature in 400µl elution buffer for 30minutes. Afterwards, the suspension is centrifuged at 3000rpm at RT for 5minutes. The SN is transfered to a new eppi. The crosslinking between DNA and protein is reversed in presence of 20µl 4M NaCl at 65°C horizontally shaking at 300rpm over night.

Day 5

To degrade proteins, including antibodies and protein of interest, a proteinase K digestion is set up.

Therefore, 8µl 0.5M EDTA, 16µl 1M Tris pH 6.5 and 2µl proteinase K solution [20mg/ml] are added to immuno-precipitations (IPs). The degradation occurs during incubation horizontally shaking at 55°C for one hour. To remove degraded proteins, IPs are subjected to phenol-chloroform-isopropanol (PCI) extraction with 600µl PCI. The PCI-IP emulsion is vortexed to mix. To separate DNA-containing aqueous phase from protein-containing phenol phase the samples are centrifuged at 14krpm at 4°C for 10 minutes. The aqueous phase is transfered to a new eppi. To collect the DNA, it is precipitated by addition of 600µl –20°C 96% EtOH, 12µl 3M NaOAc, and 1µl glycogen [20mg/ml]. The samples are kept at –20°C for an hour. To pellet the precipitated DNA, the samples are centrifuged at 14krpm at 4°C for 20 minutes. The supernating EtOH is discarded and pellets are washed with 500µl –20°C 70% EtOH to rehydrate DNA. After another 10minutes of centrifugation at 14krpm at 4°C, the SN is taken of and the DNA pellets are air-dried. Then, the DNA pellets are allowed to dissolve in

200µl nuclease-free dH_2O. To get rid of RNA, 1µl RNase A is added and the samples are incubated at 37°C for 30 minutes. Finally ChIP samples were stored at –20°C until use for PCR.

ChIP PCRs

A PCR master mix for ChIP samples differs from common PCR master mixes: per sample 4µl less water is added. In contrast to common PCRs where usually the input of DNA template does not exceed 1µl, in ChIP PCRs 5µl of antibody-purified ChIP samples are used as templates. Therefore, the amount of ddH_2O in the master mix is reduced by 4µl per antibody-purified sample.

A PCR master mix for 19 samples is prepared as following:

	1x ChIP PCR MM	19x (-6x 4µl ddH_2O)
DdH_2O	38.75 µl	712.25 µl
10x Taq buffer	5 µl	95 µl
$MgCl_2$	3 µl	57 µl
dNTPs (0.1 mM)	1 µl	19 µl
Primer fwd	0.5 µl	9.5 µl
Primer rev	0.5 µl	9.5 µl
Taq Polymerase	0.25 µl (1unit)	4.75 µl

For input samples 49µl MM+ 1µl template and for antibody-purified ChIP samples 45µl MM and 5µl template were used.

The polymerase-containing MM is laid in sterile 0.5ml PCR tubes, then the templates are added, and the PCR is performed in following conditions:

Temperature	Time	Cycles
95 °C	4 min	
50.5 °C (dependent on primers)	4 min	
72 °C	45 sec	
95 °C	1 min	40
50.5 °C (dependent on primers)	45 sec	40
72 °C	45 sec	40
72 °C	4 min	
4 °C	Infinitely	

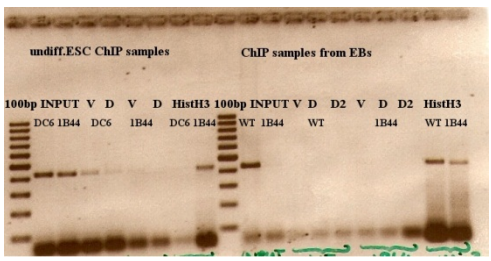


Fig.4.2.4*: shows a 2% agarose gel to visualize the results of the ChIP PCR (20µl of the PCR products)*

This gel electrophoresis of the ChIP PCR products shows input bands for both of the undifferentiated ESC lines, which proves that the region of interest is present in the sonicated samples and that the PCR works. In this PCR analysis, the DC6 (desmin overexpressing ESC line) shows binding of vimentin (another type 3 IF) and weaker binding of desmin to the genomic region of a minimal cardiac enhancer (Lien et al. 1999). As a positive control, we use IP samples, which were prepared with histone H3 IgG, because histones usually are present non-selectively on chromatin.

4.3 Cloning

4.3.1 The Generation of competent E.coli

To generate competent E.Coli, the bacteria of the selected strain (most often E.coli XL1 blue or E.coli TOP 10) are plated to an LB-plate, either directly from a frozen stock or from a small volume of a bacterial suspension. On the next day, one colony is picked and used to inoculate 1ml of liquid LB medium. After incubation on a horizontal shaker at 37°C for three or four hours (or over night), the liquid culture is diluted with 49ml of fresh LB medium in a 250 ml Erlenmeyer flask. The liquid culture then is incubated at 37°C until it reaches an optical densitiy OD_{600}= 0.3-0.6, which usually takes about two to four hours. Then the E.coli culture is centrifuged in a 50 ml Falcon tube at 1000 x g and 4°C for 10 minutes. The supernatant is removed and the bacterial pellet is resuspended in 5ml TSB. The suspension is then incubated on ice for ten minutes. The competent bacteria are then aliquoted (10x0,5ml) to pre-cooled (4°C) reaction tubes, with screw lids. Finally, the competent E.coli are shock frozen in liquid nitrogen and are stored at -80°C until use in bacterial transformation.

TSB-Medium

10% PEG_{6000} or PEG_{8000} 25.0 g
5% DMSO 12.5 ml
10mM $MgCl_2x6H_2O$ 0.51g
10mM $MgSO_4x7H_2O$ 0.62g

The solution is filled with LB medium to a final volume of 250ml, is then sterile filtered and stored at 4°C.

4.3.2 Glycerol stocks of bacteria

For long term storage of transformed bacteria, glycerol stocks are frozen from fresh over night liquid cultures under sterile conditions. Therefore, 150µl of sterile 87% glycerol p.a. is laid in sterile cryo-vials using tips with cut ends, as glycerol is highly viscous. Then 850µl of an

over-night (O/N) liquid bacterial culture is added, resuspended and vials are closed in sterile conditions. After shock-freezing in liquid nitrogen (N_{LIQU}) glycerol stocks are stored at -80°C.

4.3.3 Retrieving plasmids

4.3.3.1 Wizard® Plus SV Minipreps DNA Purification System

1-10ml of a bacterial overnight culture is centrifuged in a table top centrifuge at full speed for 5 minutes. The supernatant is removed and the pellet is resuspended in 250 µl cell resuspension solution. Then, 250 µl cell lysis solution is added. To mix, the suspension is inverted for four times. To degrade proteins, 10µl alkaline protease is added. To mix, the solution is inverted for four times and then incubated at room temperature for five minutes. To stop the lysis, 350 µl neutralization solution is added. To mix, the suspension is inverted for four times. To pellet unwanted proteins, the suspension is centrifuged in a table top centrifuge at full speed for ten minutes. Then, the cleared lysate is decanted into a spin column, which is provided with the kit. The DNA is bound to the column by centrifugation at full speed for one minute. The flow through is discarded and the column is inserted into a collection tube. The column with the DNA bound is then washed with 750 µl wash solution (with EtOH added). To wash, the column is centrifuged at full speed for one minute. The flow through is discarded. Then, the column is washed again with another 250 µl wash solution and is centrifuged at full speed for two minutes. After washing, the spin column is transferred to a sterile 1.5 ml Eppendorf tube. It is recommended to mark the sample identity on the wall of the tube, because it happens that the tubes are de-capped during the following centrifugation. To elute the DNA from the column, 100µl (or 70µl) of nuclease-free water is added to the spin column and incubated at room temperature for one minute. Thereafter, the DNA is eluted by centrifugation at full speed for one minute. The column may be discarded. The concentration of the DNA is measured in a Nanodrop device. Then the DNA is stored at -20°C or below.

4.3.3.2 Promega´s Pure Yield Midi Plasmid Preparation

This type of plasmid preparation is needed when the plasmid DNA is meant to be sequenced. For sequencing relatively high DNA concentrations and high quality is needed. The plasmids, which are prepared in this Midi prep kit are also suitable for transfection.

An over night liquid culture of the selected clones in 50ml LB+amp is centrifuged 20.000 rotations per minute (rpm) at room temperature (RT) in a Sorval SS34 rotor for 15 minutes. The supernating LB-medium is aspirated and the pellet is re-suspended in 3ml

resuspension solution. After adding 3ml cell lysis solution, it is inverted 5 times to mix. Then the solution is incubated at RT for 3min. To stop the lysis, 5ml neutralization solution are added. The tube is inverted 5 times to mix and incubated at RT for 3 minutes. To clear the lysate, it is centrifuged in a pure yield clearing column 3000rpm in a table top centrifuge for 5 minutes. For DNA binding, the cleared lysate is poured into a provided column and centrifuged 3000 rpm for 3 minutes. To get rid of bacterial toxin, the column is washed with 5ml endotoxin removal solution and twice with 20ml column wash. Finally, the plasmid DNA is eluted with 600µl nuclease-free H_2O by centrifugation at 3000 rpm for 5minutes. The concentration of the plasmid DNA is measured in a NanoDrop photometer.

4.3.3.3 QIAGEN Maxi Plasmid Preparation

For large scale plasmid preparation a liquid starter culture of a single colony of transformed E.coli is inoculated in 2ml of LB, which contains the appropriate antibiotic. It is incubated shaking at 37°C for two hours. Then the starter culture is used to inoculate a larger over night liquid culture in 100ml LB. The large scale liquid culture is incubated shaking at 37°C over night. To harvest, the cultures are centrifuged in the next morning at 6000x g at 4°C for 15 minutes. In the meantime the buffer P3 is pre-cilled to 4°C on ice. The pellet is resuspended in 10ml buffer P1. Then 10ml of buffer P2 are added and the solution is inverted for 4-6 times to mix. Then the solution is incubated at room temperature (RT) for 5 minutes. Thereafter, 10ml of chilled buffer P3 are added to stop lysis. The solution, which contains cloudy white material, is poured into the barrel of a QIAfilter cartridge, which is provided in the kit. The material is allowed to settle for 10 minutes at RT. Then the lysate is filtered into a 50ml Falcon tube, by insertion of the plunger. To the filtered lysate, 2,5ml of endotoxin removal (ER) buffer is added and incubated on ice for 30 minutes. In the meantime, a QIAGEN-tip 500 is equilibrated by adding 10ml of buffer QBT, and allowing the column to empty by gravity flow. The filtered lysate then is poured into the equilibrated QIAGEN-tip. The material is allowed to enter the resin by gravity flow. The column is washed twice by adding 30ml buffer QC and allowed to empty by gravity flow. Discard the flow through and use a new 50ml falcon tube. To elute the DNA from the column, 15ml of buffer QN are added to the column. The plasmid DNA now is precipitated by adding 10,5 ml of RT isopropanol to the eluate. The suspension is inverted to mix and aliquoted to sterile 1,5 ml Eppendorf tubes. These Eppendorf tubes are centrifuged at 15.000 x g at 4°C for 30 minutes. The supernatant is carefully decanted or removed with a gilson pipette. The pellets from the different eppies are combined in four eppies and washed with 5ml 70% ethanol (RT). After washing, the pellets

are centrifuged at 15.000 x g at 4°C for 10 minutes. The supernatant is carefully decanted and the pellet is allowed to air-dry for 5 to 10 minutes. The pellet is allowed to dissolve in an appropriate volume of sterile endotoxin free water (e.g. 200µl). The DNA concentration is measured using a NANODROP device.

4.3.4 The production of a DNA-insert for cloning

To produce a DNA-insert for cloning, a polymerase chain reaction is set up with a gDNA template (e.g. C3H mouse genomic DNA) and *Pyrococcus furiosus* polymerase (*Pfu* pol). The *Pfu* polymerase exhibits 3´→5´ exonuclease activity, which means that this enzyme does not simply extend a primed DNA-sequence, but it also moves back to correct reading mistakes ("proof reading"). Therefore, the *Pfu* polymerase needs longer extension times in a PCR cycle (at least 2 minutes per kilobase). For cloning, the PCR primers are generated to introduce two different restriction sites, which are unique within the future plasmid backbone. They serve to introduce these restriction sites to the DNA insert, which will be cloned. The backbone plasmid and the insert then will be cut with the same restriction enzymes, to generate compatible ends for later ligation.

1x Pfu master mix	
DdH_2O (nuclease free)	38 µl
10x Pfu buffer	5 µl
10 mM dNTPs	1 µl
Primer fwd	2 µl
Primer rev	2 µl
Pfu polymerase	1 µl
Template (C3H genomic DNA)	1 µl

Temperature	Time	Cycles
95 °C (melting of gDNA)	4 min	
95 °C (melting)	1 min 30 sec	35
63 °C (annealing)	45 sec	35
72 °C (extension)	2 min 30 sec	35
72 °C (final extension)	5 min	
4 °C (cooling)	Infinitely	

The DNA-insert is purified in a preparative agarose gel, which is prepared with 1x TAE buffer instead of 1x TBE.

4.3.5 QIAquick Gel extraction

Under UV light, the DNA-fragment is excised from the preparative agarose gel with a clean and sharp scalpel. To minimize damage from the exposure to the UV light, the excision must be done as quick as possible. Then, the size of the gel slize is minimized by removing extra

agarose, which does not harbour DNA. Then, the gel slice is weighted in an Eppendorf tube. To extract the DNA from the agarose gel, three volumes of buffer QG is added to one volume of gel (100mg ~ 100µl). The gel slice is dissolved by incubation at 50°C for ten minutes. If the gel slice failed to dissolve completely, the incubation may be extended. While the gel slice dissolves, it is vortexed every two to three minutes, to mix and to accelerate the reaction. After the gel slice completely dissolved, the color of the suspension must be yellow, which indicates that the pH did not change. To precipitate the DNA, one gel volume of isopropanol is added to the sample. Then, a QIAquick spin column is added to a provided 2 ml collection tube. To bind the DNA to the spin column, the sample is applied to the QIAquick column and centrifuged at 13.000 rpm for one minute. The flow-through is discarded. The spin column is placed back in the same collection tube and the column is washed with 0.75 ml buffer PE by centrifugation at 13.000 rpm for one minute. The flow-through is discarded. To get rid of remaining liquid the QIAquick column is centrifuged for one additional minute at 13.000 rpm (~17,900g). Before elution of the purified DNA from the QIAquick column, it is put into a clean 1.5 ml Eppendorf tube. To elute the DNA, 50µl of buffer EB (10mM Tris-Cl, pH 8.5) or nuclease-free ddH_2O is added to the center of the QIAquick membrane. Then, the column is centrifuged at 13.000 rpm for one minute. Alternatively, to increase the DNA concentration, the amount of elution buffer may be reduced (30 µl). In addition to that, it is recommended to let the column stand for one minute before the centrifugation. The DNA may be stored at +4°C or at –20°C for long term storage.

4.3.6 The restriction analysis of DNA

For all restriction digests, the enzymes and corresponding buffers were chosen from New England Biolabs. On the website of NEB a program is provided, which assists in finding single cutters in a known DNA-sequence (at the NEB tools tab).
http://www.neb.com/nebecomm/default.asp

NEBuffer 1: 10mM Bis-Tris-Propane-HCl, 10mM $MgCl_2$, 1mM Dithiothreitol

NEBuffer 2: 10mM Tris-HCl, 50mM NaCl, 10mM $MgCl_2$, 1mM Dithiothreitol

NEBuffer 3: 100mM Tris-HCl, 50mM NaCl, 10mM $MgCl_2$,1mM Dithiothreitol

NEBuffer 4: 50mM Potassium acetate, 20mM Tris-acetate, 10mM Magnesium acetate, 1mM Dithiothreitol

Restriction

1µg	DNA (approx. 10µl of Midiprep DNA)
2µl	of NEB Buffer 10x
1µl	enzyme 1
1µl	enzyme 2
0.2µl	BSA 100x (if necessary)
ad 20µl	with dH_2O

The restriction digests are incubated at 37°C for one to three hours, or at room temperatue over night. After the incubation the restriction digest may be stopped by addition of 6x loading dye and separation of the DNA fragments in an agarose gel electrophoresis. If the restriction was done for cloning, the restriction enzymes need to be heat inactivated at least at 65°C for 15 minutes. For cloning the digested DNA is subsequently separated on a 1-1.2% agarose gel, which was prepared with 1xTAE buffer. The DNA fragments are cut out of the gel and are purified, as described in chapter 4.3.5. Alternatively the heat inactivated samples may be stored at -20°C until use.

4.3.7 5' Dephosphorylation of DNA

For cloning of a DNA insert into plasmid, it is often necessary to de-phosphorylate the vector backbone, to prevent a self re-ligation. This is achieved by the addition of alkaline phosphatase (calf intestinal phosphatase, CIP). The CIP catalyzes a removal of the 5'phosphate groups from DNA. These 5'phosphate groups are used by ligases to re-ligate DNA. The future insert DNA is not treated with CIP to enable the ligase to ligate the DNA insert and the vector. Therefore, the 5'dephosporylation is an essential strategy to decrease the vector background in cloning strategies.
To 5'dephosphorylate a vector for cloning, it is suspended in 1xNEBuffer 3 (0.5µg/10µl). Then 0.5 units CIP per µg vector DNA are added. The suspension is incubated at 37°C for one hour. Thereafter, the DNA is purified by gel purification, spin-column purification or phenol extraction. The purified vector DNA is used for cloning.

4.3.8 The ligation of the DNA-insert and a cloning vector

Usually when a DNA insert will be cloned into a vector, the several different ratios of vector and insert are tried out. To convert molar ratios to mass ratios a formula is used:

$$\frac{\text{ng of vector}}{\text{kb size of vector}} \times \frac{\text{kb size of insert}}{\text{vector}} \times \text{molar ratio of insert} = \text{ng of insert}$$

Usually ratios of 1:1, 1:2, and 1:3 are tried out.
To assemble a ligation reaction, the following components are combined in a sterile microcentrifuge tube:

Vector DNA	100 ng
Insert DNA	e.g. 17 ng (1:1)
Ligase 10x buffer	1 µl
T4 DNA ligase	0.1- 1 unit
Nuclease-free water to a final volume of	10 µl

The reaction mixture is incubated at room temperature for three hours or at 4°C over night, or at 15°C for 4-18hours. The time of incubation depends on the restriction enzymes, which were used to cut the DNA and on the type of cut they made. While so-called “sticky ends” are effectively ligated at room temperature (22°C) for three hours or at incubation between 4°C-8°C over night, the ligation of so-called “blunt ends” is most efficient at temperatures between 15 and 20°C for 4-18 hours (over night).

4.3.9 The transformation of competent E.coli

To transform competent E.coli, the bacteria are thawed on ice. Immediately after thawing, 10µl of ligated vector is added to the suspension of bacteria. A suspension of competent bacteria must not be resuspended. Therefore the bottom of the vial is tipped with a finger to gently mix. Then, the transformation mix is incubated on ice for 30 minutes. Thereafter, the E.coli are heat-shocked in a 42°C heated water bath for 30 seconds and right afterwards put back on ice for about 5 minutes. Then, the transformed bacteria are fed with 250µl of preheated LB-medium without antibiotics. It is important to use LB-medium without antibiotics, because the bacteria need some time to express the antibiotic resistance gene, which they just received with the plasmid. The transformed bacteria are then incubated at 37°C horizontally shaking for 1.5 hours. In the mean time, LB-plates containing an appropriate antibiotic (e.g. ampicilin) are dried in a (semi-)sterile environment. To receive single colony transformants, 100µl, 50µl, 20µl, and twice 10µl of the transformed E.coli are plated. Finally, the plates are incubated at 37°C over night.

4.3.10 Analysis of the clones

In the next morning, the plates are examined. The colonies of transformants are counted. The plate is stored at 4°C.
Of the transformed bacterial colonies, some single colonies are selected for mini-preps and restriction analysis. The selected clones are picked with a tooth pick and are then plated in

separate lines on a recovery plate, which contains an appropriate antibiotic. The same tooth pick is used to inoculate a liquid culture in 10ml LB-medium containing the appropriate antibiotic (e.g. ampicillin [100µg/ml]). The liquid cultures are incubated for several hours or over night. In the next morning, the plasmids of the clones are retrieved with an SV Wizard mini prep system from Promega (as described in chapter 4.3.3 Retrieving plasmids). Then, the plasmids are digested in a qualitative restriction and are analyzed in an agarose gel electrophoresis, as described in chapter 4.3.6 Restriction digest.
From the clones, which carry the insert in the right orientation, one or two are chosen for sequencing at VBC Biotech. Therefore, a plasmid midi preparation should be set up.

4.4 Dual Luciferase® Reporter System

The Dual Luciferase Reporter System is a powerful tool to assess information about a genomic region of interest. For instance, a putative promoter or enhancer region may be tested for its ability to induce the expression of a *luciferase* reporter gene. Therefore, the genomic region of interest must be cloned into a reporter plasmid upstream of the *firefly luciferase* gene. Thereafter, eukaryotic cells are transiently transfected with this reporter plasmid. To correct for deviations in the reporter activity, which arise from different transfection efficiencies, a phRL-TK internal control reporter is co-transfected. From *firefly luciferase* reporter vector (pGL-variants), a 61kDa monomeric protein is synthesized. The firefly reporter protein does not require post-translational processing for enzymatic activity. From the *renilla luciferase* reporter vector (phRL-TK), a 36kDa protein is synthesized. Similar to the firefly luciferase, the renilla luciferase does not require post-translational processing for enzymatic activity *(Promega)*. After transient transfection, the eukaryotic cells can be tested for luciferase activity. As various transfection methods often result in inconstant transfection efficiency, phRL-TK an internal control reporter plasmid is included in this reporter system. It is co-transfected with the *firefly luciferase* plasmid and expresses the *renilla luciferase* gene under the control of the Herpes simplex virus (HSV) thymidine kinase (TK) promoter. As a result, the activity of the *firefly* luciferase may be normalized to the activity of the *renilla* luciferase, which reflects the transfection efficiency. This serves to minimize the risk of evaluation artefacts, because all transfected wells receive the same amount of the control plasmid (phRL-TK). So deviations, which arise from different transfection efficiencies or differences in the vitality of the cells, are corrected.

To assay the reporter activity, eukaryotic cells are transfected with both luciferase reporter plasmids (NKE24, which contains the firefly *luciferase* gene downstream of the *nkx*

2.5 promoter and the phRL, which serves as an internal control). 48 hours after transfection, the cells are harvested with passive lysis buffer. According to the protocol from Promega, the cell layer is scratched with the tip of a pipette and 1ml of the 1x passive lysis buffer (provided by Promega) is applied. To lyse the transfected cells, they are incubated at room temperature for 30 minutes. Thereafter, the cells easily can be harvested by resuspension. For the luciferase assay, 50µl of the cell lysate is pipetted into a special 96-well reading plate. The luciferase catalyzes an oxidative decarboxylation of the luciferase substrate, which is provided by Promega in the reaction kit (DLR™). The reaction requires ATP, Mg^{2+} and O_2 and leads to release of energy in form of photons. Photon emission is achieved through oxidation of beetle luciferin via a luciferyl-AMP intermediate that turns over very slow. The intensity and stability of the luminescent signal is significantly enhanced by the addition of Co-enzyme A (CoA) to the substrate buffer.

The evaluation of luciferase reporter activity is performed automatically in a Berthold Centro plate-reader, with MicroWin software.

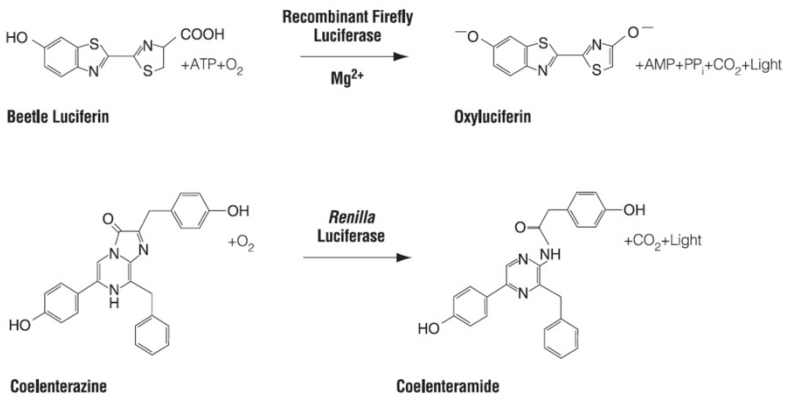


Fig.4.4: *Luciferases catalyze an oxidative decarboxylation of the substrates shown below. The oxidative decarboxylation of the substrates leads to a release of energy in form of photons. (oxyluciferin λ 530-635nm; coelenteramide λ 482nm)*

5. Appendix

5.1 References

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5.2 Abstract

On the role of desmin in cardiomyogenesis

Desmin is a type III intermediate filament protein, which has a well known role in the maintenance of the structural integrity within muscle cells. It was shown to be vital in the regeneration of muscle *in-vivo* and *in-vitro*. Studying the role of desmin during *in-vitro* differentiation in the embryoid body (EB) model revealed that the presence of ectopically expressed desmin boosts the differentiation of embryonic stem cells toward a cardiogenic fate *(Hofner et al.2007),* whereas the ectopic expression of an amino-terminally truncated version of desmin severely hampers cardiomyogenesis *in-vitro*. In addition to the boost of cardiomyogenesis, which is observed as earlier onset of cardiomyogenesis, the ectopic expression of desmin led to significant upregulation of the cardiac-specific TF gene *nkx2.5* and the mesodermal TF gene *brachyury*. To elucidate the mechanism behind the upregulation of the cardiac-specific TF gene *nkx 2.5*, desmins ability to physically interact with regulatory sequences in genomic DNA was assessed in chromatin-immuno-precipitation (ChIP) of *in-vitro* differentiated embryonic stem cells (ESC). Desmin binds to the *nkx 2.5* promoter at day 6, 7, 8, and 9 in $des^{+/+}$ EBs. In $des^{+/+/ect}$ EBs desmin binds to the *nkx 2.5* promoter at day 5, 6, 7, and 8. The fact that desmin binds earlier to the *nkx 2.5* promoter when it is ectopically expressed compared to $des^{+/+}$ EBs, is in line with the observed earlier onset of cardiomyogenesis in the EB *in-vitro* differentiation model. Desmin also binds to a minimal cardiac enhancer sequence, which is located in the 5'-UTR of the *nkx 2.5* gene *(Lien et al.1999).* To test if desmin not only binds to regulatory genomic elements but also modulates transcription, luciferase reporter assays were performed with three different cell types (10T ½ fibroblasts, C2C12 myoblasts, and primary cardiomyocytes). These cells types differ in their natural expression levels of desmin. The luciferase reporter assays revealed that desmin might act inhibitory or "non-permissive" on the *nkx 2.5* promoter in 10T ½ fibroblasts and C2C12 myoblasts. In primary cardiomyocytes desmin was rather found to activate the *nkx 2.5* promoter.

5.3 Zusammenfassung

Über die Rolle von Desmin bei der Entwicklung von Herzzellen

Desmin ist ein Typ III Intermediär Filament Protein, das bisher vorallem für seine Funktion in der Erhaltung der zellulären Integrität von Muskelzellen bekannt war. Das Protein ist von enormer Bedeutung in der Regeneration von Muskelzellen nach Belastung, wie in vielen *in-vitro* und *in-vivo* Studien gezeigt wurde. Studien über die Rolle von Desmin in der *in-vitro* Differenzierung im "Embryoid body" (EB) Modell zeigen, daß die Anwesenheit von überexprimiertem Desmin die Differenzierung von embryonalen Stammzellen (ESC) der Maus zu Herzzellen fördert. Im Gegensatz dazu konnte gezeigt werden, daß die Expression einer Desmin-variante mit fehlendem Amino-terminus, zu erheblicher Verzögerung der Herzzellen-entwicklung führt *(Hofner et al.2007).* Zusätzlich zu dem beobachteten förderlichen Effekt von überexprimiertem Desmin auf die Differenzierung von Stammzellen zu Herzzellen wurde gezeigt, daß gleichzeitig die Expression des Herz-spezifischen Transkriptionsfaktor (TF) Gens *nkx2.5* und des mesodermalen TF Gens *brachyury* signifikant erhöht wurde. Um weiteren Einblick in die zugrundliegenden Mechanismen dieser Aktivierung zu gewinnen, wurde die physische Interaktion von Desmin mit regulatorischen Elementen der genomischen DNA mittels Chromatin Immun-Präzipitation (ChIP) getestet.

Die ChIP Ergebnisse zeigen, daß Desmin Protein an Tag 6, 7, 8 und 9 der EB *in-vitro* Differenzierung an den *nkx 2.5* Promoter in $des^{+/+}$ EBs bindet. In $des^{+/+/ect}$ EBs bindet Desmin den *nkx 2.5* promoter an Tag 5, 6, 7, and 8. Die Tatsache, daß Desmin früher an den *nkx 2.5* Promoter bindet, wenn es in ESC überexprimiert wird, ist in Übereinstimmung mit der Beobachtung der "verfrühten" Herzzellenentwicklung in EBs. Die Bindung von Desmin konnte auch am „Minimal Cardiac Enhancer" *(Lien et al.1999)* in der 5'-UTR des *nkx 2.5* Gens nachgewiesen werden.

Weiters, um zu testen ob Desmin nicht nur genomische regulatorische Elemente binden kann, sondern auch ihre Aktivität beeinflusst, wurden Luciferase Reporter Assays in drei verschiedenen Zelltypen (10T ½ Fibroblasten, C2C12 Myoblasten, and primäre Cardiomyocyten) durchgeführt. Die drei Zelltypen unterscheiden sich durch das Ausmaß ihrer natürlichen Desmin Expression. Die Luciferase Reporter Assays ergaben, daß Desmin als Inhibitor oder "Aktivierungs-verhindernder" Faktor auf die Aktivität des *nkx 2.5* Promoters in 10T ½ Fibroblasten und C2C12 Myoblasten wirkt. In Reporter Assays mit primären Cardiomyocyten zeigte sich Desmin eher als Aktivator des *nkx 2.5* Promoters.

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Eckhart, L., Ballaun, C., Uthman, A., **Gawlas, S.**, Buchberger, M., Fischer, H., and Tschachler, E. (2009). Duplication of the caspase-12 prodomain and inactivation of NLRC4/IPAF in the dog. Biochemical and biophysical research communications *384*, 226-230.

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Thema der Diplomarbeit: „On the role of desmin in cardiomyogenesis“

7/2010-6/2011 Labortechnikerin des Laura Bassi Centre **OCUVAC** am Institut für spezifische Prophylaxe und Tropenmedizin, Kinderspitalgasse 15 1090 Wien.

Im Rahmen dieser Tätigkeiten konnte ich mir Kenntnisse über Methoden der Zellkultur, wie auch im Bereich der Molekularbiologie aneignen. Ich verfüge über Erfahrung in der Kultivierung von Fibroblasten, embryonalen Stammzellen der Maus, und primären Zellen. Ich bin zudem vertraut mit gängigen Methoden der Bakterienkultur, PCR, Plasmidpräparationen, Transfektion und Lipofektion von Zellkulturmaterial. Im Rahmen meiner Tätigkeit am Tropeninstitut erlernte ich zusätzlich die Kulturtechnik für obligat intrazelluläre Bakterien (Chlamydien).

e.h Sonja Gawlas