Aus der Chirurgischen Klinik und Poliklinik – Innenstadt,
der Ludwig-Maximilians-Universität München
Direktor: Prof. Dr. med. Wolf Mutschler

Establishment and investigation of tendon-derived cell lines immortalized by the human telomerase reverse transcriptase gene.

DISSERTATION

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vorgelegt von: Sophia Amina Poppe
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der Universität München

Berichterstatter:	Prof. Dr. med. Matthias Schieker
Mitberichterstatter:	Prof. Dr. Günther Eißner Prof. Dr. Peter Müller
Mitbetreuung durch die promovierten Mitarbeiter:	Dr. rer. nat. Denitsa Docheva
Dekan:	Prof. Dr. med. Dr. h.c. M. Reiser FACR, FRCR
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1. INTRODUCTION

1.1. General background

1.1.1. Anatomical and molecular structure of tendons

1.1.1.1. Tendinous tissue

Tendon is the essential connection between muscle and bone, whereas ligaments form the interconnection between bone and bone. Nevertheless both tissues are similarly structured and belong to the group of dense connective tissue and more precisely to the group of tendinous tissue. In research these tissues receive little attention. As opposed to cartilage or bone, very little is known about the microscopic and molecular structure of tendons and ligaments.

In the following, a detailed description of the anatomical structure of tendons with reference to different review articles is given. Tendons and ligaments do not have a consistent macroscopic appearance. Depending on the region of the body they can be flat, round, short or as drawn-out as the Achilles tendon stretching from the calf muscles to the heel bone. Some particularly flattened tendons for example in the ventral abdominal region are called aponeurosis (anterior abdominal aponeuroses). Other tendons need a synovial sheath (e.g. around the tendons of the extensor muscles at the hand) that has a protective function and reduces friction to ensure their functionality. Every tendon shows a structural hierarchy. From the outside inwards these are: tertiary bundles, fascicles (secondary bundles), fibrils, subfascicle (primary bundle), microfibrils (collagen fibre) and collagen fibril (tropocollagen). The epitenon surrounds the whole tendon, while the endotenon runs between the fascicles and tertiary bundles and contains blood vessels (see Figure 1). Yet under physiological conditions a fully developed tendon is a poorly vascularised tissue (Schieker et al. 2004).

In every tendon the force that is regularly applied to it determines the main orientation of the fibres. When the tendon is in a resting state the fibrils lie in a wavy conformation, defined as crimp, while when force is applied, fibrils straighten out and the tendon becomes stiffer. Thus under tensile stress and strain, tendons are resilient but flexible enough to adapt to the mechanical demands of their environment.

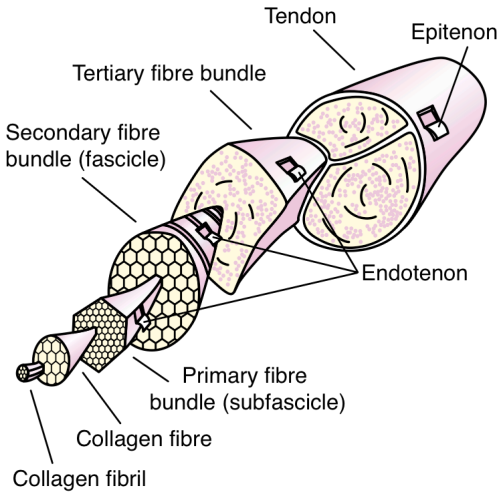


Figure 1. Structural hierarchy of tendons (Riley G, Expert Reviews in Molecular Medicine, Cambridge University Press 2005).

On a molecular basis tendinous tissue can be divided into a cellular part, consisting mainly of mesenchyme-derived tenocytes and tenoblasts, and of an extracellular part (extra-cellular matrix, ECM), which is produced and maintained by the cells. In a mature tendon less than 5 % of the whole volume consist of cells. Cells that do occur are mainly tenocytes but also chondrocytes, synovial cells and vascular cells, which exhibit low mitotic activities.

Besides the high percentage of water (65-75 %), the dry mass of a tendon contains mostly collagen I, which is found in all connective tissues in different quantities. Other components are elastin, collagen type III, IV, V and VI as well as proteoglycans (decorin, aggrecan etc.) and structural glycoproteins. The latter two form the so-called ground substance that stabilizes the tenocytes within the collagen fibres. Very importantly some of the constituents serve as markers for a tenogenic profile, as will be further elaborated in chapter 4.7 (O'Brien et al. 1997, Tozer and Duprez 2005, Sharma et al. 2006, Hoffmann et al. 2006).

1.1.1.2. Osteotendinous junction - enthesis

Other than the above mentioned main structure, there are areas where the tendon meets the demands of a different kind of strain. This means tendons do not only directly transmit the force from the muscle to the bone but they also have to sustain increased pressure as a hypomochlion e.g. the Patellar tendon where the tendon transfers the force from the thigh to the lower leg via the patella. This can only be achieved by a change of the composition of the ECM. One very important part of

the tendon where this change of ECM is essential is the osteodendinous junction which is also called 'enthesis'. This is the area where the tendon anchors to the bone. Generally speaking the change of ECM is a change of connective tissue. As cartilage is the tissue that is primarily specialized to sustain pressure and cyclic loading, the enthesis contains fibrous cartilage in some regions. Although this attachment site is schematically divided into four zones: tendon, fibro-cartilage, calcified fibrocartilage and bone, it is merely a smooth transition (Benjamin et al. 1986). Knese and Biermann 1958 were among the first to describe the enthesis in greater detail. Based on their definition Benjamin and Ralphs 1995 later classified the entheses as fibrous and fibro-cartilaginous. The fibrous enthesis is additionally divided into bony and periosteal segments, depending on the attachment site of either bone or periost. Opposedly, the fibrocartilaginous enthesis consists of the four zones already described above.

Obviously the attachment site is a zone of increased stress. Not only do two different connective tissues, tendon and skeletal tissue, have to interact here, but also does the enthesis have to balance the deviant elastic moduli of both (Knese and Biermann 1958). Tendons and ligaments are able to decrease their diameter in response to distension to a certain extent. At attachment sites this deformation would lead to high shear stress, which is not tolerable as it would compromise the connection between bone and tendon. Fibrocartilage does not give way to distension and therefore entheses contain fibrocartilage in order to withstand mechanical strain (Milz et al. 2005).

As aforementioned, the ECM in the enthesis is altered due to functional adaptation. On a molecular basis this denotes not only a change in the molecular composition of the ECM but also a change of the cells themselves. While molecules typically present in hyaline cartilage and dense connective tissue increase, the ECM components of tendon decrease. This correlates with a rise of collagen II, aggrecan, link-protein and chondroitin-6-sulfat for hyaline cartilage and collagen I, collagen III, dermatan sulfate, keratan sulfate and versican for dense connective tissue (Milz et al. 2005). Entheses are easily integrated in a clinical context when being aware of the molecular composition. Because of the fact that entheses are poorly vascularised their healing potential is marginal. Yet there are other reasons for the entheses to be clinically relevant and therefore represent an interesting object for investigation. Entheses appear to be objectives in the clinical manifestation of rheumatoid arthritis. They contain comparatively high amounts of aggrecan, link

protein and collagen II. These three molecules have been detected to be autoantigens during the autoimmune response in patients with rheumatoid arthritis (Glant et al. 1998, Guerassimov et al. 1998, Myers et al. 2001).

1.1.1.3. Myotendinous junction

Tendons, unlike ligaments, form a connection between bone on the one and muscle on the other side. They are the middle piece of the force transmitting entity, consisting of myotendinous junction (connecting muscle and tendon), tendon and enthesis. Therefore the myotendinous junction performs complex tasks which is reflected in its equally complex structure. Muscle and tendon virtually interdigitate. The fibres of the muscle are surrounded by plasma and basement membranes while they form a network with collagen fibres, fibroblasts and their processes. Accordingly there is no direct contact between collagen fibril and muscle fibres. Furthermore, the terminal parts of the fibres have electron dense zones of thickened plasma membrane to which the filaments of the I-bands (surrounding the Z-line of the sarcomere) are attached (Mair et al. 1972). From a physical point of view, the interdigitation amplifies the surface area of the 'contact zone' and thus reduces the shear stress that is transmitted through contractile forces. More precisely, the degree of surface amplification is proportional to the amount of stress reduction (Trotter et al. 1985).

As seen in the entheses, the ECM composition changes in response to a different mechanical need. The same phenomenon occurs in the myotendinous junctions. Especially the expression of focal adhesion kinase, paxillin, integrin-linked kinase, mitogen-activated protein kinase and talin is increased. The increase in paxillin and talin was as well used and seen when tendon-skeletal muscle constructs engineered in vitro were evaluated (Larkin et al. 2006). In embryonic development the formation of a myotendinous junction passes through five different stages: a. close association of the cell with tendon collagen fibres, b. basement membrane formation, c. sarcolemmal folding, d. appearance of subsarcolemmal densities, and e. myofibril association with subsarcolemmal densities. Afterwards, the junction only gains quantity during its embryonic development. Furthermore the number of cells near the junction decreases over time while the associations between basement membrane and collagen fibres increase (Tidball and Lin 1989).

In general, injuries at this site represent a major clinical problem. It is essential to diagnose the patients with this kind of injury correctly, especially because

musculotendinous junction injuries are rather common (Noonan et al. 1992). These patients face unspecific symptoms such as pain, oedema and ecchymosis. Compared to other injuries in this area, musculotendinous injuries need different treatment regimes. Since suturing muscular tissue is often associated with complications such as re-rupture, common surgical interventions are not recommended and other conservative treatments such as rehabilitation and a home exercise program are preferred and present with better results (Schamblin and Safran 2007).

1.1.2. Development of tendons

Tendon development is a precondition to enthesis or myotendinous junction formation, while the (embryonic) development is probably the least known aspect of them. As the healing or intrinsic repair of injured tissues often resembles the development, in that some processes on a cellular and molecular basis are being repeated, the knowledge of the development is crucial for the understanding of tendon repair. Generally, a distinction is made between the development of limb tendons (proximal and distal) and axial and ventrolateral wall tendons. The different kinds of tendons do not only have different localizations but they also differ in their development especially relating to their behaviour in absence or presence of muscles (Schweitzer et al. 2001). D'Souza et al. 1999 examined two genes in chicken, follistatin and EphA4, which are both expressed during tendon development. The removal of an interdigital apical ectodermal ridge led on the one hand to formation of cartilage and on the other hand to induction of EphA4 and follistatin which are both associated with tendon formation. As a result of their study, they concluded that cartilage and tendon development are coordinated.

To date, only Scleraxis (Scx), a basic helix-loop-helix transcription factor, is known as a single marker for tendons and ligaments. It is expressed in all of the developing tendons and therefore represents an adequate marker for tracking tendon development in general. The embryogenesis of the musculoskeletal system is well understood: The axial skeleton evolves from the sclerotome, the skeletal muscle from the myotome and the dorsal dermis from the dermotome. By analyzing the expression of Scleraxis it was possible to identify a fourth somitic compartment named the 'syndetome' from which tendons and ligaments evolve (Brent et al. 2003). More recent studies of Brent et al. partially identified the molecular interactions between the four somitic compartments. Myotomal Fibroblast growth factor (FGF) acts on the somitic tendon progenitors through the activation of the mitogen-activated protein kinase (MAPK) pathway and subsequently of the

transcription factor Pea3 (ETS family transcription factor) which then stimulates Scleraxis expression in tendon progenitors of the syndetome. Figure 2 illustrates the molecular interactions among the four somitic compartments, dermomyotome, myotome, syndetome and sclerotome. The myotome signals positively to the syndetome, while the sklerotome actively represses the syndetome by Sox5/Sox6-dependent transcription. Moreover, the authors demonstrated in their study that when the development of cartilage is blocked by a Sox5/Sox6 double mutation or knockout, ectopic Scleraxis expression is induced in the sclerotome. This leads to the assumption that tendon development is repressed under normal conditions by chondrogenic development, indicating that chondrogenic development influences tendon development and vice versa (Brent et al. 2004 and 2005).

At a later stage of the development, collagen fibrillogenesis plays a substantial role. This does not only hold true for the embryological development, but also for all healing processes in the adult tendon. Tenocytes are responsible for the assembly of the collagen fibre network. In the case of tropocollagen, which is the basic protein unit of collagen fibres, this process takes place intracellularly. For a long time it was assumed that in vivo tropocollagen monomeres self-assemble into collagen fibrils as seen in vitro. But this opinion was revised when the collagen fibril segment as a basic component of the collagen fibril was discovered. These segments are assembled to collagen fibrils in deep extracellular recesses located on the cell surface of tenocytes. When the collagen fibril increases in length, the plasma membrane walls of the recesses are retracting. In order to form collagen macroaggregates and to allow a growth in diameter, more than one tenocyte takes part in the assembly (Ingraham et al. 2003). Subsequently the association of the glycosaminoglycan decorin stabilizes the fibrils (Zhang et al. 2006). Ingraham et al. 2003 proposed that tendon fibrillogenesis during repair is related to the fibrillogenesis of embryonic tendon development by repeating the process that was described above in mature injured tendon.

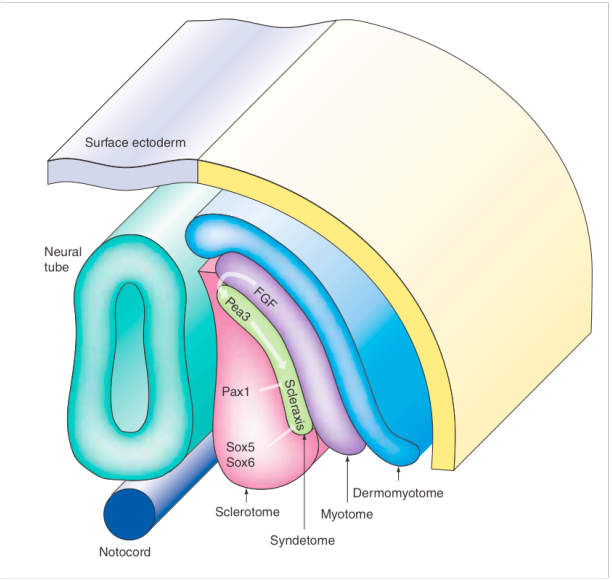


Figure 2. Proposed model of the development of somitic tendon progenitors. (Hoffmann and Gross, Regenerative Medicine 2006)

1.1.3. Principles of tendon healing

In general, tendon is a tissue with low regenerative capacity. The process of tendinous tissue repair is divided in three merging phases, which are illustrated in Figure 3. Phase one, the inflammatory phase, directly follows the injury. It is characterized by the migration of inflammatory cells such as leukocytes, neutrophils and macrophages together with fibroblasts from the epitenon attracted by pro-inflammatory factors from mast cells. These cells remove cellular debris, collagen fragments and help to dispose of the haematoma that is formed because of the damage of the surrounding blood vessels. The increased vascular permeability permits a release of vasoactive and chemotactic factors. In addition, angiogenesis is initiated by upregulation of platelet derived growth factor (PDGF), epidermal growth factor (EGF) and TGF-β1 and tenocytes are stimulated to proliferate. A higher deposit of DNA, fibronectin, glycosaminoglycan, water and collagen type III stabilizes the ECM at the repair site.

The next phase - called the fibroblastic/collagen synthesis phase - is predominantly marked by the further proliferation of epitenon and endotenon fibroblasts and the synthesis of collagen type III (Maffulli et al. 2000).

At the beginning of the third phase - remodelling/maturation phase - fibroblasts decrease in size and reduce the production of ECM. Collagen fibres commence to align in the direction of stress and more collagen type I is produced. It takes up to

one year for the healing fibrous tissue to change into a permanent scar-like tissue. The tissue that is formed after repair leaves a functional gap to healthy tendinous tissue (reviewed by Fenwick et al. 2002; Ingraham et al. 2003; Lin et al. 2004; Sharma and Maffuli 2006).

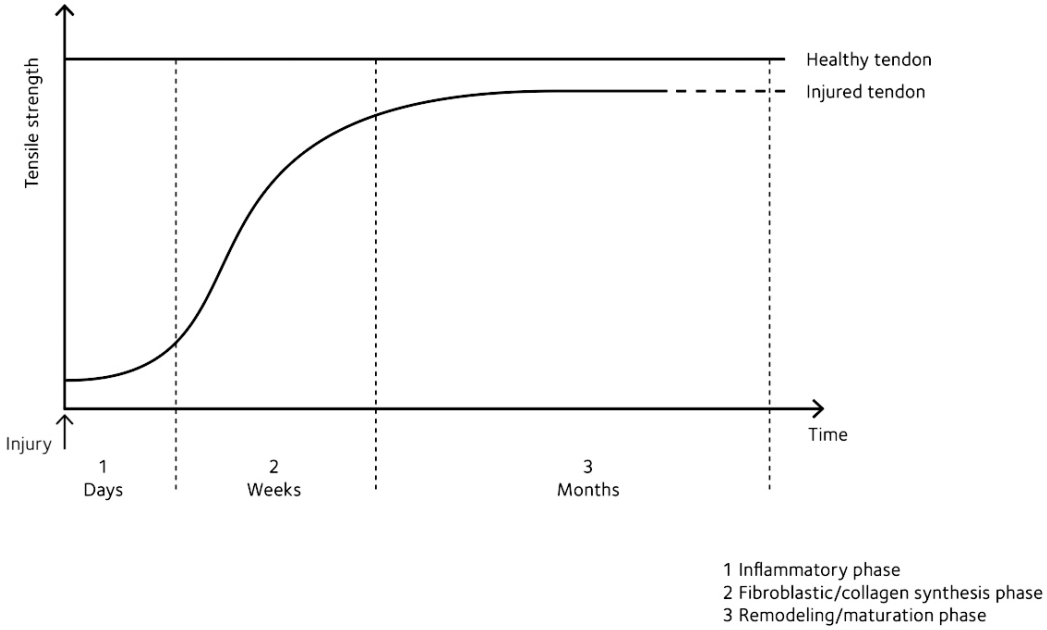


Figure 3. The different phases of tendon healing. (1) Inflammatory phase, (2) Fibroblastic/collagen synthesis phase, (3) Remodelling/maturation phase
(adapted from Gomez M, 1995)

Tendon can either heal intrinsically or extrinsically. During intrinsic healing the fibroblasts within the tendon proliferate, cells from the epitenon layer of fibroblasts differentiate into cells that resemble phagocytes and endotenon originated cells synthesize collagen. For intrinsic healing intratendinous blood supply is sufficient and therefore the risk of adhesion formation is diminished. By contrast the extrinsic healing depends on an extratendinous blood supply and the infiltration of inflammatory cells and fibroblasts from the surrounding sheath and synovium. When cells from outside the tendon migrate into the repair site and vessel ingrowth is induced, adhesions form and impair the normal function of the tendon gliding. Thus, extrinsic healing is considered to be inferior to intrinsic healing. Taking this into account, surgery after intrasynovial tendon injury needs to be deliberate as it disrupts the synovial sheath, enhances extrinsic healing and therefore adhesion formation. Most likely tendon healing occurs as a combination of intrinsic and extrinsic healing and is dependent on the location, the severity of the trauma, the

blood supply and some minor factors. Still a better understanding of this process would help to improve surgical methods or to advance non-invasive therapies (Manske et al. 1984; Sharma and Maffuli 2006; Lin et al. 2004). Wiig et al. 1997 attempted to accelerate tendon healing by reducing adhesions with hyaluronan injections between tendon and surrounding sheaths. Unfortunately this method had no effect on the healing process. To date there are few methods to repair tendon injuries, some using autografts, allografts or synthetic prostheses with the most widely used approach being tendon sutures. Newest approaches even include gene therapy, but they are still experimental. So far none of the therapeutic options has provided a successful long-term solution and at best the restored tendons are about 60 % functional (Rees et al. 2006).

1.2. Clinical aspects

1.2.1. Tendon injuries

Tendon injuries can result from diverse causes and lead to various symptoms. They may be classified as acute or chronic and be either direct or indirect. Intrinsic and extrinsic factors such as growth factors, hypoxia, ischaemic damage, inflammatory mediators, trauma and treatment also exert their influence. Acute and direct injuries with an immediate impact are found in contusions, injuries from accidents, sports injuries or lacerations by sharp objects. By contrast, indirect tendon injuries are most likely the result of tensile overload and repetitive microtrauma due to overuse (for an overview see Figure 5). In general, the connective sites, osteotendinous and myotendinous junctions, are more susceptible to ruptures and other forms of injury than healthy tendons, because they cannot withstand as much tensile load. Common examples are the so-called tennis elbow (tendinosis of extensor carpi radialis brevis and longus tendons usually within 1-2cm of their attachment to the lateral epicondyle of the humerus), the golfers elbow (tendinosis of the flexor carpi radialis tendon within 1-2 cm of its attachment to the medial epicondyle of the humerus) or the baseball-pitchers shoulder (insertion sites of tendons of rotator cuff muscles). Therefore, ruptures in the middle of a healthy tendon occur much less frequently (reviewed by Lin et al. 2004; Sharma and Maffuli 2006).

In the US, Patients sustain more than 32 million traumatic and repetitive motion injuries to tendons and ligaments every year. One of the most common is the rotator cuff injury of the shoulder. It is estimated that more than half of the population over the age of 80 years experience rotator cuff injuries (Schoen DC 2005, Tempelhof et al. 1999, Loehr et al. 2007). Sometimes tendons rupture spontaneously. In these

cases the ultrastructure is presumably altered. Kannus and Jozsa 1991 evaluated specimens of spontaneously ruptured tendons of 981 patients. Not one of the evaluated ruptured tendons had a healthy structure (Figure 4). 97 % of the pathological changes were degenerative, including hypoxic degenerative tendinopathy, mucoid degeneration, tendolipomatosis and calcifying tendinopathy, either alone or in combination. In the remaining 3 % the pathological change was one of the following: intratendinous foreign body, rheumatoid tendinitis, a xanthoma, a tumour or a tumour-like lesion.

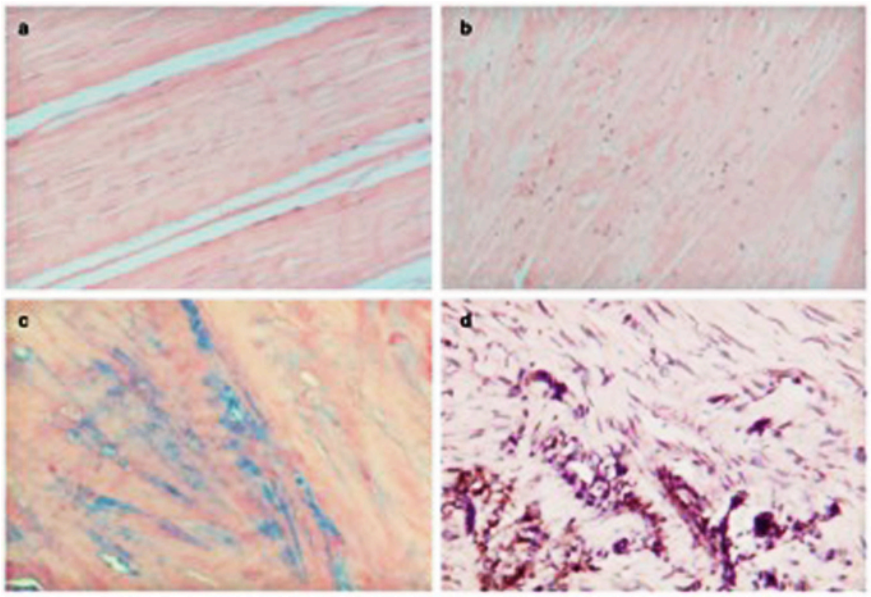


Figure 4. Histopathology of tendinopathy. (a) Normal flexor tendon histology, showing organised parallel fibre bundles and long thin tenocytes dispersed throughout the matrix. (b) Ruptured supraspinatus tendon, showing hyaline appearance, loss of matrix organisation and rounded, shrunken nuclei. (c) Glycosaminoglycan (GAG, blue) accumulation in supraspinatus tendon, GAG is surrounding rounded cells in the matrix. (d) 'Angiofibroblastic' change in painful Achilles tendinopathy, showing an increase in cell number and blood vessels. Staining: a,b and d stained with H&E, c stained with Alcian Blue and H&E (Riley G, Expert Reviews in Molecular Medicine, Cambridge University Press 2005).

Figure 5. Interdependence of tendon pathology factors. Different intrinsic and extrinsic factors as well as certain diseases and advanced age lead to decreased tendon strength.

Tendons as a connective tissue have the ability to adapt, remodel and repair within certain limits. In order to do so, ECM must be degraded and reestablished. Degenerative pathological changes may be the result of an imbalance in factors that are involved in this degradation and assembling process. These 'factors' are considered to be serine proteinases and particularly metalloproteinases such as MMP (matrix metalloproteinases), ADAM (A distegrin and metalloproteinases) and ADAMTS (A distegrin and metalloproteinases with trombospondin motifs). It is assumed that metalloproteinases are able to degrade major components of the tendon ECM and that their opponents, the tissue inhibitors of metalloproteinases (TIMP 1-4 in human), balance their activity. Nevertheless, TIMP are not the only antagonists of MMP as they also play a key role in normal tendon physiology e.g. in cell proliferation and apoptosis (Clegg et al. 2007). Another modulator that seems to be playing a decisive role in tendon healing is nitric oxide (NO). This free radical is synthesized by NO synthase from L-arginine. A study from Murrell et al. 1997 investigated that NO synthase activity is induced during rat Achilles tendon healing. NO synthase activity increases five-fold until day 7 after tenotomy of rat Achilles tendons and returns to almost normal level on day 14. Inhibition of the NO synthase led to a significant reduction in cross-sectional area and failure load, indicating an inhibition of tendon healing when no or a reduced amount of NO is present.

Interestingly, fluoroquinolone antibiotics can influence the expression of MMPs and hence promote tendon pain and rupture (Corps et al. 2005). For example, in Australia until 2002, 112 patients suffered from tendon disorders after fluoroquinolone therapy (mainly Ciprofloxacin). 30 cases involved tendon rupture (Australian Adverse Drug Reactions Bulletin 21:15, Dec 2002). Pasternak et al. 2006 showed that doxycycline (a tetracycline) also inhibits MMPs. Transacted tendons from rats treated with doxycycline showed significantly decreased force at failure and energy uptake concluding that tendon healing can be affected by doxycycline at clinically relevant serum concentrations.

However, the question which initial stimulus for the degradation process leads to tendon pain and rupture has yet to be answered. Arnoczky et al. 2007 discussed another aspect of the mechanobiological aetiopathogenesis of tendinopathy, whether it has its origin in the over-stimulation or the under-stimulation of tendon cells. Although they favour the hypothesis of under-stimulation as main source of tendinopathy, the topic remains object of further investigations.

1.2.2. Tendon repair

Tendon injuries can severely impair a patient's quality of life. Moreover, the mean age of our population will increase and thus problems with tendon degeneration and injury will increase as well. For these reasons effective therapies of tendon lesions must be found.

First of all, tendon repair should take place as early as possible after the injury. Within 12 h of injury it is called primary repair, within 14 days delayed primary, secondary would be between 2 and 4 weeks and late secondary after this period (Wright P, 1999). Depending on the severity of the injury, the demands of the patients and the feasibility, an appropriate therapy is chosen. Currently the most common therapy remains surgery. However there are vast varieties of different suturing techniques (Amlang and Zwipp, 2006). In general, the advantages or disadvantages of a technique are hard to judge, but what is most important is the number of strands crossing the repair site since it was proven that the repair strength is directly proportional to this (Lin et al. 2004). There is no consistent agreement when and to which extent the repaired tendon should be permitted to move after the intervention. A complete immobilization leads to reduced mechanical performance, impaired function and promotes the generation of adhesions. Opposed to this, remobilization can cause the formation of tears or in the worst case can cause re-rupture while at the same time antagonizing the bad effects of immobilization (Hoffmann et al. 2006; Lin et al. 2004). Taken together, stating a guideline for the optimal post-surgical treatment regimen is not (yet) possible.

Furthermore, there are cases where the tendon suffers from a critical size defect. Under certain circumstances (for instance in anterior cruciate ligament ruptures) the use of an autograft presents a possible solution although the restoration of tendon-bone interfaces could prove to be complicated. Recently, there have been numerous studies concerning tendon replacement and tissue engineering of tendon (Ahmed et al. 2008; Annovazzi et al. 2009; Omae et al. 2008; Tsiridis et al. 2008).

Tissue engineering has the aim to augment the healing response by means of delivering cells seeded on a supporting scaffold to the repair site. Therefore three of the crucial targets of these studies are to select an adequate scaffold material, appropriate cell type as well as suitable environmental stimuli such as growth factors. Growth factors regulate a variety of cellular processes and are often indispensable for cell proliferation or differentiation of cells especially on scaffold

material. EGF and bFGF for example are factors promoting proliferation of fibroblasts during the tendon healing process (Table 1). Great demands are made on suitable scaffolds including biocompatibility, biomechanical stability, resilience and porosity. Regarding the cells, no unanimous consent has yet been found regarding the most suitable type for tissue engineering purposes. Undisputedly important, however, remain properties that are crucial for a successful outcome of any healing process such as a long life-span and high metabolic activity of differentiated cells. Particularly mesenchymal stem cells (MSCs) have been exhaustively tested. In most studies MSCs are used because of their relatively easy isolation and their potential to proliferate well in vitro. Also they do progress from an immature progenitor cell through various lineages e.g. the osteogenic, chondrogenic, adipogenic and especially tenogenic lineage into specific cells of the respective path (Pittenger et al. 1999, Kuo et al. 2008).

Table 1. Growth factors involved in tendon healing

Factor	Biological effects
bFGF	Proliferation of fibroblasts Enhanced collagen deposition Increase in wound tensile strength
EGF	Proliferation of fibroblasts Enhanced collagen deposition Increase in wound tensile strength
EGR-1	Upregulation of TGF-β, PDGF, HGF and VEGF Enhanced angiogenesis Enhanced collagen deposition
HGF/SF	Enhanced angiogenesis Enhanced collagen deposition
PDGF-B	Influx of mononuclear cells and fibroblasts Enhanced collagen deposition Enhanced Angiogenesis Increase in wound tensile strength
TGF-β	Migration of mononuclear cells and fibroblasts Enhanced collagen deposition Increase in wound tensile strength
VEGF	Enhanced angiogenesis Enhanced granulation tissue deposition

In the field of tendon tissue engineering various combinations of matrices, cells and stimulating factors were tested. Different scaffold materials like polyglycolic acids, gels based on collagen type I or cross-linked collagen fibres served as matrix for

different types of cells ranging from tenocytes to MSCs. Butler et al. 2007 for example used a type I collagen sponge seeded with MSCs to repair defects in the patellar tendon in a rabbit model in vivo. Furthermore the constructs were mechanically stimulated prior to implantation into the defect site. This implant successfully enhanced the repair of the injured tissue and the dynamic load capacity significantly compared to untreated controls. Some factors indispensable for a positive outcome of tendon tissue engineering have already been detected.
Beyond a suitable scaffold material and metabolically active cells, for instance, mechanical stimulation of the constructs has been proven to be a crucial factor for a favourable clinical outcome reflected in high biomechanical stability (Cao et al. 2006; Zeugolis et al. 2008).

1.3. Tenocytes in vitro

1.3.1. Culture of tenocytes

Concerning the cultivation of tenocytes, a critical point is the preservation of a differentiated cell state. Earlier in vivo studies of avian and rabbit tendon cells showed decreased levels of type I collagen and decorin transcripts, and a loss of differentiated function during prolonged monolayer culture (Bernard-Beaubois et al. 1997, Schwarz et al. 1976). Another study performed with human tenocytes suggested that the differentiated state of the tenocytes can be preserved when they are grown in a three dimensional culture system after initial monolayer expansion. A combination of ultrastructural, biochemical and molecular tools showed that the phenotypic identity of the tenocytes was retained. Taken together, high-density culture may be useful as a new method for autologous tenocyte transplantation (Schulze-Tanzil et al. 2004). However, this approach does not allow extensive, long-term molecular analysis of tenocyte metabolism and signalling due to the use of primary cells, which have a limited lifespan. Also the high number of experimental cells needed presents a problem due to their limited availability. Besides, it would be interesting to further investigate the potential of tendon-derived progenitors to differentiate into other mesenchymal cell lineages such as osteocytes, chondrocytes and adipocytes, claiming that tenocytes or tendon-derived fibroblasts have a mesenchymal stem-cell-like character, thus supporting the hypothesis that there might be a role for altered tendon-cell differentiation in the pathophysiology of tendon injuries (de Mos et al. 2007).

1.3.2. Immortalization of tenocytes

1.3.2.1. Immortalization by using SV40 large T antigen gene

A recent publication using murine tenocytes has demonstrated that after immortalization with SV40 large T antigen gene, several cell clones (TT-E4, TT-G11 and TT-D6) maintained tendon phenotype-related gene expression (such as Scx, Six 1, EphA4, COMP and type I collagen transcripts) during one year of continuous cultivation (Figure 6).

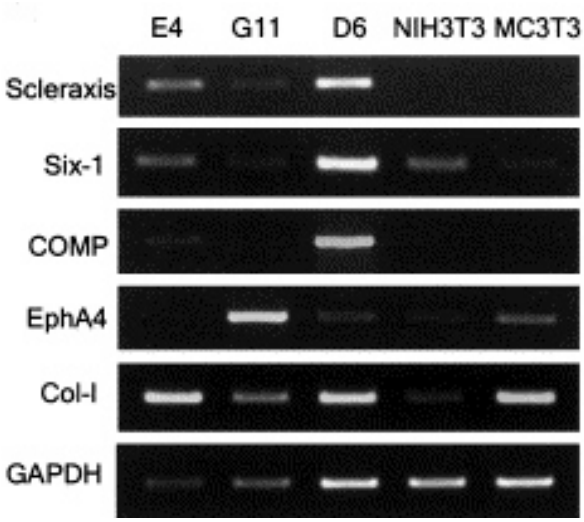


Figure 6. Tenogenic profile. RT-PCR analysis of expression of genes related to tendon phenotype (Salingcarnboriboon et al. 2003).

Furthermore, these clonal cell lines formed tendon-like tissues when they were implanted into defects made in murine patella tendon. Interestingly, these cells expressed genes related to osteogenic, chondrogenic and adipogenic lineages at low levels when examined by RT-PCR, postulating the existence of mesenchymal stem cells in tendon tissue (Salingcarnboriboon et al. 2003).

1.3.2.2. Immortalization by using hTERT

The human telomerase reverse transcriptase gene (hTERT) encodes the catalytic subunit of human telomerase (Figure 7). This enzyme is a ribonuclear protein complex which maintains the telomere length. A telomere is a repeating sequence of double-stranded DNA located at the ends of chromosomes. As cells divide and differentiate throughout the lifespan of an organism or cell line, the telomeres become progressively shortened and lose the ability to maintain their length.

Telomerase is an enzyme that lengthens telomeres by adding on repeating sequences of DNA. Telomerase binds to the ends of the telomere via an RNA template that is used for the attachment of a new strand of DNA. Telomerase adds several repeated DNA sequences. A second enzyme, DNA Polymerase, attaches the opposite or complementary strand of DNA completing the double stranded extension of the chromosome ends (Figure 8). High levels of telomerase activity are detected in immortalized cell lines such as embryonic stem cells and cancer cells, whereas little or no telomerase activity is present in most mature, differentiated cell types. In the absence of hTERT, telomeres shorten during cell division resulting in cellular senescence and growth arrest (Harley et al. 1990).

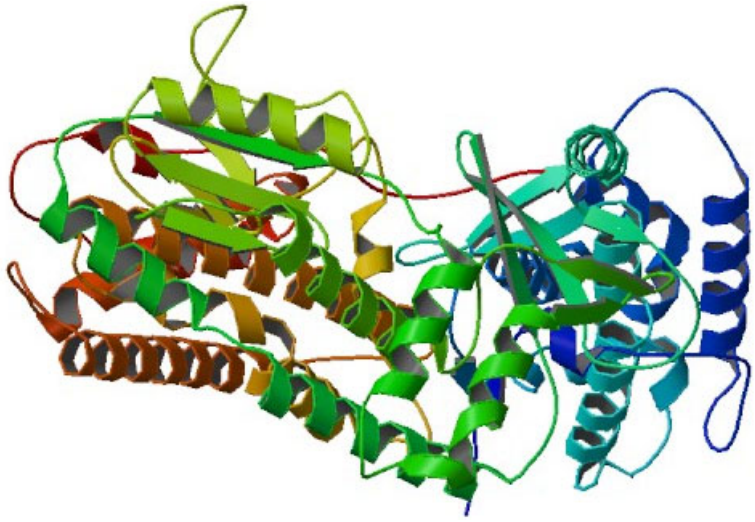


Figure 7. Structure of the catalytic subunit of telomerase, TERT (Protein Data Bank)

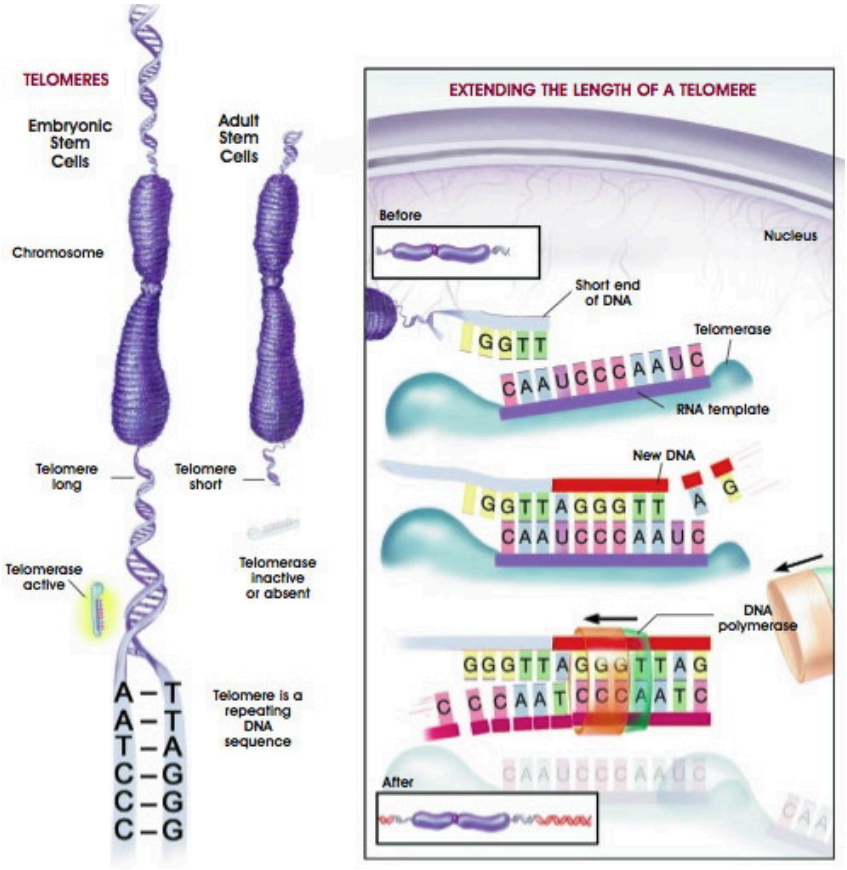


Figure 8. Telomeres and Telomerase. (Winslow T 2001, Stem Cell Information, Appendix C)

Successful immortalization of human mesenchymal stem cells (hMSCs) by retroviral as well as lentiviral overexpression of hTERT was previously reported. The immortalized cells retain their original characteristics, maintain functional activities and have an extended lifespan (Figure 9) (Simonsen et al. 2002, Boecker et al. 2008).

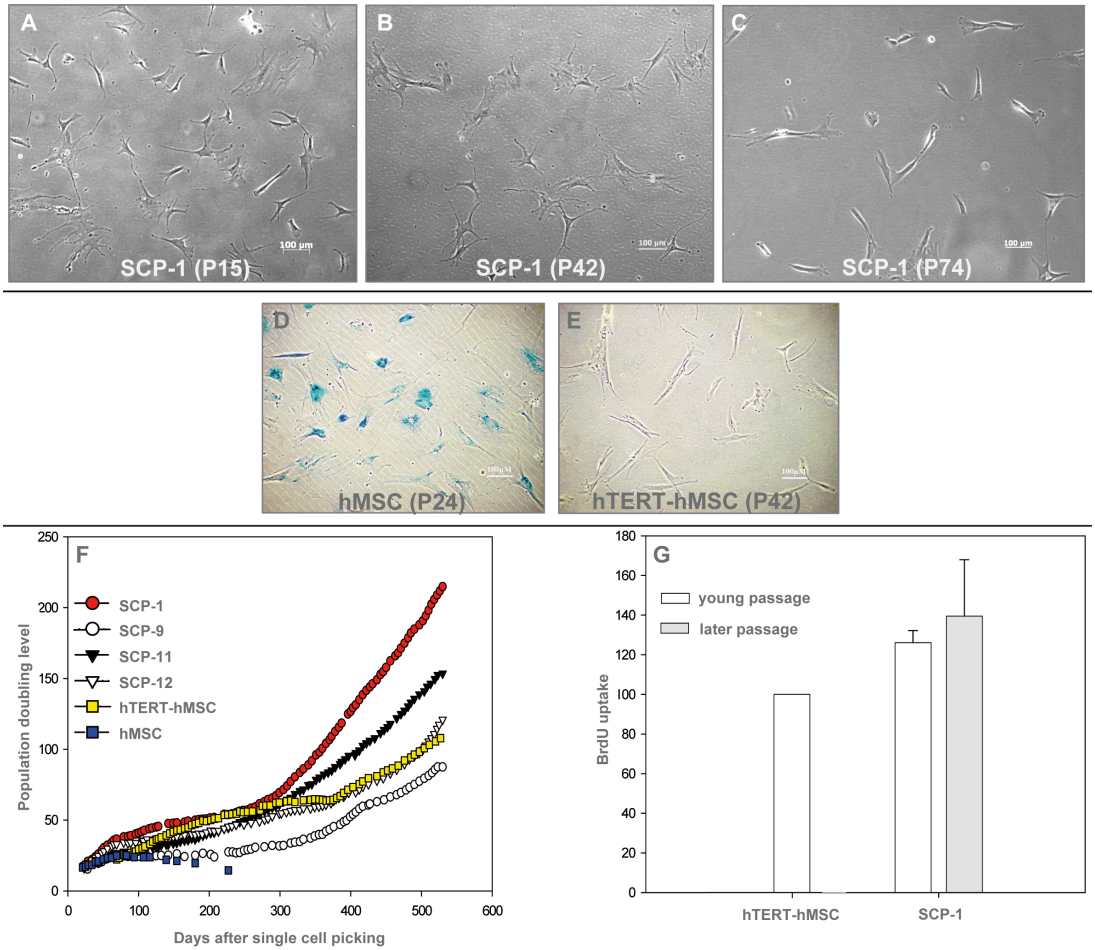


Figure 9. Important characteristics of the human mesenchymal stem cell line (SCP-1) expressing hTERT after lentiviral gene transfer. (A–C) Cell morphology at population doubling level (PDL) 31, 121 and 224 of SCP-1. (D, E) Senescence-associated β-galactosidase assay. Untransduced hMSC show pronounced β-galactosidase activity at PDL 24. In contrast, hTERT-transduced hMSCs had no β-galactosidase activity even at higher passages. (F) Growth curve of untransduced hMSC, heterogeneous hTERT-hMSCs and single-cell picked hMSC clones (G) BrdU assay of heterogeneous hTERT-hMSCs and SCP-1 showed a persistently higher proliferation of SCP-1 at young and later passages (Boecker et al. 2008).

However, in one of the studies few lines adopted a cancer stem cell-like phenotype and led to tumour formation in vivo probably due to spontaneous genetic changes (Burns et al. 2005). Opposedly, all of the obtained clonal cell lines from Boecker et al. 2008 had preserved their three-lineage differentiation potential, did not form tumours, and had normal karyotypes. In cases where neoplastic transformation of immortalized cells was reported it was mostly dependent on the viral vector used. The most common vectors used for immortalization of cells are retroviral and lentiviral vectors (see also chapter 5 Immortalization of cells - Transduction of tenocytes with hTERT avoids senescence).

1.4. Significance of performing research with tenocytes

From the tendon developmental and molecular biological point of view, a number of questions remain to be answered in this current situation where tendon related injuries are becoming more common and no entirely satisfying treatment regimen has been developed as yet. First, in order to find and conceptualize promising treatment strategies, the ontogeny of the tenocyte lineage has to be analyzed in more detail as today it is only beginning to be understood. Secondly, only recently have markers been found enabling scientists to discriminate between tenocytes and other mesenchymal cell types. Now due care has to be exercised using this knowledge in order to analyze healing processes and cell-based therapeutic approaches in closer detail. Thirdly, tendon cell research has been hampered by the lack of appropriate (especially human) cell lines allowing long-term molecular characterization of the tenocytes in vitro. This problem can be overcome by the establishment of immortalized tenocyte lines allowing studies on live cells over prolonged periods of time. Taken together, research on tendon tissue and the reparation of tendon defects has not tapped its full potential yet. Using approaches such as the ones described above, combining more sophisticated analytical methods with cell lines appropriate for analyses of long range, both the biological properties of tendons and factors positively influencing their repair can be understood better, helping researchers to come up with new and innovative treatment options for tendon defects that are so far difficult to tend to.

2. DEFINITION OF THE PROJECT

2.1. Main aim of the project

The main aim of the project was to establish and characterize a human tenocyte cell line by overexpression of the human telomerase reverse transcriptase gene using lentiviral transduction.

2.2. Milestones of the project

- A first goal was to culture and expand primary tenocytes originating from human tendon specimens.

- In a second step, primary tenocytes were to be infected with lentivirus expressing hTERT and hTERT over-expression and activity was to be determined.

- Thirdly, immunohistochemical analysis for the protein expression of hTERT was to be performed.

- Another crucial goal was to obtain and expand tenocyte clonal cell lines.

- Consecutively, the growth capacities of the hTERT-transduced tenocytes by quantifying population doubling times (growth curve) were to be determined.

- RT-PCR analysis for tenocyte specific markers such as Scleraxis, tenomodulin, type I and III collagen, COMP etc. had to be performed.

- Senescence of the cells had to be analyzed by performing senescence associated β-galactosidase assay.

- Finally, to test tumourigenicity of the cell line by applying in vitro tumour soft agar assay was one of the major goals of the project.

3. MATERIALS AND METHODS

3.1. Establishment of a primary culture of tenocytes

3.1.1. Isolation of human tenocytes

3.1.1.1. Tendon samples

Our laboratory is part of the Department for Surgery of the University Hospital in Munich. All our tendon samples were acquired during regularly scheduled operations in the hospital. The samples originate from several types of tendons (mostly of the foot) such as Flexor hallucis longus tendon and were taken from leftover material. Before the operation, informed consent from all patients was obtained. The ethical grant was accepted by the ethical commission of the medical faculty of the LMU Munich (ethical grant-nr. 166-08).

3.1.1.2. Isolation of tenocytes

The tendon samples were prepared with two different methods as published by Schulze-Tanzil et al. 2004 and Salingcarnboriboon et al. 2003.

Method 1: Immediately after the surgery the tendon sample was collected from the operation theatre in a sterile Falcon tube. First, it was transferred to 100 mm culture dish containing phosphate-buffered saline solution (PBS). Blood, other tissue remains and the epitenon membrane sheets were removed using sterile forceps and a scalpel. Using sterile scissors, the tendon was cut into small pieces of approximately 0,3 cm^2. All pieces were placed in 60 mm culture dishes containing 2 ml tenocyte growth media. The pieces were equally distributed in the dish. Dishes were placed in an incubator at 37° C and 5 % CO_2. The medium was changed every three to four days by carefully sucking off the old medium with a sterile glass pipette and replacing it.

Material: cell culture incubator (Ser. No. 39709355, Jouan, France), 100 mm culture dish (Falcon, USA), 60 mm culture dish (Falcon), Falcon tubes (Sarstedt, Germany), PBS - phosphate buffered saline (NaH_2PO4 H_2O; Na_2H_2PO4, $2H_2O$; NaCl Fa. Merck, Germany), surgical instruments, tenocyte growth medium (see 3.1.2.1.)

Method 2: All samples were acquired and prepared as described above. Instead of placing the tendon pieces in culture dishes they were put in a sterile 15 ml Falcon tube containing 5 ml of tenocyte growth medium + 1,5 mg/ml Collagenase II. The

tube was placed in an incubator at 37° C for 16-20 h depending on the size of the individual pieces. Subsequently, the digested sample was filtered through a sterile nylon mesh (70 μm) into a new sterile Falcon tube. Next, the filtrate was centrifuged at 1000 rpm for 5 min. The supernatant was discarded and the cell pellet was resolved in tenocyte growth medium. Finally, cells were counted and plated in culture flasks at about 30-40 % density.

Material: 100 mm culture dish (Falcon), 60 mm culture dish (Falcon), Falcon tubes (Sarstedt), nylon mesh (70 μm; Falcon) PBS (Merck), tenocyte growth medium (see 3.1.2.1.), Collagenase II (Worthington, USA)

3.1.2. Cultivation of human tenocytes

3.1.2.1. Cell culture

Tenocytes were cultivated in growth medium (see Table 2) in an incubator at 37° C and 5 % CO_2. Partricin was only used in the early culture period for about 2 weeks. As soon as the cells were proven to be negative for fungi, bacteria and mycoplasms Partricin was omitted.
Fetal Bovine Serum (FBS) was heat-inactivated prior to being added. Hence, 500 ml of FBS was slowly thawed at 4° C and subsequently inactivated for 20 min in a water bath at 56° C. After the heat-inactivation the serum was aliquotted again and stored at -20° C until utilization.

Table 2 – Growth medium of tenocytes

Supplements	Concentration	Volume for 500ml	Company
DMEM/HAM'S	84 %	420 ml	Gibco, USA
Fetal Bovine Serum (FBS)	10 %	50 ml	Sigma, Germany
MEM-AS	2 %	10 ml	Biochrom, Germany
Ascorbic acid-phosphate	1 %	5 ml	Sigma
Glutamine	1 %	5 ml	Biochrom
Penicilline/Streptomycin	1 %	5 ml	Gibco
Partricin	1 %	5 ml	Biochrom

3.1.2.2. High density cell culture

For high density cell culture 5000 tenocytes were seeded per cm^2 (6-well plate) and cultivated in tenocyte growth medium in an incubator at 37° C and 5 % CO_2. Medium was changed every 3-4 days. Cells were not passaged for more than 90 days.

3.1.2.3. Cell passaging

When a confluency of approximately 90 % was reached cell passaging was carried out as the cells were detached, counted and replated in cell culture flasks. All quantities refer to T75 (75 cm^2) culture flasks. For 60 mm (21 cm^2) and 100 mm (56 cm^2) dishes or T25 (25 cm^2) the amounts of cells were adapted according to the dish area.

Protocol for passaging tenocytes

1. Remove medium, wash cells with PBS
2. Detach cells with 2 ml of a trypsin/EDTA-Solution
3. After 7 min in the incubator at 37° C and 5 % CO_2 control detachment of cells by microscope
4. When all cells are detached, add 3 ml of tenocyte growth medium, mix thoroughly with a pipette and put solution in a 15 ml Falcon tube
5. Centrifuge at 500 rpm for 5 min
6. Suck off supernatant in order to remove the entire trypsin
7. Resuspend in growth medium, count the cell number and replate

Material: T75/T25 culture flask (Nunc, Denmark), pipette tips (Eppendorf, Germany), trypsin/EDTA-Solution (10x) (Cat. No. L11-003, PAA, Austria), Phosphate-buffered saline (PBS) w/o Ca^{2+}, Mg^{2+} (Cat. No. H15-002, PAA)

3.1.2.4. Determination and plating of a defined number of cells

Passaging, calculation of growth curves and other test procedures required the exact determination of cell number in cell solutions. Therefore as soon as the cells reached subconfluency they were trypsinized with 1x trypsin/ EDTA, stained by Trypan blue and counted with a Neubauer counting chamber. Trypan blue is a diazo dye and selectively colours dead cells blue, because it can not traverse the intact membranes of viable cells. The dye is used to distinguish viable from dead cells, so that the number of dead cells can be excluded.

After trypsinization, the cells were resuspended in growth medium (V: cell suspension volume). 50-100 µl of the cell suspension were transferred in a 1.5 ml

Eppendorf tube. Then 10 µl of the cell suspension was mixed with 5 µl of Trypan blue and 10 µl of this mixture was put under the cover glass of the counting chamber at the sample introduction point (see Figure 10).
Cells were counted using a microscope (10x magnification) while blue cells were excluded. Cell numbers of quadrants 1, 2, 4 and 5 and on the upper and left border of 1, 2, 4 and 5 were added together.
Cell number = [(1, 2, 4, 5)/4] × 10000 x 1,5 × V

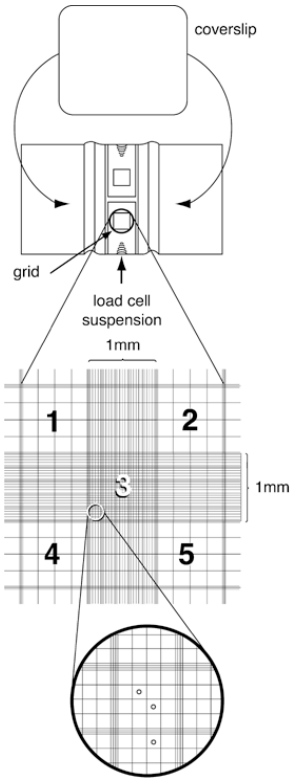


Figure 10. Improved Neubauer counting chamber, cover slip and counting grid with cells (Phelan 2007, Current Protocols)

Material: Trypsin/EDTA (Cat. No. L11-003, PAA, Austria), Improved Neubauer counting chamber (Brand, Germany), 0.4 % Trypan blue (Gibco, Cat. No. 15250-061, USA), tenocyte growth medium, Invert Microscope (Diavert, Leitz Wetzlar, Germany)

3.1.2.5. Cryoconservation of cells

Tenocytes can be long-term cryopreserved by storing them in a freezer at -80° C or in liquid nitrogen at -196° C. For these purposes the cells were resuspended directly after passaging in a special pre-cooled freezing medium Table 3.

Table 3. Freezing medium

Ingredients	Concentration	Company
Tenocyte growth medium	80 %	see Table 2
Dimethylsulfoxide (DMSO)	10 %	Sigma
Fetal bovine serum	10 %	Sigma

The cell number in the freezing medium did not exceed 0,5 x 10^5 - 1 x 10^6 cells/ml. As a last step the solution was pipetted in 2 ml cryovials (Nalgene, USA), then stored at -20° C for 2 h and at -80° C over night. The cells were stored for different periods of time at -80° C or in liquid nitrogen.

3.1.2.6. Cell thawing

When cells had been cryoconserved and had to be thawed for plating and culturing, the vials were incubated in a preheated 37° C water bath until the solution was fluid. The cells were then transfered in 5 ml growth medium and centrifuged for 5 min at 500 rpm. The supernatant was discarded and the cell pellet resuspended in 5 ml growth medium. Finally, the cells were plated in a culture flask.

Material: Tenocyte growth medium, water bath (GFL, Germany), 15 ml Falcon tube

3.1.3. Calculation of cumulative population doubling

The long-term growth behaviour of tenocytes was investigated by counting their cell number and calculating the cumulative population doubling level (PDL).
Population doubling (PD) was used to describe cell growth in a logarithmic mode.

The population doubling that was gained at each passage was determined using the following formula:

$$PD = [\log (Nn / No)] / \log 2, \text{ or}$$

$$PD = [\log (Nn / No)] \times 3.33$$

Nn = number of cells harvested at the end of a period of growth;

No = number of cells plated initially in the growth vessel.

Cumulative population doubling level (PDL) is thus the sum of population doublings:

$$PDL\ n = \Sigma\ PD\ (1, n)$$

Population doubling time (PDT) is the period of time required for the cells to double. To calculate the PDT, the time period in which the cells are growing is divided by the population doubling value that is gained in the same time period.

$$PDT = (\text{time period}) / PD$$

3.2. Cell transduction by hTERT lentivirus

3.2.1. Lentivirus production and transduction of human cells

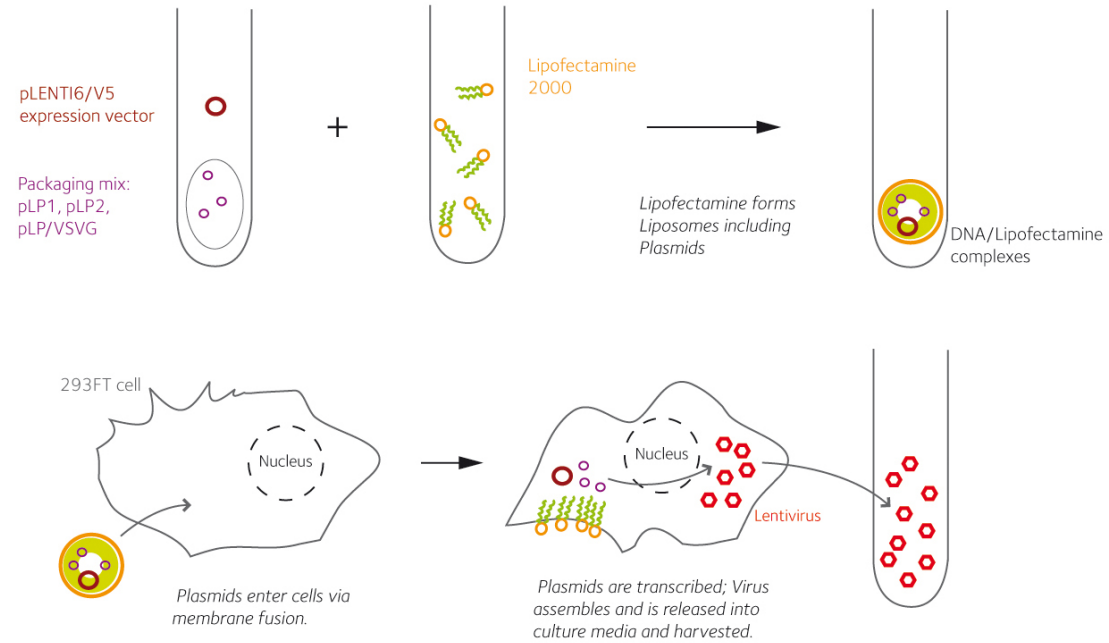
This part of the project was kindly carried out by Oliver Rossmann and Dr. Wolfgang Boecker in collaboration with Prof. Ulrich Koszinowski in the S2-laboratory facility of the Max von Pettenkofer-Institut of the University of Munich. To clone hTERT into the lentiviral vector the hTERT cDNA from pBABE-PURO plasmid was subcloned in pENTR11 plasmid (Invitrogen, Karlsruhe, Germany) by blunt end SalI/NotI ligation. Subsequently, the hTERT cDNA was transferred from pENTR11 into pLenti/V5-DEST by recombination reaction using LRClonase according to the manufacturer's protocol (Invitrogen).

The correct sequence of the resulting pLentiV6/V5-hTERT was confirmed by sequencing (Sequiserve, Vaterstetten, Germany). Figure 11 shows schematically the lentiviral expression construct.

Figure 11. Plasmid chart of pLenti6/V5-hTERT (lentiviral expression construct). LTR: long terminal repeat, RRE: rev-responsive element, P_{CMV}: CMV-promoter, P_{SV40}: SV40-promoter, EM7: prokaryotic promoter, BSD: blasticidin resistance gene.

Among the seven different tenocyte donors, two were selected, based on their morphology to be infected with hTERT. In the following scheme the basic principles of the infection are depicted (Figure 12). For the lentivirus production the ViraPower lentiviral expression system (Invitrogen) was used and carried out according to Boecker et al. 2007, J Gene Med. Briefly, plasmid DNA-Lipofectamine complexes were added to a 293FT cell suspension (23.76 x 10^6 total cells) in a T-225 tissue culture flask. Two days after transfection, the virus-containing supernatant was harvested. The transduction of human tenocytes was accomplished with hTERT lentivirus (MDI: 5 x 10^4) in the presence of 6 µg/ml polybrene (hexadimethrine bromide, Sigma). Afterwards the infected cells were selected by adding growth medium containing blasticidin (10 µg/ml). After 7 days of selection, the cells were cultured in normal tenocyte growth medium and the cell culture supernatant was tested to be viral particle free by p24 ELISA.

A



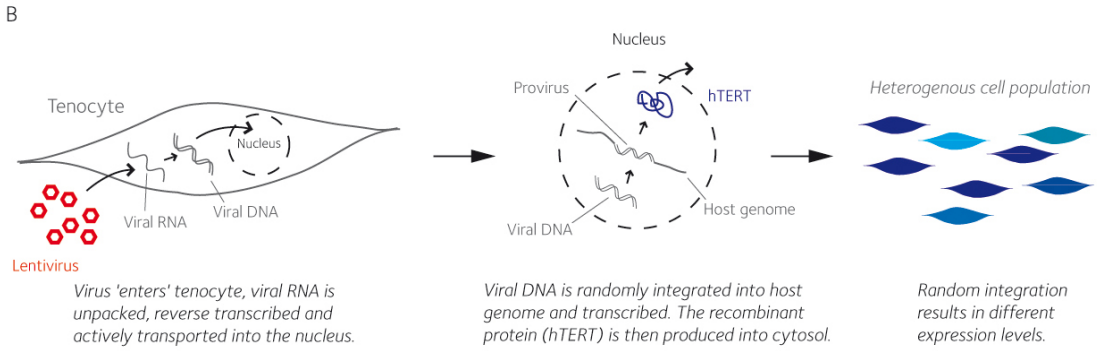


Figure 12. Lentiviral production using a lentiviral vector. (A) Transfection. (B) Transduction. The details of each process are depicted in the figure.

Materials: 293FT cell line (Cat. No. R700-07; Invitrogen, Germany), ViraPower lentiviral packaging mix (Cat. No. K4975-00, Invitrogen), blasticidin S HCl (Invitrogen), 293FT culture medium [10 % (v/v) FBS ,1 % Pen/Strep, 0,1 mM MEM non-essential amino acids (Invitrogen) ad D-MEM (PAA)], tenocyte culture medium, Falcon tubes (Sarstedt), culture flasks (Nunc), Lipofectamine 2000 (Cat. No. 11668-027, Invitrogen)

3.2.2. Single cell cloning

In order to obtain hTERT tenocyte clones out of the heterogeneous cell culture, single cell cloning was performed. Three different methods were applied:

Single cell picking: Five days prior to picking, cells were seeded at a concentration of 75 cells per cm² in a T25 culture flask. At the beginning of the procedure 100 µl of medium was added to each well of a 6-well-plate. Cells were trypsinized for 5 min at room temperature and resuspended in 5 ml of medium. 1 ml of the suspension was further diluted in 9 ml of PBS (1:10 dilution). Subsequently, 5 ml of the dilution were pipetted into a 100 mm petri dish. Under a microscope single cells were picked with a 10 µl pipette (adjusted to 3 µl) and placed in the 6-well-dishes.

The single cells were let to attach to the surface of the culture dish, 4 hours later the wells were controlled under the microscope and the region where the cell had attached was marked on the bottom of each well. Finally, 2 ml of medium was added to each well. This procedure was kindly performed by Tzvetan Popov.

Colony forming: The cells were seeded at a very low density of 15 cells per cm^2. Every day the dish was checked for single but dividing cells. As soon as a colony evolved from one single cell, a plastic ring was put around the colony and was locked into position with sterilized Vaseline. The colony in the ring was then trypsinized and moved in a well of a 6-well-plate for further expansion.

Serial dilution: Briefly, this method is based on an increasing dilution of a tenocyte cell suspension, until 1 cell/200 µl is obtained and it is placed in one well of a 96-well plate. For further details see the protocol below:

1. Remove culture medium and wash cells with 5 ml PBS per T-75 flask.
2. Trypsinize cells with 2 ml 1x Trypsin/EDTA solution for 5 min at RT.
3. Take up the cells with 5-6 ml culture medium and resuspend well in 50 ml Falcon tube -> solution S (starting solution).
4. Count cells as described in 3.1.2.3.
5. Dilute solution S to 1000 cells/ml in a new 50 ml Falcon tube -> solution A (include an additional dilution step of 1:10 if the number of cells in solution S exceeds 100 000/ml -> Solution S1).
6. In a new 15 ml Falcon tube add 1 ml of solution A to 9 ml of culture medium (1:10 dilution) to achieve a cell concentration of 100 cells/ml -> solution B.
7. In a new 50 ml Falcon tube add 2 ml of Solution B to 38 ml of culture medium (1:20 dilution) to achieve a cell concentration of 5 cells/ml = 1 cell/200 µl -> solution C
8. Transfer solution C to a reagent basin and with the help of a multi-channel pipette, add 200 µl of the solution to each well of the 96-well dish. Retain well A1 (positive control) to add non diluted cell suspension for the adjustment of the microscopic level during the observation of the plate.
9. Grow cells in cell culture incubator under standard conditions.
10. The 96-well plate can be examined the day after. Wells with one cell are identified and marked. Cells from these wells are expanded as an individual clones.

Materials: 6-well-plate (Nunclon, Denmark, Cat. No. 140675), 96-well-plate (Nunclon), 15 and 50 ml Falcon tubes, 1x trypsin/EDTA-solution PBS, vaseline, plastic rings, 100 mm and 60 mm petri dishes, reagent basin, multi-channel pipette, 10 µl and 200 µl pipette tips, cell counting materials

3.3. Methods for analysis

3.3.1. RT-PCR

3.3.1.1. RNA isolation

The RNA isolation was performed following the standard protocol from Qiagen (RNeasy Mini Handbook 04/2006) using the provided materials in the RNeasy Mini Kit from Qiagen. As recommended in the handbook cells were lysed with 600 µl lysis buffer per T75 at a confluency of approximately 80 - 90 % then scraped from the surface of the culture flask and thoroughly mixed with the buffer. Not more than 1 x 10^6 cells were used for the procedure.

Material: RNeasy Mini Kit (Cat. No. 74106, Qiagen, USA), cell scraper (Sarstedt), lysis buffer (Buffer RLT + 14.3 M β-mercaptoethanol)

3.3.1.2. cDNA Synthesis

For cDNA synthesis the Cloned AMV First-Strand cDNA Synthesis Kit for RT-PCR from Invitrogen was used. The procedure was carried out according to the manufacturers instructions. 1 µg of total RNA was used for cDNA synthesis and a volume of 20 µl cDNA solution was obtained.

Material: Cloned AMV First-Strand cDNA Synthesis Kit (Cat. No. 12328-040, Invitrogen), PCR thermocycler PTC-200 (Bio-Rad, Germany)

3.3.1.3. Polymerase chain reaction

For Polymerase chain reaction primer sequences were either self-designed with the Clone Manager program (Scientific & Educational Software, USA) or adopted from publications.

The primers were ordered from Invitrogen diluted to a concentration of 10 pmol/µl. The components of a basic PCR set up are depicted in Table 4.

Table 4. PCR components

Reagent	Volume per sample (µl)
10x Puffer	2
MgCl	0,6
dNTP	0,4
Primer forward	0,5
Primer reverse	0,5
Q-Solution	4
Taq Polymerase (Cat. No. 10342-020, Invitrogen)	0,2
H_2O	10,8
cDNA	1

Table 5. Standard PCR program (the steps in the box were repeated for 30-40 cycles)

Temperature	Time	Step
95°C	3 min	denaturation
95°C	30 sec	
45-65°C	30 sec	annealing
72°C	1 min	elongation
72°C	10 min	
4°C	forever	

The PCR was performed in the PCR thermocycler PTC-200 using standard PCR program (Table 5). The program (annealing temperature and cycle number) was adapted individually for each primer set (Table 6).

Table 6. Primer sequence and reaction conditions

Target gene	Primer (forward/reverse)	Annealing temp.(°C)	Product size (bp)	Cycles	Reference
Collagen I	5'-CCAGTCACCTGCGTACAGAA-3'	54	223	40	Gronthos et al. 2006
	5'-GAGACCACGAGGACCAGAAG-3'				
Collagen III	5'-CCC ACT ATT ATT TTG GCA CAA CAG-3'	50	99	38	Shigeyama et al. 2005
	5'-GCA TGG TTC TGG CTT CCA GA-3'				
COMP	5'-GCT CTG TGG CAT ACA GGA GA-3'	53	145	40	Tian et al. 2006
	5'-CAT AGA ATC GCA CCC TGA TG-3'				
Decorin	5'-GCT GGA CCG TTT CAA CAG AGA G-3'	54	437	40	Chung et al. 2002
	5'-TCA TTC TCA TGG GCA CGC AG-3'				
EphA4	5'-CCA ACC AAG CAG TGC GAG AG-3'	54	385	35	self-designed
	5'-CCA GAC CCA ATG CCA CGA AG-3'				
Eya1	5'-GTT CAT CTG GGA CTT GGA-3'	46	229	40	Okada et al. 2006
	5'-GCT TAG GTC CTG TCC GTT-3'				
GAPDH	5'-CAA CTA CAT GGT TTA CAT GTT C-3'	50	181	30	Takahashi et al. 2003
	5'-GCC AGT GGA CTC CAC GAC-3'				
hTERT	5'-CTA CGG CGA CAT GGA GAA C-3'	54	413	35	Boecker et al. 2008
	5'-GAC ACT TCA GCC GCA AGA C-3'				
Scleraxis	5'-CCT GAA CAT CTG GGA AAT TTA ATT TTA C-3'	48	294	38	Schulze-Tanzil et al. 2004
	5'-CGC CAA GGC ACC TCC TT-3'				
Six1	5'-TGC TTG TTG GAG GAG GAG TT-3'	55	206	35	Ng et al. 2006
	5'-AAG GAG AAG TCG AGG GGT GT-3'				
Tenascin C	5'-GAG AAA GGC AGA CAC AAG AG-3'	57	395	35	self-designed
	5'-GCA GTC CAG TTG AGT TTG AG-3'				
Tenomodulin	5'-GGA TAC ACT GGC ATC TAC TTC G-3'	48	381	45	self-designed
	5'-GTG ACG GGT CTT CTC TAC TTT C-3'				

3.3.1.4. Agarose gel electrophoresis

For analysis of PCR products gel electrophoresis was used. Agarose gels (0,8 – 1,2 %) were produced and 6 µl of ethidium bromide dye was added. The solid gel was covered with TAE buffer. The PCR samples (20 µl) were mixed with 6x Orange loading dye, loaded on the gel and run at 100 V current. Ethidium bromide intercalates into DNA and allows visualization of the separated PCR fragments. After the electrophoresis, the gel was pictured with a gel imager (Transilluminator, Vilber Lourmat, Germany).

Material: Agarose (Biozym Scientific, Germany), TAE buffer 50x [242 g/l tris-base, 57,1 ml/l acetic acid, 100 ml/l EDTA (500mM, pH 8,0)], ethidium bromide (Merck), 6x Orange loading dye (Fermentas, Canada), 100 bp DNA ladder (Invitrogen), gel chamber (Peqlab, Germany), machine for electrophoresis (Consort E132, Peqlab)

3.3.2. Immunocytochemistry

3.3.2.1. Fixation of cells

Cells were cultivated in plastic chambers affixed to a glass microscope slide. When the cells had grown to the desired confluency (50-60 %) they were fixed with 100 % methanol. Tenocyte growth medium was sucked off, the cells were washed with 2 ml of PBS and 2 ml of methanol was added for 10 min at -20° C. Afterwards the cells were washed with PBS for 10 min at room temperature. Finally, the methanol was removed and the slide with the cells was dried at room temperature. The fixed cells were then stained immediately or stored at -80° C.

Material: BD Falcon CultureSlides, methanol (Merck, Germany), PBS

3.3.2.2. Immunofluorescence

The slides were subdivided into fields using a Dako pen. Thus, different concentrations of primary antibody were applied and part of the slide was used as a negative control where only secondary antibody was applied. The staining is described below:

1. Rehydrate cells 5 min in PBS supplemented with 0.1 % Tween
2. Blocking with 3 % bovine serum albumin (BSA) in PBS for 60 min at room temperature
3. Remove blocking solution by tapping it off
4. Application of primary antibody Anti-hTERT 1:10 in PBS (200 µl per field); negative control just with PBS or blocking solution
5. Incubate over night at 4° C in humidified chamber
6. Wash 3x5 min in PBS supplemented with 0.1 % Tween
7. Application of secondary flourescent antibody (diluted in PBS) – Alexa 546 1:500
8. Incubate for 60 min at room temperature in a humidified dark chamber
9. Wash again 3x5 min in PBS supplemented with 0.1 % Tween
10. Application of 200 µl DAPI (1:10000 dilution with aqua dest.; nuclear staining)
11. Incubate for 1 min at room temperature protected from light
12. Wash 2x5 min in PBS supplemented with 0.1 % Tween
13. Place mounting medium on the slide and with the help of a preparative needle put the cover glass in place (try to avoid trapping of bubbles). Keep the slides at 4° C
14. Perform microscopical analysis and take photographs

Material: Histological jars, primary antibody: Anti-hTERT (348-358) Rabbit pAB (Calbiochem, Germany, Cat.No.PC563), secondary antibody: Alexa Fluor 546 goat anti rabbit (Invitrogen, Cat.No.A11035), Dako pen (Dako, Denmark), 3 % BSA (0,3 g/100 ml; Sigma), DAPI (4,6-diamino-2-phenylindole, dihydrochloride; Molecular Probes), Tween 20 (Merck, Germany), mounting medium (6 g glycerin, 2.4 g Mowiol 4-88 (Calbiochem), 6 ml aqua dest., 12 ml 0.2 M Tris pH 8.6, 0.024 g, 1,4-Diaza-bicyclo[2.2.2]octane (DABCO; Fluka, Germany), Axioskope microscope (Carl Zeiss MicroImaging GmbH, Germany)

3.3.3. Senescence-associated β–galactosidase assay

In order to detect β-galactosidase activity a Senescent Cells Staining Kit (Sigma) was used. The histochemical stain is based on the artificial substrate X-gal that forms a local blue precipitate upon cleavage with β-galactosidase at pH 6 (Itahana et al. 2007). For this experiment untransduced tenocytes, hTERT-transduced tenocytes and hydroxyurea-treated hMSCs (positive control) were used. Yeo et al. 2000 positively tested hydroxyurea as an inductor of senescence in vitro. During H_2O_2-dependent peroxidation of hydroxyurea, NO is generated which inhibits ribonucleotide reductase and as a consequence of this a lack of deoxynucleotides required for DNA sythesis. In the end this results in DNA single strand breaks leading to cell cycle arrest (Sato et al. 1997, Jiang et al. 1997, Mata et al. 1989). HMSC were treated with 2 mM hydroxyurea in growth medium for 48 hours according to Yeo et al. 2000.

Operating procedure:

1. Aspirate the growth medium from the cells (6 well dish, 60 % density).
2. Wash the cells twice with 1 ml of 1x PBS per well.
3. Add 1.5 ml of 1x Fixation Buffer per well and incubate the plate for 6–7 minutes at room temperature.
4. Rinse the cells 3 times with 1 ml of 1x PBS per well.
5. Add 1 ml of the Staining Mixture per well. Incubate at 37° C without CO_2 for approximately 12 hours.
6. Replace Staining Mixture with 70 % glycerol solution and seal the plate with Parafilm to prevent it from drying out.
7. Observe cells under a microscope, count all blue stained cells as well as the total number of cells and take example pictures.

Material: Senescent Cells Staining Kit (Sigma, Cat. No. CS0030, USA)
Fixation Buffer 10x (Product Code F 1797) Solution containing 20 % formaldehyde, 2 % glutaraldehyde, 70.4 mM Na2HPO4, 14.7 mM KH2PO4, 1.37 M NaCl, and 26.8 mM KCl, Reagent B (Product Code R 5272) 400 mM Potassium Ferricyanide, Reagent C (Product Code R 5147) 400 mM Potassium Ferrocyanide, X-gal Solution (Product Code X 3753) 40 mg/ml, Ultrapure water (Sigma, Cat. No. W3500-1L, USA), 6 well plate (Nunclon, Denmark, Cat. No. 140675), PBS, hydroxyurea (Sigma), hMSC growth medium AlphaMEM (Invitrogen)

3.3.4. Telomerase activity test

To demonstrate the telomerase activity in hTERT-transduced tenocytes, a photomeric enzyme immunoassay for quantitative determination of telomerase activity (Telo TAGGG Telomerase PCR ELISA^{PLUS}, Roche; Figure 13) was utilized.

1. Telomerase adds telomeric repeats (TTAGGG) to the 3' end of the biotin-labelled synthetic P1-TS-primer (artificial template).
2. Elongation products are amplified by PCR using the primers P1-TS and P2, generating PCR products with the telomerase specific 6 nucleotide increments.
3. An aliquot of the PCR product is denatured and hybridized to a digoxigenin-(DIG)-labelled, telomeric repeat-specific detection probe.
4. The resulting product is immobilized via the biotin labelled primer to a streptavidin-coated microplate. The immobilized PCR product is then detected with an antibody against digoxigenin (anti-DIG-POD) that is conjugated to peroxidase.
At last, the peroxidase metabolizes TMB (3,3',5,5'-tetramethylbenzidine) in order to form a coloured reaction product. The extinction can be measured at 450 nm (with a reference wavelength of approximately 690 nm).

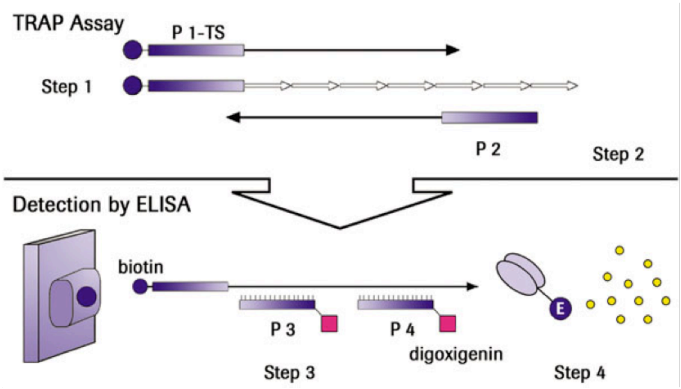


Figure 13. Scheme of the different steps of the telomerase activity test. TRAP: Telomeric Repeat Amplification Protocol - the telomerase-reaction product is amplified by PCR (Roche Applied Science, Cat. No.11854666910 2008)

All procedures of the assay were carried out accurately following the protocol provided by the company. Measurements were performed in triplets.
A pellet of 2 x 10^5 cells (HTD2, HTD5, HTD2 hTERT and HTD5 hTERT, all at passage 10) was used per single reaction. After washing in PBS the cell pellet was resuspended in 200 µl of lysis reagent before performing the TRAP reaction from 3 µl of the cell extract. 293FT cells served as a positive control as this is a permanent cell line (established from primary embryonal human kidney transformed with sheared human adenovirus type 5 DNA) and exhibits a high telomerase activity (Graham et al. 1977).

Materials: Telo TAGGG Telomerase PCR ELISA^{PLUS} (Roche Applied Science, Cat. No.11854666910, Germany), ELISA reader (Microtek Laborsysteme GmbH, Germany)

3.3.5. Soft agar assay

The soft agar assay was performed according to Boecker et al. 2008 using 6-well plates. In total 4 x 2500 cells per donor were seeded. HT1080 cells (fibrosarcoma cell line) were employed as positive controls and untransduced tenocytes as negative controls.

1. Prepare 0.5 % base agar solution: mix DMEM/tenocyte culture medium (+ 20 % FBS) with agarose and microwave until the agarose has dissolved. Let it cool down in water bath at 40°C.
2. Fill in 6-well plate (1 ml/well).
3. Prepare 0.35 % top agar solution: mix DMEM/tenocyte culture medium (+ 20 % FBS) with agarose and microwave until the agarose has dissolved. Let it cool down in water bath at 37°C.
4. Trypsinize and count cells, harvest 2500 cells/well.
5. Resuspend harvested cells in 0.35 % top agar solution (37° C) and aliquot on top of base agar layer (0.5 ml/well).
6. Incubate cells for 28 days at 37° C with 5 % CO_2.
7. Feed cells 1-2 times per week with 0.5 ml of culture medium per well.
8. After 28 days stain colonies with 0.005 % crystal violet for 1 h.
9. Observe cells under a microscope, count cells/colonies and take pictures.

Material: agarose (Biozym Scientific), 6-well-plates (Nunclon), tenocyte growth medium, DMEM culture medium (PAA), FBS, crystal violet (Sigma), light microscope (Axiovert 100, Carl Zeiss), water bath (GFL, Germany), microwave (Siemens, Germany)

3.4. Microscopy

Cell morphology was observed throughout the culture period by the inverted phase-contrast microscope Axiovert S100 (Carl Zeiss, Germany). Immunocytochemistry was analysed with the epifluorescence microscope Axioskope2 (Carl Zeiss). Images were acquired with a Zeiss black and white digital camera (AxioCam MRm; 1388 x 1040 pixels) and processed with the Zeiss AxioVision software (AxioVs40 V 4.7.1-08-2008).

3.5. Statistical analysis

Statistical analysis was performed using SigmaPlot version 8 (SPSS, Germany). Significances were calculated using Student's t-test. A value for $P < 0.05$ was considered significant.

4. RESULTS

4.1. Primary culture of tenocytes

4.1.1. Tendon donors

The properties of the tendon donors, such as the age of the patients, the type of tendon and the size of the sample are shown in Table 7. An anonymisation of the samples was implemented, giving each a new specific identification code: HTD, with H standing for human, T for tendon and D for donor. In total 10 specimens were obtained but only from 7 donors we gained cells. Two donors were used in the following part of the study (HTD2 and HTD5).

Table 7. Donor properties

Donor	Age	Type of tendon	Size [mm]
HTD2	58	Flexor tendon toe	2x5
HTD3	43	Flexor tendon toe	3x10
HTD4	62	Extensor hallucis longus	4x15
HTD5	60	Achilles tendon	5x15
HTD6	62	Flexor tendon toe	4x20
HTD7	52	Peronaeus longus	10x15
HTD9	50	Cruciate ligament	15x10

4.1.2. Tenocyte outgrowth and tendon digestion

Approximately 14 days after plating the tendon pieces in culture dishes, tenocytes started to migrate out (Figure 14). Once the area around the tendon pieces was filled with a dense layer of cells, the tissue was removed from the culture dishes. Subsequently, the adherent cells in the dish were trypsinized and replated after counting in a cell culture flask (T25) at a density of around 1000 cells/cm². Further, the cells were cultivated and passaged as shown in 3.1.2.2.

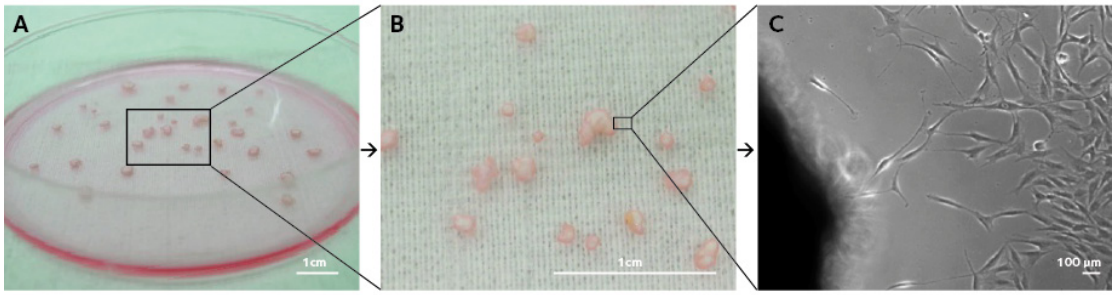


Figure 14. Culture dish with tendon pieces (A and B) and outgrowth of tenocytes (C).

The digestion of tendon tissue allowed the extraction of tenocytes within a period of approximately 4 hours. However, the cells were less homogeneous than the ones gained by outgrowth. We therefore refrained from using the cells extracted from the tendon tissue by digestion for further investigation.

4.2. hTERT-expression

4.2.1. hTERT RT-PCR

Lentiviruses containing the gene of hTERT were generated from the lentiviral expression construct pLenti6/V5-hTERT. The pLenti6/V5-DEST-vector contains a blasticidin resistance gene for blasticidin-S deaminase allowing a selection of transduced cells, proving the successful infection. At passage 4 two donors, HTD2 and HTD5, were infected.

Analyzing the infected cells on RNA-level principally proved the successful outcome of the HTERT-transduction of the primary tenocytes. To assess the functionality of the inserted gene, overexpression of hTERT in tenocytes was also proven on a protein level. First, the hTERT expression was proven by RT-PCR in both donors (HTD2 hTERT and HTD5 hTERT).

To show that the transduced tenocytes do not lose the hTERT transgene over time, hTERT-tenocytes at passage 8 and 16 were tested as well. In the RT-PCR, at both time points a strong expression of hTERT mRNA was detected in hTERT-transduced tenocytes, while no hTERT expression was detected in untransduced tenocytes (Figure 15).

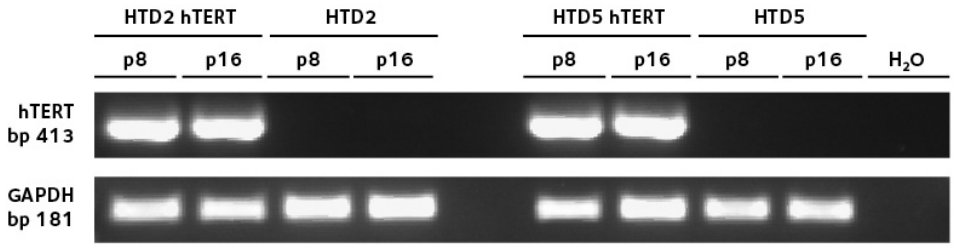


Figure 15. hTERT expression was detected in hTERT-transduced tenocytes, but not in untransduced control tenocytes.

4.2.2. hTERT immunocytochemistry

Using immunocytochemistry the expression of hTERT was proven on a protein level (Figure 16).

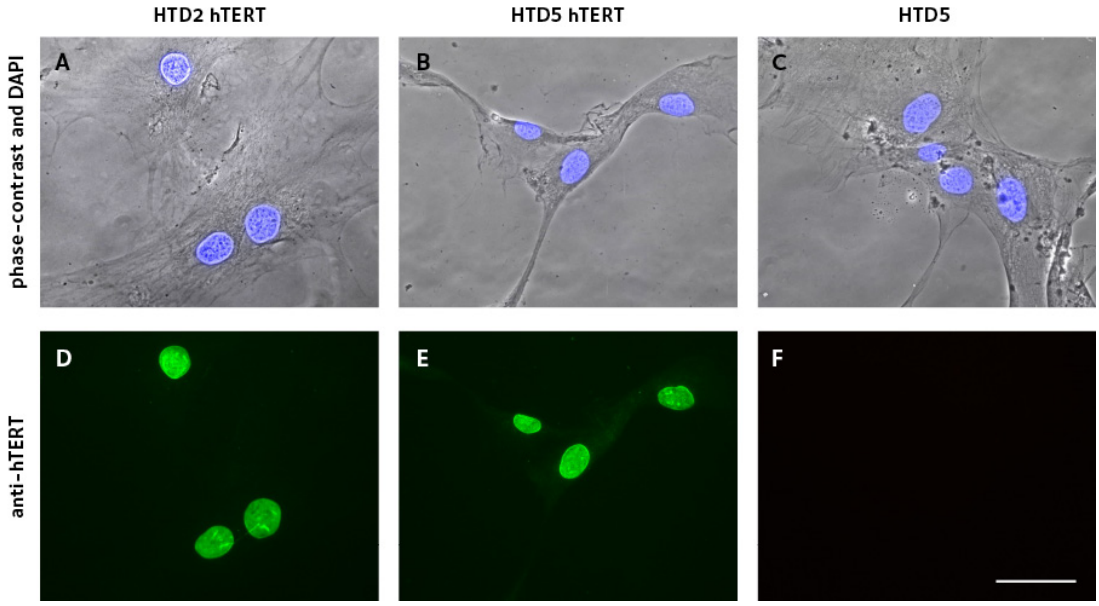


Figure 16. Immunocytochemical staining of HTD5 (p 9) and HTD2 hTERT (p 10) and HTD5 hTERT (p9). A, B, C phase contrast and DAPI nuclear staining (blue). D, E, F hTERT staining (green). Secondary antibody: Alexa 546 goat anti rabbit.
Bar: 100 µm.

HTERT protein was primarily located within the nucleus of the hTERT-transduced tenocytes, while no hTERT expression was detected in untransduced control tenocytes.

4.2.3. Analysis of telomerase activity

The telomerase activity assay revealed consistent results with RT-PCR and immunocytochemistry. HTD2, HTD5, HTD2 hTERT and HTD5 hTERT (2 x 10^5 cells, all passage 10) were tested for telomerase activity with a photomeric enzyme immunoassay (Figure 17).

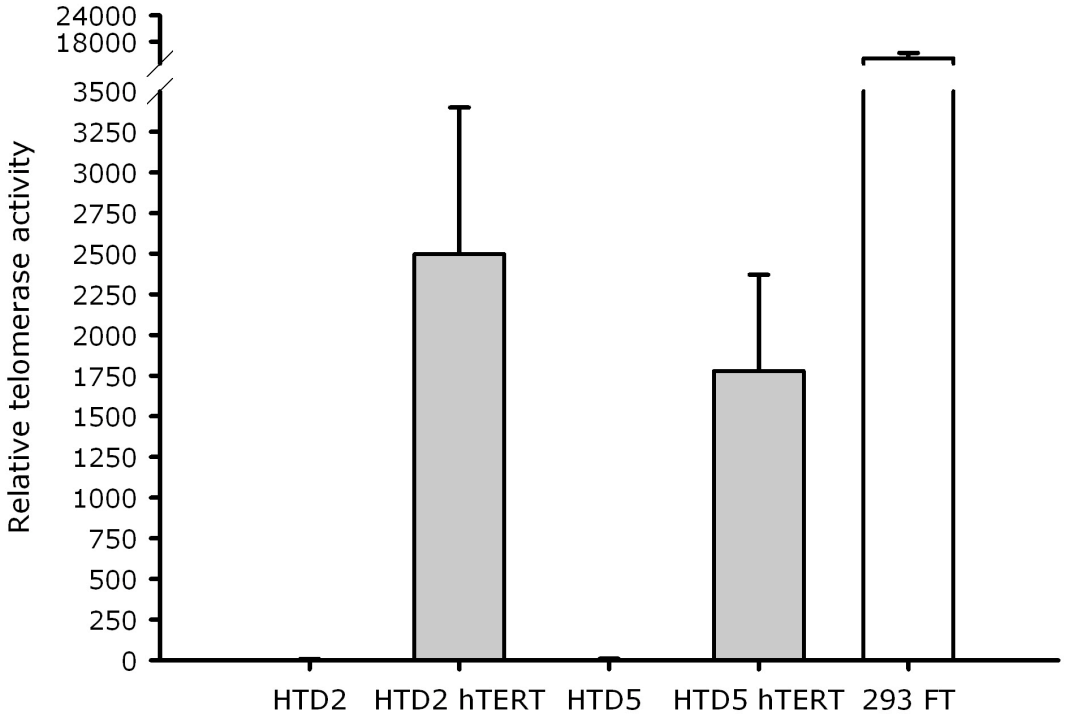


Figure 17. Telomerase activity assay. HTERT-tenocytes (HTD2 hTERT and HTD5 hTERT) have significant higher relative telomerase activity ($P < 0,05$). Untransduced tenocytes (HTD2 and HTD5) show merely a background level of telomerase activity. 293 FT cells: positive control. Error bars: standard mean of samples in triplicates.

The results demonstrate that untransduced human tenocytes had only background levels of telomerase activity, while hTERT-overexpressing cells showed significantly higher levels of telomerase activity. 293FT cells served as positive control.

4.3. Single cell cloning

In order to obtain a single cell-derived tenocyte cell line, three different methods were applied: single cell picking, colony forming unit assay and serial dilution. Under standard culture conditions isolated tenocyte clones did not proliferate (see Table 8). Therefore different approaches were used to improve the attachment, growth and survival of single cells:

As a first approach FBS was increased to 20 % in tenocyte culture medium in order to supply the single cell with more nutrients. Another strategy was coating the surface of the culture dishes with type I collagen (approximately 4 $\mu g/cm^2$) in order to aid adherence of the single cell. Thirdly, a special 6-well culture dish insert was

implemented. The insert has a porous ground area on which cells adhere while factors that are excreted by the cells can pass the membrane and are dissolved in the medium. Thus, certain (growth) factors secreted by a heterogeneous hTERT-transduced tenocyte population can reach the single cell on the bottom of the well (Figure 18).

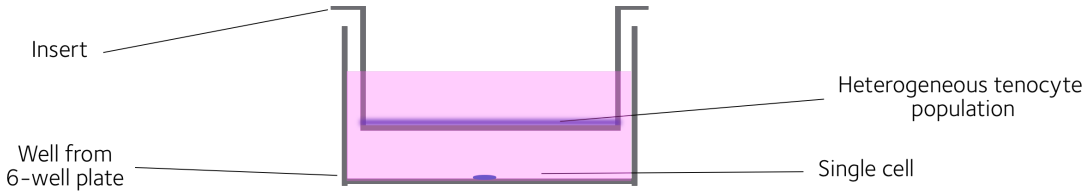


Figure 18. Scheme of culture dish inserts. A single cell was picked and seeded on the bottom of a 6-well culture dish. Heterogeneous tenocytes were seeded on the insert. The well is filled with culture medium.

Table 8. Summary of single cell cloning

Method	Nr. of experiments	Efficiency	Clonal production (>12 cells)	Clonal production (< 12 cells)
Single cell picking	5 (206 single cells picked)	> 85 %	none	10
Colony forming	3	-	none	none
Serial dilution	3	45 %	none	5

All runs of single cell picking, colony forming and serial dilution experiments did not lead to a proliferating single cell clone. If the cells divided at all, they ceased to proliferate after 2-3 divisions (Table 8). After approximately 14 days the cell morphology changed from laterally stretched and spindle shaped to round and flattened before the cells detached.

4.4. Growth kinetics

Whereas both donors of the tissue samples were almost the same age the disparity of tendon type and sex might lead to different growth behaviour. The growth characteristics of heterogeneous hTERT-expressing human tenocytes (HTD2 hTERT and HTD5 hTERT) and untransduced human tenocytes (HTD2 and HTD5) during prolonged culture were monitored, by calculating the population doubling level

(PDL). Both untransduced donors showed initially a higher proliferation rate than the hTERT-tenocytes. After 18 (HTD2) and 9 (HTD5) PDL the growth curve reached a plateau phase indicating the transition into a senescent state. The proliferation rate of hTERT-transduced tenocytes in the beginning was lower (12 PDL HTD2 hTERT and HTD5 hTERT) but both donors continued to proliferate for a time period of 300 days (Figure 19 and Figure 20).

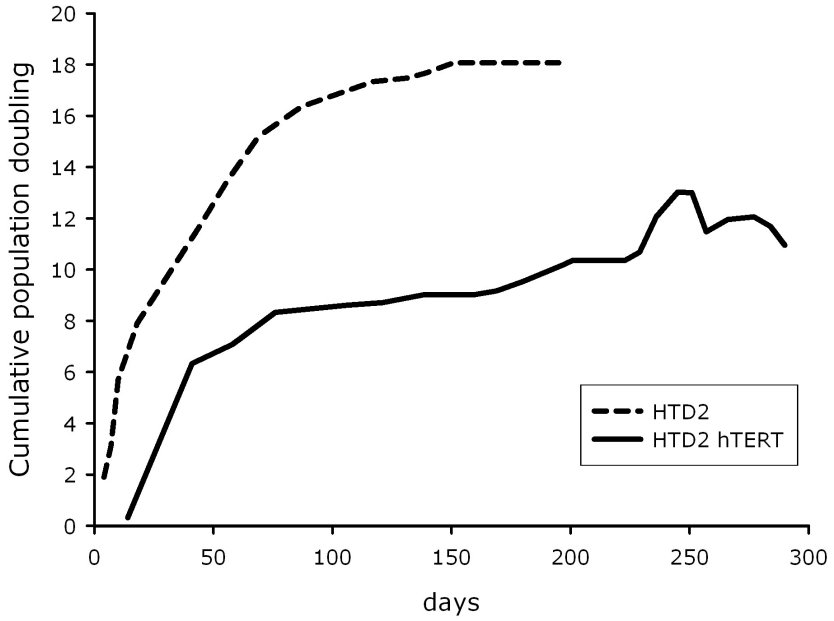


Figure 19. Growth curve of HTD2 and HTD2 hTERT. Complete growth arrest and senescence of HTD2 cells after around 160 days.

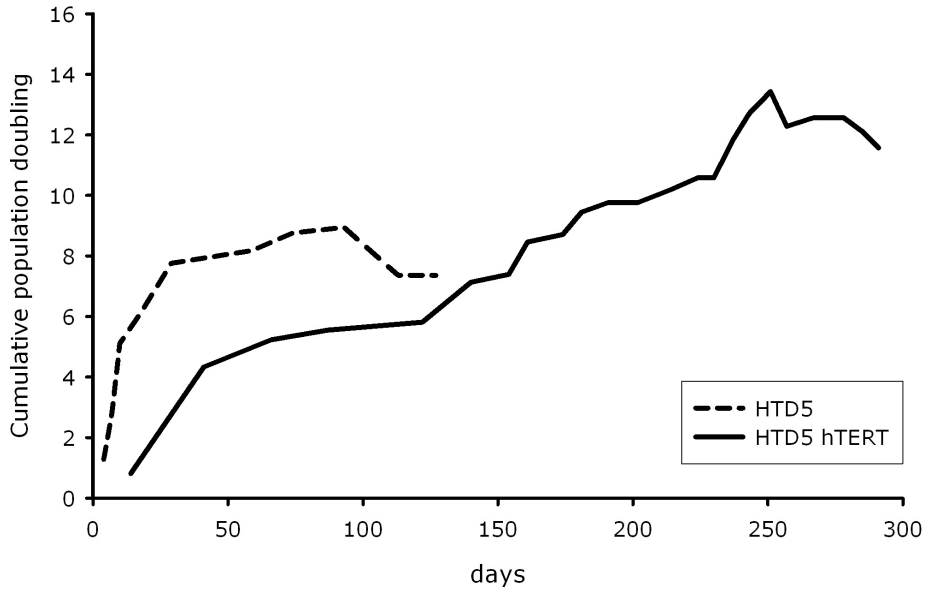


Figure 20. Growth curve of HTD5 and HTD5 hTERT. Complete growth arrest and loss of HTD5 cells after 100 days.

4.5. Cell morphology of primary and hTERT-transduced cells

The cell morphology of hTERT-expressing and control tenocytes was monitored throughout the culture period as cells were photomicrographed regularly (Figure 21). For a better classification the individual cell passages were linked to three growth stages: early (≤10 passages), middle (11-19 passages) and late (≥ 20 passages).

Human tenocytes in early passages appeared thin, bi-polar and spindle-shaped. During prolonged culture, the cell shape changed into a flat and outstretched appearance suggesting that the cells underwent senescence. The untransduced human tenocytes started to alter their morphology at 8 (HTD5) or 18 (HTD2) PDL respectively. In contrast HTD2 hTERT and HTD5 hTERT retained a spindle-shaped appearance for nearly one year (12 PDL). At the end of this period hTERT-transduced tenocytes altered their morphology from a slender spindle-shaped form to a more flattened appearance, while at the same time, their growth capacity decreased markedly (Figure 22).

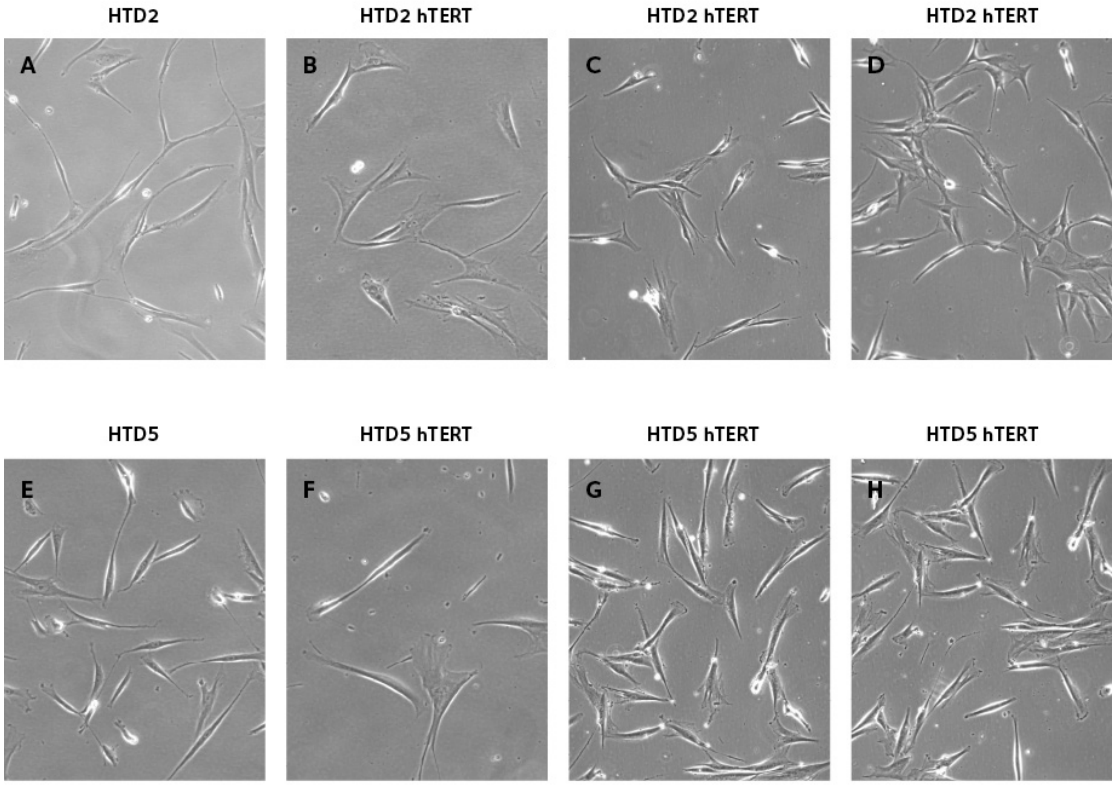


Figure 21. Cell morphology at different phases: early, middle and late. (A and E) early untransduced tenocytes (B and F) early, (C and G) middle, (D and H) late passages of hTERT-transduced tenocytes. 10x magnification. Bar: 100 μm.

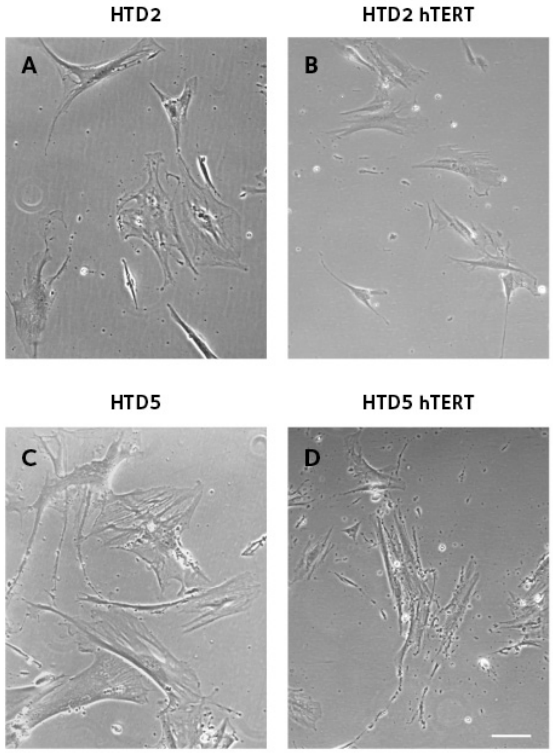


Figure 22. Senescence-associated morphological changes in untransduced and transduced tenocytes. (A and C) untransduced tenocytes which exhibited senescence features already at passage 13. HTERT-transduced tenocytes showed cell flattening at passage 28 (B and D). 10x magnification. Bar: 100 μm.

4.6. Senescence analysis

In order to objectify senescence of the cells a specific assay, the β-galactosidase assay, was performed. With the β-galactosidase assay senescent cells can be distinguished from viable, quiescent, tumourigenic or immortal cells. For the experiment 20000 cells of HTD2, HTD5, HTD2 hTERT, HTD5 hTERT and control hMSC per well (6-well plate) were seeded and cultivated for 7 days. 48 hours prior to the staining, control hMSCs were treated with 2 mM hydroxyurea (HU; Figure 23).

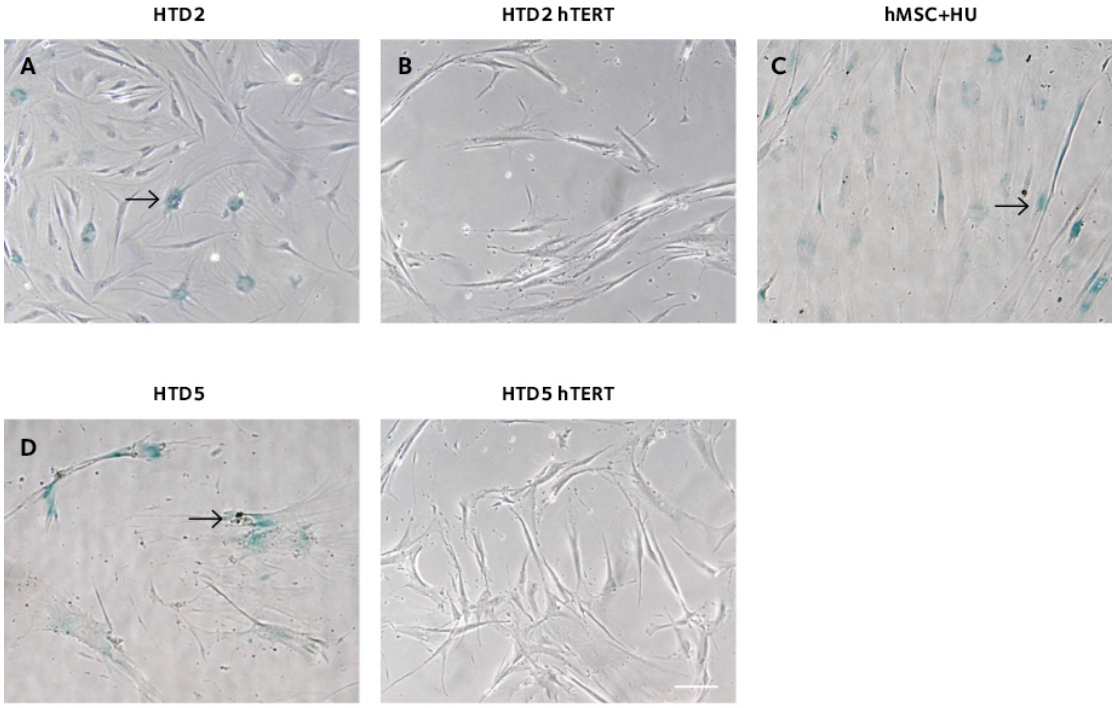


Figure 23. Senescence associated β-galactosidase assay. Blue cells are senescent cells (examples →). A and D depict untransduced tenocytes with pronounced

β-galactosidase activity at passage 18. B and E show hTERT-transduced tenocytes with almost no

β-galactosidase activity at passage 20. HU- treated hMSCs: positive control (C). 10x magnification.

Bar: 100 μm.

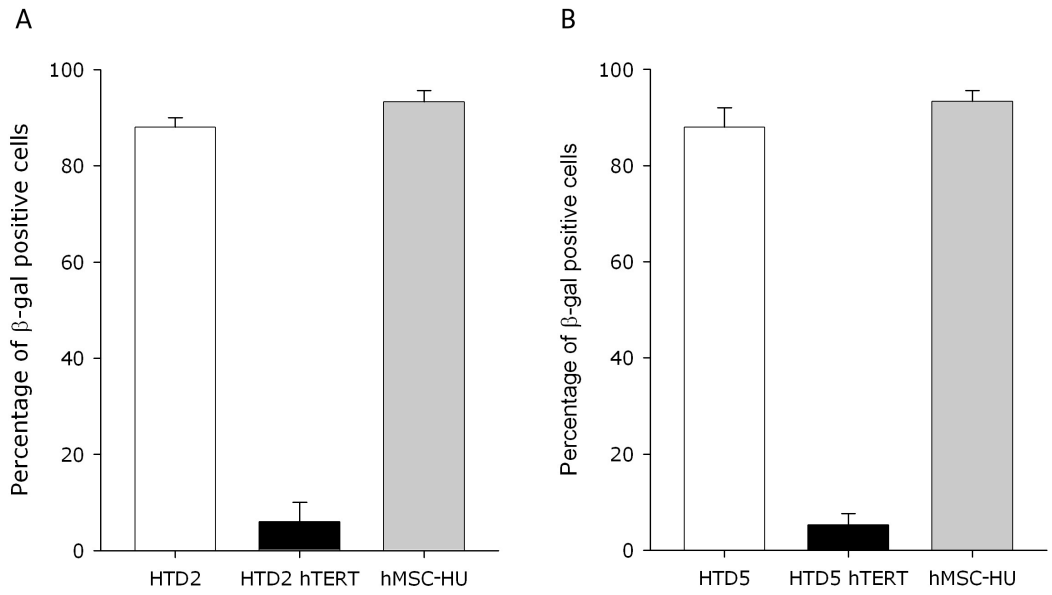


Figure 24. Quantification of the senescence associated β-galactosidase assay.
A: Donor 2. B: Donor 5. Untransduced tenocytes (white bars) had noticeably more β-galactosidase positive cells compared to hTERT-tenocytes (black bars). Positive control: hydroxyurea pretreated human mesenchymal stem cells (hMSC-HU; grey bars). Error bars: standard mean of samples in triplicates.

Untransduced human tenocytes exhibited significant senescence-associated β-galactosidase activity similar to the positive control around passage 18. In contrast, introducing hTERT into human tenocytes resulted in an extension of their lifespan as seen in their growth curve over time. Moreover, this was reflected by the lack of detectable senescence-associated β-galactosidase activity even at late passages (passage 20; Figure 24).

4.7. Tendon-related gene expression

Tendon-related gene expression was investigated by RT-PCR and served to confirm that the hTERT-transduced tenocytes did not loose their tenogenic nature during prolonged culture time.

Therefore, a combination of tendon specific gene markers was selected (Figure 25). These markers were sub-divided into three groups: A) fibrillar proteins (collagen I, collagen III), B) matrix proteins (Decorin, Tenascin C, COMP and TNMD) and C) transcription factors (Six 1, EphA4, Eya 1). All markers were tested at two different passages (early and middle) by PCR for untransduced tenocytes HTD2 p4 and p15, HTD5 p4 and p12 and hTERT-transduced tenocytes HTD2 hTERT p 8 and p 16,

HTD5 hTERT p8 and p15. Some markers were strongly expressed in early passages (e.g. Eya 1, EphA4), others in middle passages (e.g. Decorin, COMP). Both cell types showed no downregulation of genes over prolonged in vitro passaging. Among all genes tested only TNMD mRNA was not expressed after passage 1 (see 4.8.).

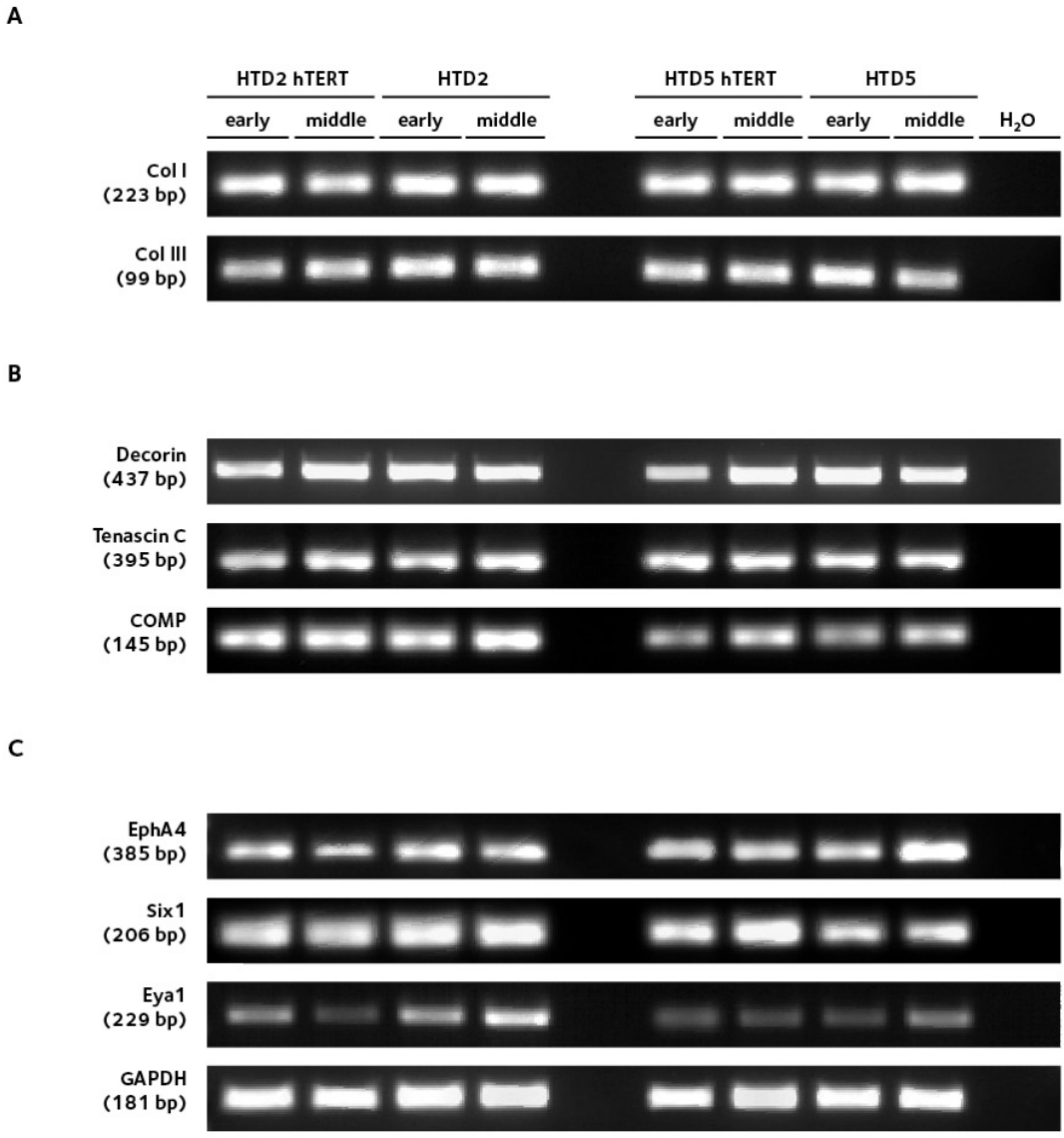


Figure 25. Tenogenic profile of HT D2 hTERT, HT D5 hTERT, HT D2 and HT D5 (early: ≤10 passages, middle: 11–19 passages). (A) fibrillar proteins, (B) matrix proteins and (C) transcription factors.

4.8. Long-term high density culture

Cells cultured over long periods of time started to rearrange themselves within the culture dishes. After 30-40 days the previously exclusively two-dimensional growth pattern turned into the formation of three-dimensional strands (Figure 26 A). Furthermore, after 90 days in culture, the cells started to deposit matrix within the culture dishes.

Tenomodulin is expressed in the first cell passage (HTD7 p1) and afterwards it is rapidly lost (HTD5 hTERT p5). Interestingly, after 90 days in high density culture, tenomodulin expression can be detected again by RT-PCR (HTD5 hTERT p5 90d; Figure 26 B).

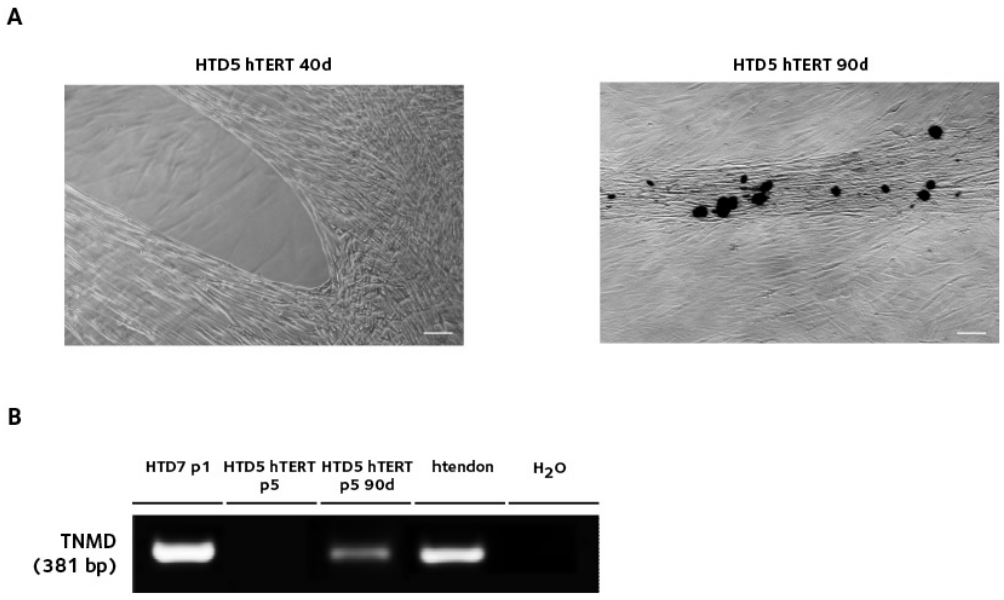


Figure 26. Long term high confluence culture. (A) Cell morphology after 40 and 90 days. (B) Tenomodulin expression. Human tendon (htendon) served as positive control. Bar: 100 μm.

4.9. In vitro tumourigenicity analysis

To evaluate the tumour forming potential of hTERT-transduced human tenocytes a functional in vitro assay was performed (Figure 27). Tumour cell lines like HT1080 are, amongst other things, characterized by the loss of anchorage dependency. This is a key feature of neoplastic transformation. Therefore, cancer cells like HT1080 are able to form colonies when seeded in soft agar and served as a positive control in this assay.

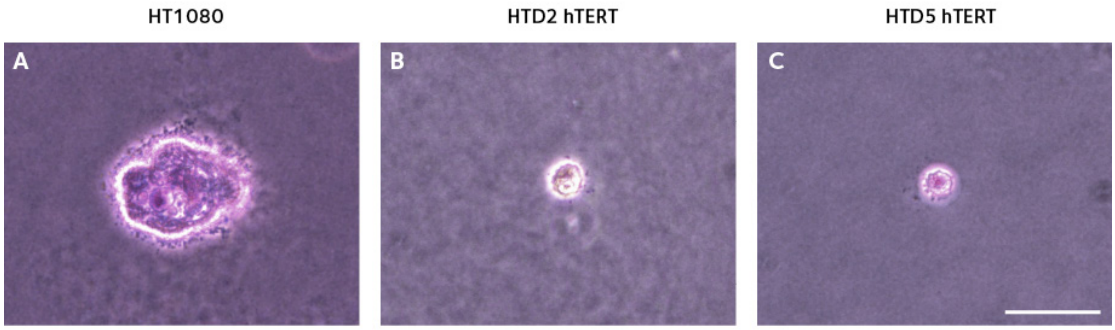


Figure 27. Soft agar assay. Colony forming of control tumour cell line HT1080 (A). No colonies formed in hTERT-transduced tenocytes (B and C). Bar: 100 µm.

In this investigation the top agar was inoculated with 2500 cells of HTD2 hTERT p20 and HTD5 hTERT p28 per well. After 28 days the tumour cell line HT1080 had formed colonies, while hTERT-immortalized tenocytes showed contact inhibition and remained single cells. This indicates that hTERT-immortalized cell lines did not undergo malignant transformation in vitro.

5. DISCUSSION

Tendinous tissue and its interaction with adjacent tissues like bone or muscle is a complex subject. Not only that the embryonic development of tendon is still poorly understood but also, clinically more relevant, there is not much known about tendon pathology and possible treatment strategies in case of tendinopathy or tear. Research concerning tendon repair is only standing at the beginning of the possibilities that are arising from tissue engineering and cell- and gene-based therapy. These new approaches need suitable, stable and quite a number of cells to work with in a rather short time span so that they are actually applicable for clinical use. Therefore, a human tenocyte cell line, allowing long term molecular characterization in vitro and leadoff studies for clinical and therapeutic applications, is needed.

Isolation of tenocytes – Tenocyte outgrowth and tendon digestion

The isolation of tenocytes was one of the first milestones in this project. Two methods of tenocyte extraction are known: first, digestion of the tendon tissue with collagenase and second, culturing explants of human tendon in culture dishes until the cells actively migrate from the tendon tissue. Collagenase digestion was performed by Salingcarnboriboon et al. 2003 with Achilles tendon of transgenic mice. The second technique, tenocyte outgrowth, was applied by Schulze-Tanzil et al. 2004 and Fu et al. 2007 using human tendon and rat patellar tendon, respectively. For tissue engineering applications the time in which the cells can be obtained and expanded plays a significant role. Schulze-Tanzil et al. demonstrated that the cells started to migrate out of the tissue after 1-2 weeks while it took even more days until they formed a monolayer that could be trypsinized and further expanded. Besides digesting the tendon tissue with collagenase is not only faster but as well more cells at once can be gained. On the other hand one could speculate that the cells that are able to actively migrate out of the tendon are viable and healthy and therefore more suitable for further implementation in tissue engineering.

Furthermore, the treatment of the cells with collagenase over a prolonged period of time might cause cellular damage. In this project both methods were applied without any difficulty. In general, it has to be decided on an individual basis which method is the most convenient. For us the viability of the cells was crucial and we therefore immortalized cells gained by the outgrowth method.

Immortalization of cells – hTERT transduced tenocytes avoid senescence

In this project novel tendon-derived cell lines were established and they stably expressed hTERT on RNA and protein level as well as they showed a significant telomerase activity (Figure 16 and Figure 17).

New approaches in tendon repair, like tissue engineering and cell-based gene therapy rely on constituent cells to work with. Unfortunately the availability of tenocytes that could substitute damaged or diseased tendon tissue is limited. Theoretically, extracting the tenocytes, expanding them in vitro, processing them further and re-implanting the cells, could solve this problem. However, there are some aspects that contradict this theory. First the cell number is generally limited, secondly their proliferation in vitro often leads to dedifferentiation and thirdly in vitro culture conditions frequently result in senescence-associated growth arrest (replicative senescence) and finally cell death (Stenderup 2003).

One possible solution to this problem could be the immortalization of cells. Each cell division leads to telomere length shortening of chromosomes, which can be the cause of replicative senescence (Wright and Shay, 1992). Telomerase can synthesize telomeric repeats and therefore extend cellular lifespan. Primary tenocytes show no telomerase activity in vitro (Figure 17) and therefore ectopic expression of hTERT in tenocytes can prevent replicative senescence. In our study HTD2 hTERT and HTD5 hTERT cell lines showed an extended lifespan compared to untransduced tenocytes (see 4.4). Many other groups have shown the feasibility of cellular lifespan extension for e.g. human endothelial, fibroblast and oesophageal squamous cells by using hTERT gene therapy (Bodnar et al. 1998, Hahn et al. 1999, Yang et al. 1999, Morales et al. 2003, Cheung et al. 2010). However, when cells are generally modified it is important that not only the immortalization or transgene expression is ensured but also that the cells retain their (tenocyte) character.

Another method successfully used for immortalization of cells is the overexpression of SV40 large T antigen gene. Salingcarnboriboon et al. 2003 isolated tenocytes from Achilles tendon of transgenic mice harbouring the SV40 large T antigen gene. The authors were able to culture the harvested cells for more than one year. One important difference between the two mentioned genes is that the SV40 large T antigen gene interferes with the cell cycle making it more prone to malignat transformation. SV40 large T antigen is a primary viral gene product that is involved in both viral replication and SV40-mediated cell transformation. It promotes transformation by binding and inactivating the products of a number of tumour

suppressor genes, which include p53 and pRb. And it also stimulates cell proliferation by promoting cell-cycle progression from G1 to S phase (Carbone et al. 1997, Fanning et al. 1992).

Contrary to SV40, hTERT does not cause growth deregulation (Harley 2002; Lee et al. 2004; Toouli et al. 2002). Still it has to be considered that neoplastic transformation was reported for hTERT-transduced adult MSC after prolonged in vitro culture (Serakinci et al. 2004). Yet most of the cases reported with neoplastic transformation of immortalized cells depended largely on the viral vector used. Montini et al. 2006 compared the tumourigenic potential of retroviral vectors and lentiviral vectors. They observed that retroviral vectors triggered dose-dependent acceleration of tumour onset contingent on long terminal repeat activity. Furthermore, insertions at oncogenes and cell cycle genes were enriched in early onset tumours, indicating involvement in tumourigenesis. Contrary to these findings, tumourigenesis was unaffected by lentiviral vectors and did not enrich for specific integrants (Burns et al. 2005). This suggests that in the case of hTERT not the insert itself leads to neoplastic transformation but more likely the retroviral vector.

Recently, Boecker et al. 2008 have reported the successful immortalization of human MSC via lentiviral gene transfer of hTERT. These cells showed no sign of replicative senescence in vitro with ‚significantly higher and unlimited proliferation capacity' and ‚preserved their stem cells character as shown by osteogenic, adipogenic and chondrogenic differentiation'. At the same time the clones did not undergo malignant transformation, as confirmed by in vitro and in vivo assays. Based on their results in this project the same approach was used and carried out successfully in human tenocytes.

Single cell cloning - The establishment of a single tenocyte-derived cell line under standard culture conditions was not feasible

Primary cell cultures derived from different tissue types are often heterogeneous in standard conditions as they may contain more than one cell type. To ensure that there is no variation in cell culture characteristics and to obtain a homogeneous cell culture a single cell-derived cell line is most suitable for long term in vitro studies. Cell cloning is therefore an effective way to ensure the purity of a cell line. Many strategies of single cell separation have been reported. So far the most common ones are: single cell picking, colony forming (cloning ring and colony scrapping) and

serial dilution technique (McFarland, 2000; Saling-carnboriboon et al. 2003; Boecker et al. 2008). As well as robotic cell transfer system or manually by laser catapultation/PALM. In this project manual single cell picking, colony forming by cloning ring and serial dilution technique was performed and the single cells obtained successfully attached. However, after several doublings no colony propagated further despite hTERT-expression. Thus, it was not possible to obtain a single cell line (Table 8).

Interestingly, McFarland et al. 2000 have reported that especially cell types that grow well at high density or in mass culture may have difficulties proliferating under the same culture conditions when cultured at low density or even as single cells. Taking this into account, culture conditions were optimized in the attempt to establish a single tenocyte-derived cell line. First, the percentage of fetal bovine serum was increased to 20 % in order to supply the single cells with more nutrients. Second, the culture dish was precoated with 4 µg/cm^2 collagen I to enhance cell attachement to the matrix and proliferation. The third approach included cell culture dish inserts (Figure 18). The inserts were seeded with a heterogeneous population of hTERT-transduced tenocytes estimating that tenocytes might be stimulating growth of neighbouring single cells by secreting various cell factors. Despite the above mentioned optimizations of the culture conditions, tenocyte cloning was not enhanced and a single cell-derived cell line was not obtained. McFarland et al. 2000 encountered the same problems with bovine, ovine, hamster and turkey myogenic satellite cells. Using chick and hamster satellite cells, the authors discovered that the cells begin to differentiate and fuse at low cell densities, which hinders their proliferation. Interestingly, the clonal propagation of chick and hamster satellite cells was successfully stimulated by adding 10-20 ng/ml fibroblast growth factor. Based on this, we suggest that single tenocytes in vitro may also be lacking a certain growth factor that stimulates their proliferation and therefore are not able to expand and form a cell clone.

Another factor that may play a role results from the findings of Schulze-Tanzil et al. 2004. The group showed that human tenocytes in monolayer under normal culture conditions exhibit long cytoplasmic processes with direct contact with neighbouring cells. Furthermore, the authors observed that the expression of tenocyte specific markers such as collagen I and Scleraxis increased during culture and prior to production of ECM, the tenocytes made an intimate contact. Taken together these

results indicate that possible reasons for non-proliferating single tenocytes could be the lack of growth or other cell factors, and cell-cell contact.

Extension of lifespan and prevention of replicative senescence

In the next part of the project, the growth behaviour and the entry into senescence of untransduced and transduced tenocytes was monitored.

HTERT-transduced tenocytes were cultured twice as long as untransduced tenocytes (Figure 19 and Figure 20). Moreover, HTD2 hTERT and HTD5 hTERT cells kept their typical, spindle-shaped morphology even in late passages (Figure 21). Finally, in comparison to untransduced tenocytes, the hTERT-expressing cells showed a background level or low β-galactosidase activity (Figure 23).

At first, upon viral treatment and hTERT transduction of tenocytes we detected reduced population doubling compared to untransduced cells. This is in contrast to most of the studies where hTERT expression in hMSCs, fibroblasts, human mammary epithelial cells and lens epithelial cells led to increased cellular proliferation (Boecker et al. 2008; Gorbunova et al. 2003; Smith et al. 2003; Xiang et al. 2002). However, some groups reported similar to our findings. For example Techangamsuwan et al. 2009 transfected adult canine Schwann cells and olfactory ensheathing cells with hTERT. These cells also showed a decreased proliferation rate. Furthermore Klinger et al. 2006 reported a significant lengthening of the population doubling time of hTERT-transduced vascular smooth muscle cells compared with control cells. At the same time, in both publications the hTERT-modified cell types continued to divide markedly longer than control cells. These results go in line with the growth kinetics of the tenocyte cell lines obtained in our study.

In Figure 28 our results are compared to the findings of Techangamsuwan et al. 2009 and Boecker et al. 2008. Interestingly, in most of the studies in which hTERT enhanced cell proliferation, the transgene was introduced at a time when telomere length had already reached a critical point. In the present study ectopic hTERT was introduced in early passages, a time point when the primary cells have lost their endogenous hTERT expression. We have not examined the telomere length of the cells at the time of infection and hTERT-transduction. Thus, we could suggest that the time frame for cell treatment (infection, hTERT-transduction) might be an important factor and could explain the differences in the cell behaviour.

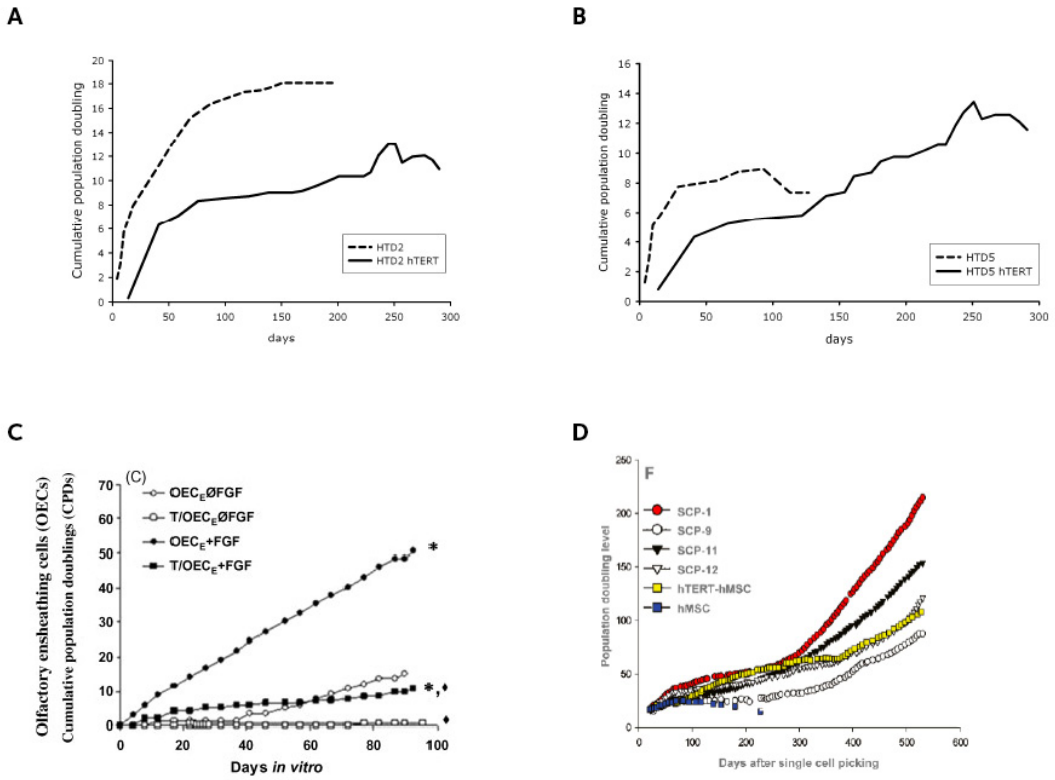


Figure 28. (A and B) growth curves of the present study. HTD2 hTERT and HTD5 hTERT had lower population doubling compared to the untransduced primary cells. Similar finding is demonstrated in (C) - olfactory ensheathing cells also showed reduced proliferation after introduction of hTERT which was independent of FGF-2 treatment (Techangamsuwan et al. 2009). An opposing finding is shown in (D) hTERT-transduced hMSCs and single cell picked clones (SCP-1,9,11,12) exhibited higher population doubling level compared to untransduced hMSCs (Boecker et al. 2008).

Another factor could be the age of the donor. The introduction of hTERT can rescue the cells from replicative senescence but can not reverse the biochemical aging of the cells such as chromosomal instabilities, methylation events and mitochondrial damages (Klinger et al. 2006). In our study, the tenocytes derived from donors that were around 60 years of age. Tenocytes immortalized by hTERT from far younger donors would supposedly show a higher proliferation rate, presuming that ectopic hTERT expression would lead to the same proliferation rate as seen in untransduced tenocytes.

Finally, a crucial factor could be the viral treatment itself. Some cell types might be more sensitive to the chemicals than others. A mock-transfected tenocyte cell line could have been good to compare with. Mock-transfected cells are cells that are

transfected with the plasmid alone, therefore the effect of the gene of interest alone could be revealed as well as the impact of the lentiviral transfection on the growth kinetics of the tenocytes.

Taken together, all these factors might lead to a noteably decreased proliferation rate in virally treated and hTERT-transduced cells. Still, hTERT-transduced tenocytes divided far beyond the entry into senescence of the control tenocytes, despite the lower proliferation rate. Furthermore, a senescence associated
β-galactosidase assay was performed. In agreement with the results of other studies (Boecker et al. 2008, Wang et al. 2005), we found that hTERT-transduced tenocytes show very low β-galactosidase activity in comparison to untransduced control cells even at later passages. In conclusion, the hTERT-transduced tenocytes overcame senescence and showed a prolonged lifespan compared to primary tenocytes.

Immortalized tenocytes retain their tenogenic profile over time

Until recently it was not possible to distinguish tenocytes from other cell types due to the lack of tendon-specific gene markers. Just now that repair mechanisms of tendon and tendinogenesis are beginning to be understood, certain genes involved with tendon development and repair are brought to light (Docheva et al. 2005, Shukunami et al. 2006, James et al. 2008). Thus for this project a tenogenic profile was selected, consisting of a set of tendon related genes like collagen I and III, matrix proteins (Decorin, COMP, Tenascin C) and transcription factors (EphA4, Six1, Eya1). In this study we analysed these genes on RNA level by RT-PCR. Based on this analysis, first, tenocytes can be distinguished from other cell types and second the expression changes of the individual genes can be monitored over time. Moreover the tenocyte cell lines express tendon related genes such as scleraxis, collagen I and III, COMP and EphA4 that are partly also expressed in developing tendon during embryogenesis.

Our results demonstrate that the isolated cells were indeed tenogenic and furthermore that neither the control tenocytes nor the hTERT-transduced tenocytes lost their expression profile over time. These findings differ from previously recorded data by Yao et al. 2006. The authors evaluated the expression of collagen I, collagen III and Decorin on protein level and observed that their expression decreased in higher passage number. Thus they concluded that the phenotype of tenocytes rapidly drifts with progressive passage of the culture. Similar to this project, Salingcarnboriboon et al. 2003 monitored SV40 immortalized

mouse tenocytes during prolonged culture. Although they did not examine the variation of the expression of the genes over time, the expressed genes encoding scleraxis, six1, COMP, EphA4 and collagen I made it apparent that these cell lines kept their tendon cell property on RNA level despite immortalization comparable to the results in this project.

Another well-known tendon-specific marker gene is Tenomodulin. It is involved in tenocyte proliferation and tendon maturation and is used as a marker for tenocytes in culture. In the study presented here, no TNMD expression was detectable neither in hTERT-transduced tenocytes nor in untransduced tenocytes when analysed by RT-PCR after passage 1. Our observations go in line with the data of Shukunami et al. 2006. Using both trypsin and collagenase digestion methods they isolated tenocytes from leg tendons at stage 41 chick embryos and analysed TNMD expression by northern blot. The authors concluded that tenomodulin transcripts are lower in avian tenocytes having been cultured over prolonged periods of time than they are in native tendons.

We therefore assumed that a long-term cultivation of tenocytes in high density, particularly with the formation of three-dimensional structures, mimics the united cell structure of the in vivo situation. This was supported by the proven restored expression of TNMD in tenocytes after 90 days in high density culture.

Taken together in our model system hTERT-transduced tenocytes maintained the expression of the selected tendon-specific genes during prolonged periods of in vitro cultivation. This suggests that HTD2 hTERT and HTD5 hTERT cells are no subject of phenotypic drift and dedifferentiation.

Tenocytes transduced by lentivirus and hTERT possess no tumourigenicity in vitro

Some genetic and epigenetic changes can lead to neoplastic transformation, thus enabling a cell population to proliferate independently of both external and internal signals that normally restrain growth. Cell populations that are transformed in this way for instance show reduced requirements for extracellular growth factors and are not restricted by cell-cell contact. Anchorage-independent growth and with it the soft agar assay which is used to assess the cells' tendency to transfer malignantly is one of the most important indications for tumourigenic transformation of cells (in vitro). As discussed above hTERT gene transfer may lead to neoplastic transformation of the cells. Even though lentiviral gene transfer makes the cells less prone to malignant transformation than retroviral gene transfer hTERT-transduced

tenocytes were tested for anchorage-independent growth by soft agar assay to rule out growth-deregulation. HTERT-transduced tenocytes in contrast to HT1080 (positive control) did not form colonies in soft agar. Our results are similar to the data of Boecker et al. 2008, who reported that hTERT-transduced hMSCs showed contact inhibition, while the control tumour cell line expanded into colonies. Furthermore, the group tested whether hTERT-transformed hMSCs show tumour formation in vivo by injecting the cells subcutaneously into nude mice. None of the tested clones formed a tumour at the injection site, whereas an extensive tumour growth was observed in HT1080 injected mice as a positive control. Hence, the next step in our project could be a similar in vivo analysis of hTERT-transduced tenocytes. In conclusion the generated tenocyte cell lines HTD2 hTERT and HTD5 hTERT did not demonstrate anchorage-independent growth in vitro suggesting no malignant transformation.

6. CONCLUSIONS

The goal of the project was to establish and characterize immortalized human tenocyte cell lines by overexpression of the human telomerase reverse transcriptase gene. Based on our results, the following can be concluded:

1. Primary human tenocytes were successfully obtained and cultured in vitro.

2. Using lentiviral approach, hTERT cDNA was transduced in the primary cells and two cell lines – HTD2 hTERT and HTD5 hTERT were generated.

3. HTERT transgene was successfully expressed in the cell lines as proven on RNA level by RT-PCR and on protein level by immunohistochemistry and quantitative telomerase activity test.

4. The establishment of a single-tenocyte derived cell line was not achieved. However, the HTD2 hTERT and HTD5 hTERT cell lines were homogeneous and demonstrated stable morphological appearance during prolonged culture period.

5. HTERT-transduced tenocytes proliferated twice as long and show extended lifespan in contrast to the control cells and no significant β-galactosidase activity was detected in hTERT-transduced tenocytes.

6. HTERT-transduced tenocytes retained their tenogenic profile over time which was evaluated by RT-PCR analysis of selected tendon-specific gene markers.

7. Tenocytes transduced by hTERT had anchorage-dependent growth in vitro suggesting no malignant transformation of the generated cell lines.

7. PERSPECTIVE

One of the main goals of tissue engineering is to create viable tissue equivalents in vitro. This can only be achieved by obtaining live and metabolically active cells that are able to repair a defect site through their continuous matrix synthesis. Until now, many questions concerning cell-based strategies for tissue engineering of tendon remain unanswered. Apart from the choice of the most suitable cell type and treatment protocol, several further aspects have to be considered, such as adequate carrier materials for the delivery of the cells, supportive treatment measures to additionally enhance tendon regeneration (e. g. the application of proteins that can promote tendon healing), and various optimizations to ensure cell survival upon transplantation.

In comparison to other connective tissues tendon cell research has been primarily hampered by the lack of appropriate human cell lines allowing the long-term molecular characterization of the tenocytes in vitro. Moreover, prior to clinical applications in most cases animal models need to be developed to ensure the safety and effectiveness of the tissue engineering application. Therefore standardized cell populations and in vivo animal models are indispensible for the development of appropriate tissue engineering strategies for tendon repair and reconstruction. Furthermore such in vitro and in vivo models can be very valuable for testing of novel tendon substitutes suitable for clinical applications or to assess new innovative regenerative strategies.

Thus, the human tenocyte cell lines established in this project will be suitable for extended molecular characterization in vitro and will help to better understand the tenocyte lineage. At the moment the project is repeated with far younger tenocyte donors to eliminate the reservations of the cell line of this project due to the reduced proliferation capacity. The immediate object is the application of the cell line in a mouse model.

8. SUMMARY

Development of the musculosceletal system requires coordinated formation of distinct types of tissues, including bone, cartilage, muscle and tendon. Compared to muscle, cartilage and bone, molecular, cellular and developmental biology of tendon have not been well understood due to the lack of tendon cell lines. In addition tissue engineering of tendon is hampered by the rather difficult retrieval of tenocytes and their senescence-associated growth arrest during culture. Therefore the purpose of this study was to establish and characterize human tendon cell lines.

Two tendon cell lines (HTD2 hTERT and HTD5 hTERT) were established using lentiviral gene transfer to ectopically express hTERT. The cell lines stably expressed hTERT on RNA and protein level. Untransduced cultured tenocytes show only a background level of telomerase activity, but it was significantly increased by hTERT transduction. Ectopic expression of hTERT led to an extended lifespan and prevented senescence while the cells kept their typical spindle-shaped morphology of young primary human tenocytes. Moreover, in comparison to untransduced tenocytes the cells possessed significantly lesser β-galactosidase activity indicating that they had not entered a senescent state. Throughout the entire culturing period the hTERT-transduced tenocytes expressed tendon-related genes such as those encoding collagen I, collagen III, Tenascin C, EphA4, Eya1, scleraxis, Six and COMP. Using soft agar assay, no malignant transformation was shown by the hTERT expressing tenocytes. In conclusion, extending the lifespan of human tenocytes by ectopic expression of hTERT using lentiviral gene transfer may be an attractive and safe way to generate cells allowing extensive molecular characterization and development of novel tissue engineering applications.

9. ZUSAMMENFASSUNG

Die Entwicklung des muskuloskeletalen Systems erfordert die koordinierte Entwicklung von verschiedenen Gewebearten wie Knochen, Knorpel, Muskel und Sehne. Im Gegensatz zu Knochen, Knorpel und Muskel weiß man bisher nicht viel ueber die Molekular-, Zell- und Entwicklungsbiologie von Sehnen. Der Grund dafuer sind fehlende Tenozytenzelllinien. Außerdem wird das Tissue Engineering von Sehnen durch die schwierige Gewinnung von Sehnenzellen und dem seneszenzbedingten Wachstumsstillstand unter Kulturbedingungen behindert. Daher war es Ziel dieses Projektes eine humane Tenozytenzelllinie zu etablieren und zu charakterisieren.

Mittels lentiviralem Gentransfer wurden zwei Tenozytenzelllinien (HTD2 hTERT und HTD5 hTERT) hergestellt welche ektop hTERT exprimieren. Beide Zelllinien exprimieren stabil hTERT sowohl auf RNA- als auch auf Protein-Ebene. Im Hinblick auf Telomerase-Aktivitaet zeigen untransduzierte Sehnenzellen in Kultur lediglich einen Hintergrundwert, waehrend die hTERT-Transduktion die Aktivitaet signifikant erhoeht. Ektope Expression von hTERT fuehrte zu einer verlaengerten Lebensdauer und verhinderte die Seneszenz der Zellen. Gleichzeitig behielten die Tenozyten ihre typische spindelfoermige Morphologie. ueberdies zeigten die Zellen im Vergleich zu untransduzierten Tenozyten signifikant weniger β-Galactosidase-Aktivitaet und dies weißt darauf hin, dass die Zellen noch nicht in den Zustand der Seneszenz uebergegangen sind. Waehrend der gesamten Kulturperiode exprimierten die hTERT-transduzierten Tenozyten sehnenspezifische Gene wie Kollagen I, Kollagen III, Tenascin C, EphA4, Eya1, Scleraxis, Six1 und COMP. Unter Verwendung eines Soft-Agar-Assays konnte gezeigt werden, dass die hTERT exprimierenden Tenozyten keiner boesartigen Transformation unterlagen.

Zusammenfassend kann gesagt werden, dass die Verlaengerung der Lebensdauer von humanen Tenozyten durch ektope Expression von hTERT mittels lentiviralem Gentransfer eine attraktive und sichere Moeglichkeit ist, um Zellen herzustellen die eine ausfuehrliche molekulare Charakterisierung und die Entwicklung von neuartigen Tissue Engineering Anwendungen ermoeglichen.

10. LITERATURE

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11. APPENDIX

List of Abbreviations

ADAM	A distegrin and metalloproteinases
ADAMTS	A distegrin and metalloproteinases with trombospondin motifs
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
COMP	cartilage oligomeric matrix protein
DAPI	4',6'-Diamidino-2-phenylindole
DMSO	Dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
e.g.	exempli gratia (for example)
EGF	epidermal growth factor
EGR-1	early growth response 1
ELISA	Enzyme Linked Immunosorbent Assay
EphA4	ephrin receptor A4
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HGF	hepatocyte growth factor
hMSC	human mesenchymal stem cells
hTERT	human telomerase reverse transcriptase
HU	hydroxyurea
MMP	matrix metalloproteinase
NO	nitric oxide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF-B	platelet derived growth factor B
PDL	population doubling level
PDT	population doubling time
rpm	revolutions per minute
SF	scatter factor
SCP-1	single cell picked clone 1 of immortalzed hMSCs
TAE	Tris-Acetate-EDTA
TGF	transforming growth factor
VEGF	vascular endothelial growth factor