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Development of an inducible transgenic mouse model that overexpresses crucial transcription factors for tendon regeneration

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Chapter 2: Materials and Methods

ABBREVIATIONS

AAV	Adeno-associated virus
ASO	Antisense oligonucleotides
BMP4	Bone morphogenetic protein 4
BMP12	Bone morphogenetic protein 12
bp	Base pairs
BFP	Blue fluorescent protein
Bgn	Biglycan
cDNA	Complementary DNA
cm	centimeters
c-Myc	MYC proto-oncogene, bHLH transcription factor
Col1a1	Collagen type1 a1
Col1a3	Collagen type1 a3
Col5a1	Collagen type5 a1
Col12a1	Collagen type12 a1
Col14a1	Collagen type14 a1
DAPI	4’6-diamidino-2-phenylindole
DCN	Decorin
dox	Doxycyclin
DMEM	Dulbecco’s Modified Eagles’s Medium
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide Triphosphates
e.g.	for example
EDTA	Ethylenediaminetetraacetic acid
EmGFP	Emerald Green Fluorescent Protein
Egr1	Early growth response 1 transcription factor
Egr2	Early growth response 2 transcription factor
EPCR	Endothelial protein C receptor
et al.	*et alii*. (and other authors)
EtBr	Ethidium bromide
EtOH	Ethanol
Fmod	Fibromodulin
GFP	Green fluorescent protein
h	Hour

-ABBREVIATIONS-

IRES	Internal ribosomal entry site
IL10	Interleukin 10
IVD	Intervertebral disc
Klf4	Kruppel-like factor 4
L	Liter
MgCl2	Magnesium chloride
min	Minute
Mkx	Mohawk Homeobox
mM	milli Molar
mRNA	Messenger ribonucleic acid
ng	Nano gram
Oct3/4	Octamer-binding protein 3/4.
OSKM	Reprogramming transcription factors: octamer-binding protein 3/4 (Oct3/4), Sox2, Krüppel-like factor 4 (Klf4) and Myc, collectively referred to as OSKM.
$p16^{INK4A}$	$p16^{INK4A}$ is one of the isoforms encoded by cyclin-dependent kinase inhibitor 2A (Cdkn2a)
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDGFRα	Platelet derived growth factor receptor, alpha polypeptide
PDL	Periodontal ligament
RNA	Ribonucleic acid
rpm	Rounds per minute
RT	Room temperature
rtTA	tetracyclin (or doxycycline)-inducible reverse tetracycline transactivator
Scx	Scleraxis Basic Helix-Loop-Helix Transcription Factor
Sox2	Sex determining region Y-box 2
TAE	Tris-acetate-EDTA buffer
TET1	Ten-eleven translocation methylcytosine dioxygenase 1
TET2	Ten-eleven translocation methylcytosine dioxygenase 2
TdAR	Tendon activator (rtTA) reporter (Scx-scarlet, Col1a1-BFP)
Td4F	Tendon four factors (Scx, MKX, Egr1, Egr2) mouse line
TE	Tris-EDTA buffer
Tgfβ-2	Transforming growth factor beta 2

-ABBREVIATIONS-

Tgfβ-3	Transforming growth factor beta 3
TIMP1	Timp metallopeptidase inhibitor 1
TNC	Tenascin-C
Tppp3	Micro-tubulin polymerization promoting protein family member 3
TSPC	Tendon stem/progenitor cells
WT	Wild Type
µL	micro liter
µM	micro molar
µmol	micro Mol
α-SMA	Smooth muscle actin-alpha
β-gal	beta-galactosidase
°C	degrees (in Celsius)

CHAPTER 1: INTRODUCTION

The tendon is the intermediate link between the weight-bearing tissue (bone) and the motor tissue (muscle). Therefore, it has the role of regulating movement by coordinating the transfer of muscle-generated forces to the bone and buffering the stresses generated by muscle contraction in the less deformable bone. Because of its unique role, the tendon is prone to injury, challenging to repair, and has a vast and irreversible impact on the patient's athletic ability after injury. Injury and ageing of the Achilles tendon are not life-threatening, but the long recovery period and loss of motion can be harrowing and financially burdensome. Therefore, research on repairing Achilles tendon injuries and anti-ageing Achilles tendons are of great clinical prospects and economic benefit.

The discovery of tendon stem/progenitor cells (TSPCs) (Bi et al. 2007), a particular cell population with the ability to repair tendons, has led to a new chapter of cellular and gene therapy for the treatment of tendon injuries and the fight against the challenging disease of tendon ageing. I constructed a transgenic model to study the mechanism of tendon injury and ageing; it provides a novel and reliable animal model to explore efficient tendon regeneration therapies for clinical application.

1.1 Importance of Tendon Regeneration

The importance of the tendon is related to its functional and compositional characteristics. Firstly, as the connecting part of the motor tissue of the whole body, its most important function is to coordinate the transmission of the forces generated by the muscles to the bones. At the same time, the failure of coordination of the tendon can lead to significant damage to cartilage and joints when movement causes stress to the bones (e.g., braking, acceleration, counteracting forces in different parts of the body due to changes in the centre of gravity). This failure can even lead to traumatic injuries such as falls. Therefore, the health of the tendons is as vital to the patient as it is to the healthy person. Because of this characteristic of tendons, approximately half of all skeletal muscle injuries are tendon injuries.

Secondly, the composition of tendons differs from that of most tissues. They are mainly made up of an extracellular matrix dominated by collagen fibres interspersed with many proteoglycans. Together, these contribute to the tendon's robust nature, allowing it to withstand large forces without damage to transmit them and be very elastic to coordinate stresses. Unfortunately, however, this structure results in the blood supply to the Achilles tendon being inferior to that of sparse tissues. The cell density in the tendon area is much lower than in loose connective tissue. This feature makes it difficult to repair tendon injuries, and the tendon becomes less functional with age as it struggles to maintain a healthy extracellular matrix and even develops tendinopathy. Because of the specificity of the tendon mechanism, it is prone to injury, ageing, and scar repair. So, research into the maintenance of tendon health and tendon regeneration is urgent.

Of the extracellular matrix that makes up tendons, collagen I is the main component of healthy tendons—accounting for more than 90% of the tendon's collagens (Schneider et al. 2018). Injured tendons form scarred tendons containing large amounts of collagen III which is difficult to be reconstructed (Maffulli et al. 2000). This alteration significantly reduces the toughness of the tendon tissue, and therefore the scarred tendon is highly susceptible to re-rupture. In addition, small proportions of collagens such as Col V, VI, XI, XII, and XIV also play an essential role in regulating the structure and function of tendon collagen (Ansorge et al. 2009; Bönnemann 2011; Izu et al. 2021; O'Brien 2005; Sun et al. 2015; Sun et al. 2020).

Collagen and protein components such as elastin (El) and tenascin-C (TN-C) together with proteoglycans such as decorin (Dcn), biglycan (Bgn), fibromodulin (Fmod), and lumican (Lum) form the fascicular (FM) and the interfascicular matrix (IFM). The IFM is the foremost bearer of tendon function. The epitenon and paratenon, which surround them, are rich in blood and lymph vessels and many different cells. The peritenon, made up of epitenon and paratenon, is the main structure for the metabolism and energy supply of the tendon (Doral et al. 2010; O'Brien 2005).

Tendon stem progenitor cells and tendon cells together form the main cellular component of the tendon. Tendon stem progenitor cells are derived from a wide

range of sources, including but not limited to perivascular TSPC and peritenon-derived TSPC. The latter may be derived from tendon fibro-adipogenic progenitor (T-FAPs) cells correctly differentiated subpopulations. In contrast, the fibro-adipogenic progenitor (FAPs) cells that fail to differentiate correctly to tenocytes will become the fibrosis cells (Harvey et al. 2019). In addition, erythrocytes, osteoblasts, endothelial cells, macrophages, and antigen-presenting cells are also present in the tendon. This complex composition suggests a tricky balance of cellular renewal within the tendon itself to maintain a healthy microenvironment to create the conditions for the constant renewal of the extracellular matrix (Figure 1).

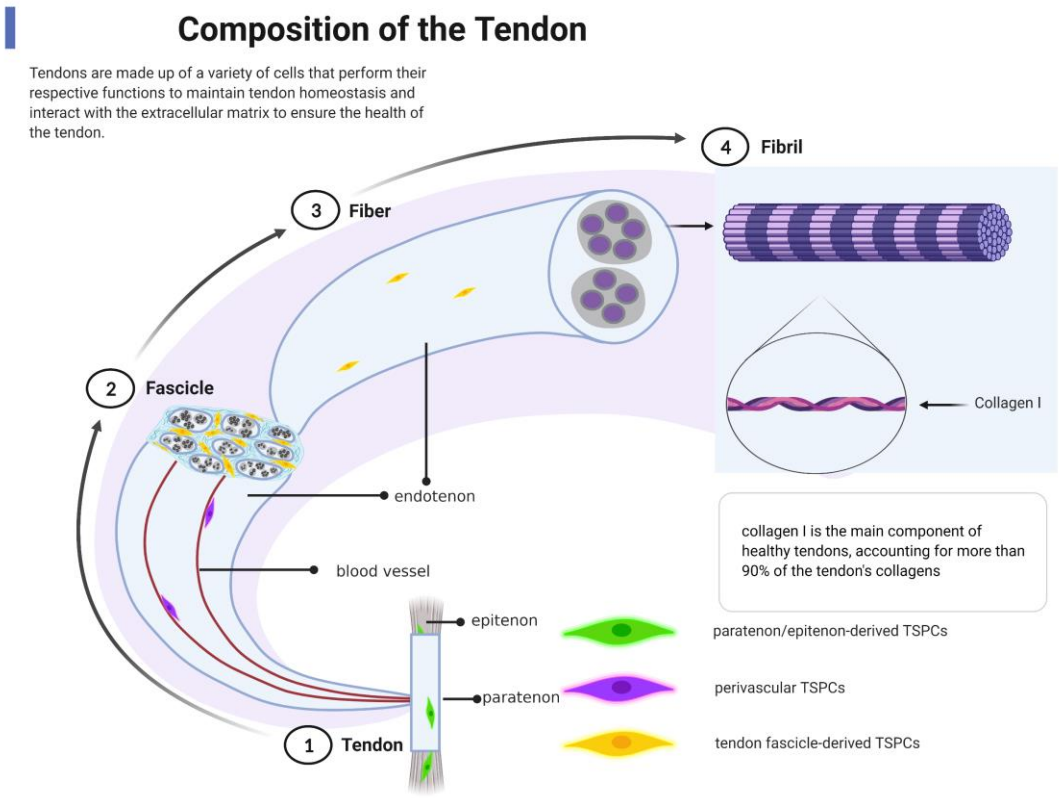

Figure 1. Schematic illustration of the tendon.
The tendon is composed of perivascular TSPC, peritenon-derived TSPC, and tendon cells interspersed in the Achilles tendon fibres. TSPC proliferate and differentiate into tendon cells, maintaining a balance of cell renewal in the tendon. The tendon cells continuously produce extracellular matrix (e.g., proteins, polysaccharides, and growth factors). This renewal ensures the health of the microenvironment of the tendon. Collagen I, which makes up the tendon fibres of healthy tendons, is the main component of the extracellular matrix and accounts for over 90% of tendon collagen (Doral et al. 2010).

1.2 The partial reprogramming strategy

Partial reprogramming of adult tendon cells following injury by establishing a temporary and locally limited pluripotent state is a novel cellular-molecular therapeutic strategy to limit scar formation following injury and promote regeneration of the affected tissue (Lu et al. 2020; Ocampo et al. 2016; Sarkar et al. 2020; Wang et al. 2021). Scaring and defective healing often occur after adult tissue injury, and their occurrence limits the regeneration and repair of affected tissues.

The basic principle of in vivo cellular reprogramming is to promote the proliferative capacity of reprogrammed cells through transformation/de-differentiation, resulting in the generation of precursor cells in the injured tissue that differentiate into the typical target cells of that tissue. Yamanaka factors generally achieve the de-differentiation process, and the redifferentiation and trans-differentiation processes can be facilitated by transcription factors that play an essential regulatory role in the development of the tissue or by transcription factors that can directly transform other cell types (stem cells or fibroblasts) into functional mature cells typical of the tissue.

Partial reprogramming is generally achieved by enabling the controlled expression of OSKM transcription factors (Oct3/4, Sox2, KLF4, and c-Myc) that place the cells in a locally limited pluripotent state, thereby enhancing their proliferative capacity and improving their cellular state (apparent epigenetic rejuvenation) (Ocampo et al. 2016). This class of partially reprogrammed cells then enhances repair and reduces scar formation by increasing the number of beneficially repairing Achilles tendon cells(Doeser et al. 2018b). Up to now, the mechanisms are not yet fully understood.

1.2.1 Yamanaka factors (Oct3/4, Sox2, Klf4, c-Myc)

As I know, most cells in the body are limited to proliferate. At the same time, they cannot differentiate into the many functional cells I need because they have lost their pluripotency. However, during early embryonic development, embryonic stem (ES) cells naturally possess both properties as pluripotent stem cells. Thus, by transferring the nucleus of a differentiated cell into an ES cell through nuclear transplantation, the developmental process can be reversed, leaving somatic cells in an embryonic-like state again, i.e., reprogrammed. The problem was that I was unable to obtain enough cells until the advent of the Yamanaka factors Oct3 / 4, Sox2, c-Myc, and Klf4 be

found, which made it possible to transform differentiated somatic cells into pluripotent stem cells (Takahashi and Yamanaka 2006). These four transcription factors have been repeatedly studied for 15 years (2006-2021) to verify the induction potential and stability of different combinations and to confirm their effectiveness and stability. In turn, their mechanisms of action have been gradually unraveled, laying the foundation for their clinical translation.

More importantly, technological advances in viral (AAV) and non-viral (small molecule, ASO) induced pluripotent stem cells have allowed their application to be no longer limited to a single strategy like in vitro acquisition of IPSC and induced redifferentiation. This advance has redefined the whole idea of damage repair solutions and anti-ageing programs.

1.2.2 The partial reprogramming in vivo

Efficient and reliable animal studies are necessary before clinical translation can take place. Good animal models can provide us with reliable data to elucidate mechanisms of action and identify possible risks (and explore strategies to avoid or address them). In this context, transgenic mice carrying fluorescent reporter genes and tendon specific inducible OSKM overexpression mice provide a valuable platform for experiments in vivo.

I constructed the tendon reporter mouse model specifically designed for in vivo studies of tendon cell differentiation and tendon repair/regeneration. The red fluorescent reporter protein will be switched on when tendon stem/progenitor cells emerge, and the blue-fluorescent reporter protein will be switched on when the tendon cells mature. This reporter system will make it possible to track the different states of the Achilles tendon cells. The red fluorescent protein will be expressed when partial reprogramming returns the cells to a progenitor-like state; the blue fluorescent protein is defined when the progenitor-like cells re-differentiate into tendon cells (playing their most important role in collagen I secretion).

The above effect is achieved through the following pathway:

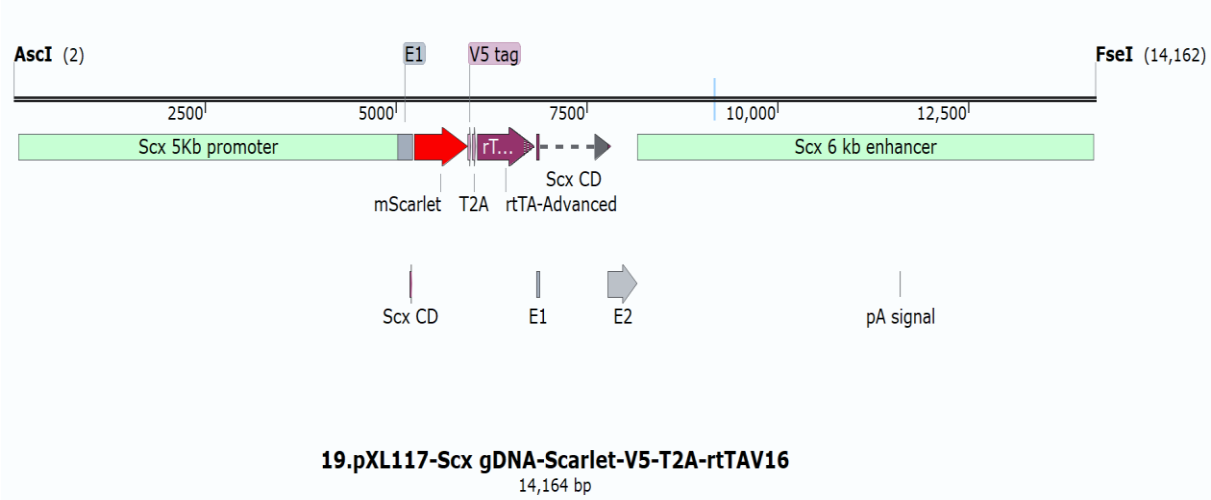

Figure 2. This transgene as tendon progenitor cell reporter and Scx-promote rtTAV16 activator
Reverse tetracycline trans-activator (rtTA) and mScarlet red fluorescent protein are expressed under the control of the Scx promoter. T2A links rtTA and mScarlet. This design means that rtTA and mScarlet will be expressed in a 1 to 1 ratio. Scx promoter is expressed in tendon stem/progenitor cells. When tendon stem/progenitor cells emerge during differentiation or regeneration, the mScarlet red fluorescent reporter will be turned on, and the reverse tetracycline trans-activator will be expressed. When tetracycline or doxycycline is provided, it can switch on a combination of transcription factors driven by the tetracycline promoter (OSKM, etc.).

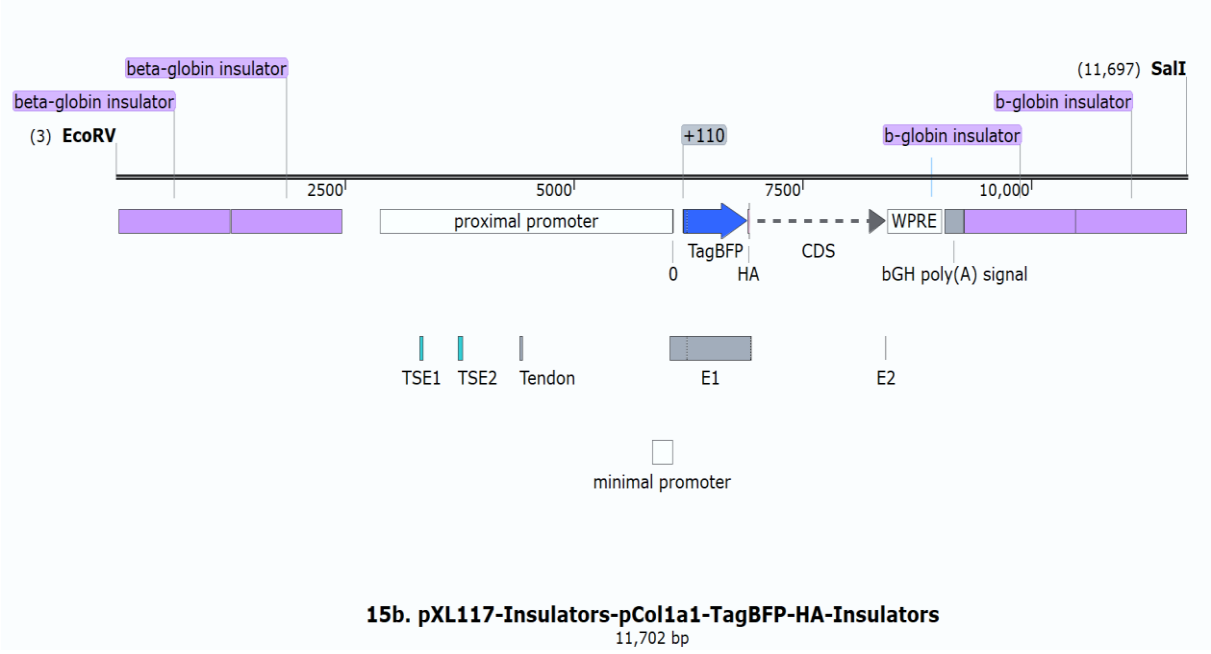

Figure 3. The blue fluorescent protein reports the mature tenocyte.
The blue fluorescent protein reporter expresses under the control of the 3.6kb Col1a1 proximal prokinetic. Two β-globin insertion sequences were added to both ends of the transgene to prevent promoter silencing. The 3.6kb Col1a1 promoter is expressed primarily in mature tendon cells. This construct will be co-injected with the Scx promoter construct. The blue fluorescent protein will be turned on when the Tendon stem/progenitor cells have differentiated and matured.

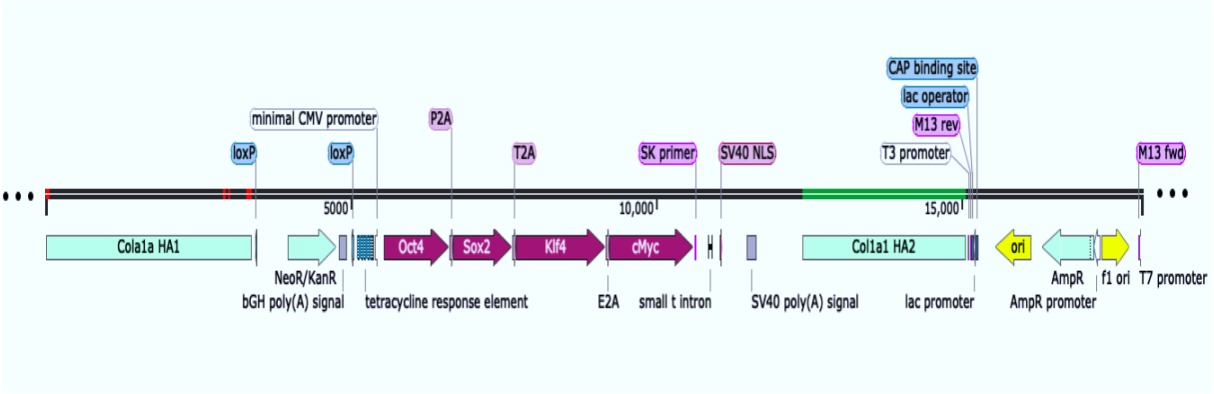

Figure 4. Tetracycline inducible four reprogramming transcription factors (Oct4, Sox2, Klf4, and c-Myc).
Tetracycline inducible four reprogramming transcription factors (Oct4, Sox2, Klf4, and c-Myc) were targeted into the 3' end of the Col1a1 gene. The endogenous Col1a1's function is intact. Without tetracycline induction, the four reprogramming transcription factors are not expressed. (Carey et al. 2010)

After activation with doxycycline, endogenous Scx-expressing (reported by Scarlet) tendon progenitor cells in the mouse line express OSKM transcription factors (Oct3/4, Sox2, KLF4, and c-Myc), resulting in a locally limited pluripotent state of the cells, their increased proliferative capacity, and improved cellular status (apparent rejuvenation), thus enhancing repair and reducing scarring (Figure 2,4). Follow-up was continued until mature tendon cells marker type I collagen (reported by BFP) was abundantly expressed and replaced type III collagen (Figure 3), the primary protein type in scar healing.

1.2.3 Regeneration and scar formation

Tendon reconstruction after injury, maintenance of health, and ageing-induced changes are associated with a population of cells with the ability to repair the tendon injury, namely tendon stem/progenitor cells (TSPC). TSPCs from the tendon sheath and extracellular matrix are a complex subpopulation. Some of the cells are regulated by the bone morphogenetic proteins induced by double-chain proteoglycans (Bgn) and fibronectin (Fmod), which hold the extracellular matrix, thus forming the homeostasis of the tendon. They are involved in reconstruction or repair after injury (Bi et al. 2007). Interestingly, neonatal tendons can be rejuvenated entirely, but their ability to regenerate gradually decreases with age. Suggesting that the diminished capacity of tendons to self-balance and renew themselves are thought to be closely related to the ageing of the TSPC and changes in the local microenvironment (Dai et al. 2020; Kohler et al. 2013; Li et al. 2019).

Another group of TSPC derived from the tendon sheath express micro-tubulin polymerization promoting protein family member 3 (Tppp3 +). The Tppp3 + Pdgfrα + cell subpopulation was identified as tendon stem cells; the Tppp3 - Pdgfrα + cell subpopulation was identified as fibroblast progenitors. The former promotes tendon repair after tendon injury by proliferating and differentiating into new tendon cells, characterized by platelet-derived growth factor receptor alpha (Pdgfrα). This subgroup generates new tendon cells when exposed to platelet-derived growth factor-AA (PDGF-AA). The latter, in turn, produces fibrotic cells that are detrimental to tendon repair, and these fibrotic cells are thought to be the origin of scarring following Achilles tendon injury (Harvey et al. 2019).

In adult stem cells, there are two subpopulations with different proliferative and differentiation abilities. A subpopulation of tendon stem cells with a high differentiation potential, capable of differentiating into various connective tissue lineages, which has a greater capacity to regenerate the Achilles tendon (Rajpar and Barrett 2020). Senescent TSPC, on the other hand, show an inadequate repair capacity, with poor Achilles tendon repair and increased scarring after injury compared to the former (Yan et al. 2020).

In conclusion, the critical point of whether an Achilles tendon injury leads to reconstruction or scarring is the state of the TSPC and the influence of the local microenvironment. Of importance is the ratio of the different TSPC subpopulations, the state/age of the cells, and the regional growth factors (niche).

Interestingly, it is possible to partially reprogram cells in vivo through epigenetic re-modelling directly by cyclic induction, thereby also reversing the senescent state of cells and enhancing their ability to repair organ damage (Ocampo et al. 2016). At the same time, the cyclic induction of OSKM allows for partial reprogramming without causing teratomas, which is vital for Achilles tendon regeneration.

Transient expression of OSKM-induced partial reprogramming of the cellular state may increase TSPC proliferation and tendon re-modeling capacity, in addition to acting on fibroblasts to reduce their α-SMA expression and thus fibrotic scar

formation. Further analysis showed that local microenvironmental factors that promote scar formation, such as transforming growth factor β1 (TGFß1), were also restricted, suggesting that transient OSKM expression after injury may inhibit scar formation and provide conditions for tissue reconstruction (Doeser et al. 2018b).

In summary, partial reprogramming of tendon tissue after transient induction injury may synergistically combat scarring and rebuild tendon tissue to improve the cellular status and create a conducive tendon reconstruction.

1.3 The critical transcription factors for tendon development

After a transient reprogramming strategy, I can obtain cells in a partially reprogrammed state, thus avoiding the emergence of a fully pluripotent state. However, this state is almost transient and difficult to capture even in the fluorescent reporter transgenic mice I have designed. Failure to modulate reprogramming would inevitably lead to the emergence of teratoma, preventing the regeneration of the Achilles tendon after injury. One potential strategy to address these problems and achieve improved control of the cell state is to induce tendon differentiation in cells in a partially reprogrammed form. To obtain target cells that resemble naturally developed tendon cells, screening induction factors focused on crucial transcription factors during tendon development.

During tendon development, axial tendons are mainly regulated by two transcription factors, Scleraxis BHLH transcription factor (Scx) and Mohawk Homeobox (Mkx). In contrast, the development of limb tendons is regulated by Scx,Mkx, and early growth response 1/2 (Egr1 / 2) (Delgado Caceres et al. 2018). The absence of any key transcription factor has a detrimental effect on tendon development and repair (Liu et al. 2010; Nichols et al. 2018). I, therefore, overexpressed these four factors in transgenic mice to regulate cell-cell reprogramming and induce redifferentiation correctly.

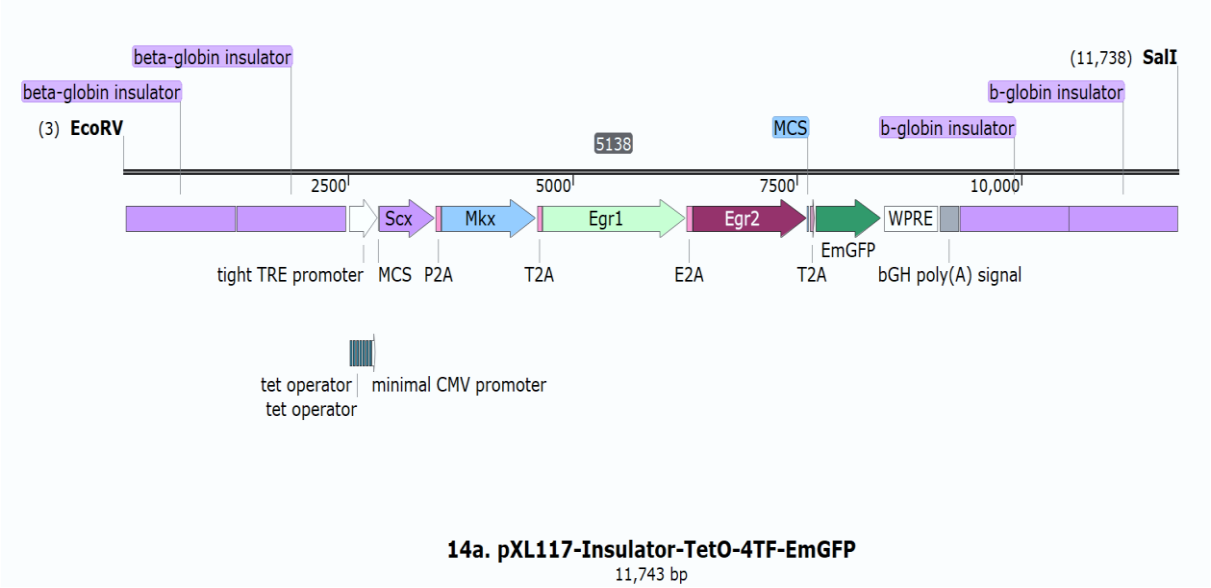

Figure 5. Tetracycline inducible four key transcription factors in tendon development (Scx, Mkx, Egr1, and Egr2).
This construct consists of a tetracycline-inducible promoter and four key transcription factors in tendon development (Scx, Mkx, Egr1, and Egr2) and GFP (Green fluorescent protein). Two beta-globin insulators are added, flanking the whole construct to prevent the silencing of the Tet inducible promoter. This DNA fragment will be randomly integrated into the mouse genome after pronuclear injection. When this line is crossed with a tendon promoter-driven reversed tetracycline trans-activator (rtTA) mouse line, the double transgenic mice will express four key transcription factors (Td4F) in tendon development and reporter GFP when doxycycline is provided in drinking water. Alternatively, when the cells are isolated from the double transgenic mice and when doxycycline is supplied in the culture medium, the four key transcription factors (Td4F) in tendon development and GFP reporter will be expressed.

I can get the double-positive mouse by crossing this transgenic mouse strain with a tendon-specific rtTA and fluorescent reporter mouse strain (TdAR). In this double positive transgenic mouse, the four most crucial transcription factors in embryonic tendon development are expressed in parallel with EmGFP after doxycycline induction (Figure 5). This new mouse line can be used to study transcription factors for anchoring cell fate, promoting the efficiency of redifferentiation into target cells, and improving functional tissue repair.

1.3.1 Scleraxis bHLH transcription factor (Scx)

The bHLH transcription factor Scleraxis is predominantly expressed in connective tissues such as tendons and is particularly highly expressed in limb tendons, axial tendons, and some cartilage mesenchymal precursor cells (Figure 6). During tendon development, Scx is first expressed in the lateral sclerotome and mesenchymal cells of limb buds in transgenic reporter mice (Scx-GFP) from E9.5-10.5. At approximately E11.5, it expresses in the developing spine to the tail rib and the tendon tissue of the

extremities. This evidence implies that scleraxis expression is vital for the early development of tendon progenitor cell populations. On the other hand, the knockout of Scx does not directly lead to the failure of tendon forming, suggesting that a single Scx does not fully control tendon formation, implying that the role of other key tendon transcription factors is also essential.

Interestingly, as ossification occurs, Scx expression begins to decrease, and this decrease in Scx expression levels continues with adulthood and ageing (Schweitzer et al. 2001). To a large extent, this also contributes to the fact that tendon repair becomes increasingly difficult with age (Howell et al. 2017).

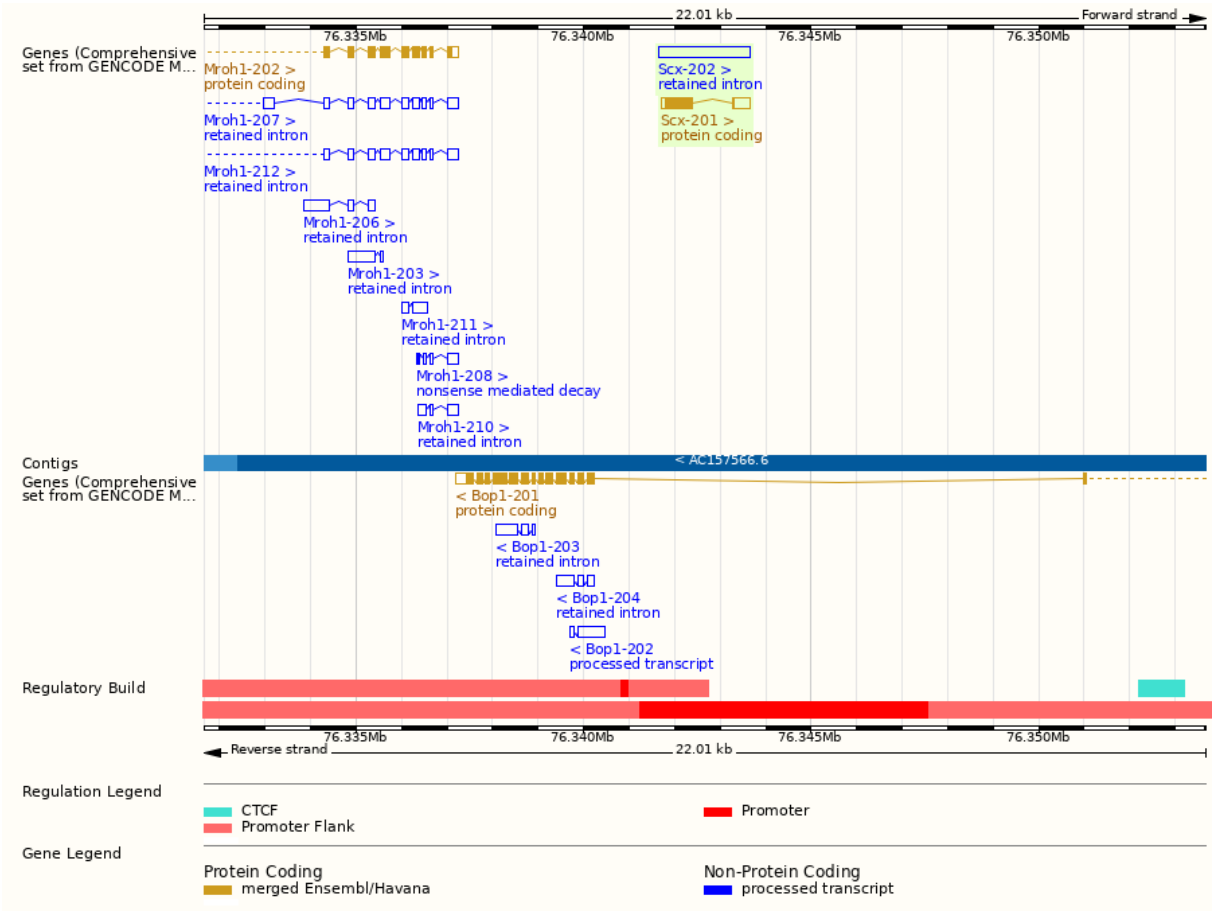

Figure 6. Schematic diagram of *Scleraxis bHLH transcription factor (Scx)*.
The Basic helix-loop-helix transcription factor scleraxis gene is located on mouse chromosome 15 and has two transcripts and is associated with 14 phenotypes. Its central role is to regulate early mesoderm formation and to induce trunk connective tissue formation. Its protein dimerizes with another bHLH protein(E12) and binds to the E-box consensus sequence to exert its regulatory effect. (Adapt from http://www.ensembl.org/Mus_musculus/Gene/Summary?db=core;g=ENSMUSG00000034161; r=15:76341652-76343658, on 6th.May 2021)

The Scx regulates the development of connective tissues such as tendons and collagen synthesis by increasing the significant cardiac isoform expression, collagen Iα2, in cardiac fibroblasts (Espira et al. 2009). Further studies have shown that Scx

deficiency causes thickening of the heart valves and disorder-organization of extracellular matrix collagen in mutant mice (Levay et al. 2008). This evidence suggests that Scx has a vital role in extracellular matrix remodeling and its role in the induction of differentiation of tendon lineages; the latter is one of the most critical factors for scar-free repair after tendon injury.

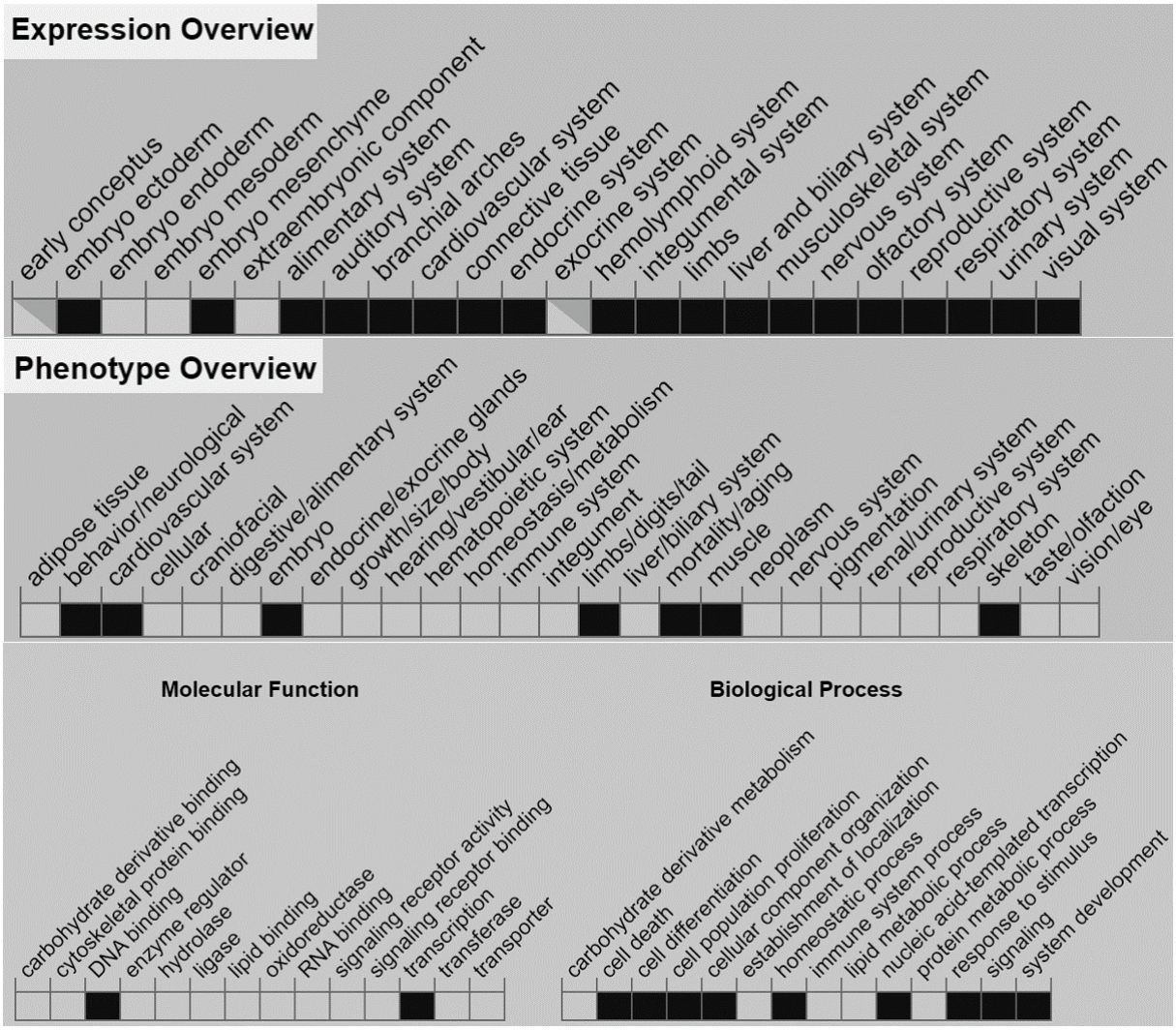

Figure 7. The General Information of *Scleraxis bHLH transcription factor (Scx)*.
An overview of the Scleraxis bHLH transcription factor gene expression, an overview of its phenotype, and an overview of its molecular biology and function are shown in the figure. The main expression sites are tendons and heart valves, and fascial-like tissues play an essential role in early tendon development. (Adapt from http://www.informatics.jax.org/marker/MGI:102934, on 6th. May 2021)

In tendon progenitors, deletion of the gene encoding Scx would result in a severe failure of dentinogenesis phenotype at embryonic day 13.5 (E13.5). Mutant mice are born with severely restricted locomotion, mainly due to the blocked differentiation of the tendon responsible for force transmission. However, the tendon, accountable for muscle anchoring, is virtually unaffected, suggesting that Scx plays a critical role in

restoring tendon force transmission function (Murchison et al. 2007). Restoring the function of force transmission after a tendon injury is one of the most critical parts of tendon function reconstruction (Figure 7).

Like its role in cardiac valves, the lack of Scx in tendon tissue leads to difficulties in extracellular matrix synthesis (especially Col1a1) and disorganized fibre bundle alignment (Figure 8). This disorder is mainly related to the failure of Scx, which is expressed explicitly in the tendon, to activate specific COL1a1 genes in tendon cells (Léjard et al. 2007).

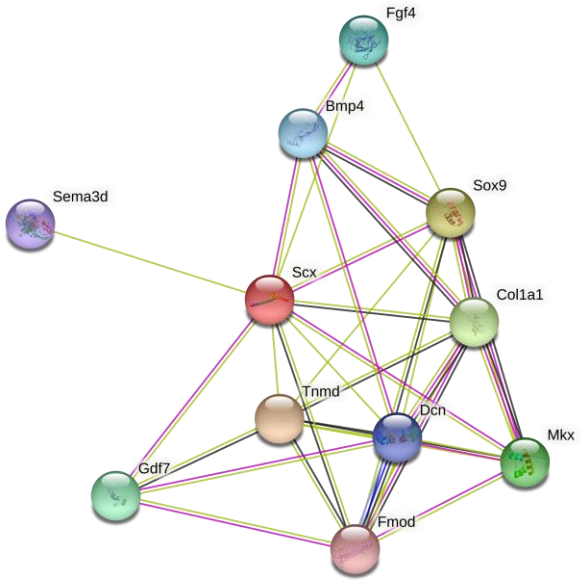

Figure 8. The protein-protein interaction network of *Scleraxis bHLH transcription factor (Scx)*. This figure is an overview of the Scx protein-protein interaction network. Tnmd, Fmod, Dcn, Col1a1 are essential proteins necessary for the tendon matrix. Bmp4, Fgf4, Gdf7 are growth factors crucial to tendon development. In contrast, Sox9 is a transcription factor that induces cartilage formation and is associated with ectopic ossification after tendon injury. In addition to Scx, Mkx is also a transcription factor essential for tendon. (Adapt from https://string-db.org/network/10090.ENSMUSP00000043668, on 6th. May 2021)

1.3.2 Mohawk homeobox (Mkx)

The Homeobox protein Mohawk is expressed in a similar region to Scx, but unlike Scx, its expression in kidney and testis development implies that it may regulate more developmental processes. The broad expression of Mkx also means that its promoter is not suitable as a promoter element for tendon-specific expression (Figure 9,10). Nevertheless, Mkx also has an essential regulatory role in the development of tendon tissue. In particular, it may play a critical role in the normal alignment of fibres,

as its absence causes wavy tails and tendon sheath abnormalities in transgenic mice. Mohawk homeobox is associated with defective development of tendon collagen fibres (particularly Col1a1) in mutant mice at embryonic day 16.5 (E16.5) (Ito et al. 2010).

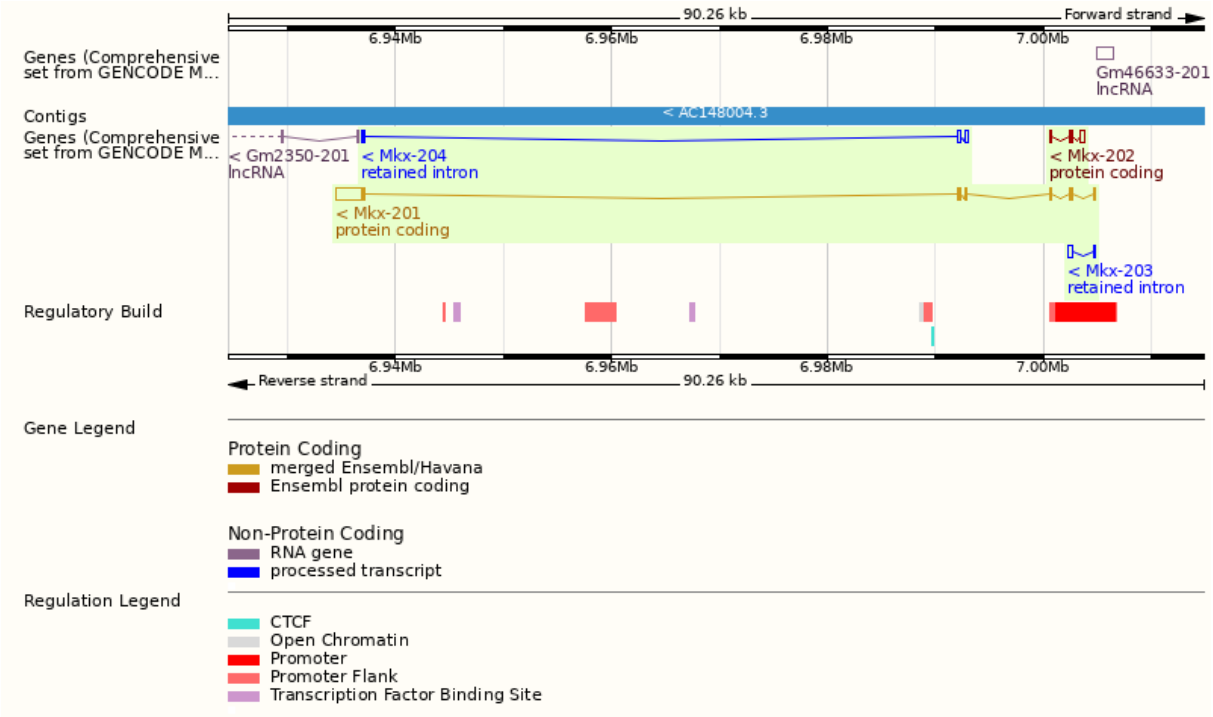

Figure 9. Schematic diagram of *Mohawk homeobox (Mkx)*.
The Homeobox protein Mohawk gene is located on mouse chromosome 18, has four transcripts, and is associated with three phenotypes. It plays a critical regulatory role in developing limb tendons, muscle, cartilage, and kidney. There are three isoforms of the proteins (one described). (Adapt from http://www.ensembl.org/Mus_musculus/Gene/Summary?db=core;g=ENSMUSG00000061013;r=18:6934518-7004780, on 6th. May 2021)

The Mohawk homology cassette (Mkx) in the periodontal ligament (PDL) is stably expressed from 10 weeks of adulthood to 12 months. Its deletion results in the loss of normal ligament cell morphology. In contrast, its overexpression increases collagen I expression and decreases osteogenic gene expression. Overexpression of Mkx prevents accidental ectopic ossification during tendon remodeling and enhances extracellular matrix reconstruction (Koda et al. 2017). Mkx expression peaks later than Scx, but its expression also decreases significantly later than Scx, suggesting that it may have a crucial role in adult tendon homeostasis (Figure 11).

Overexpression of the homologous cassette protein Mohawk (Mkx) promotes fibrous annulus (AF) tissue regeneration. By forming larger diameter collagenous protofibrils, Mkx can prevent intervertebral disc (IVD) degeneration (Nakamichi et al. 2016). This

study demonstrates the safety, feasibility, and efficacy of overexpressing Mkx at the in vivo level to reconstruct damaged tendons.

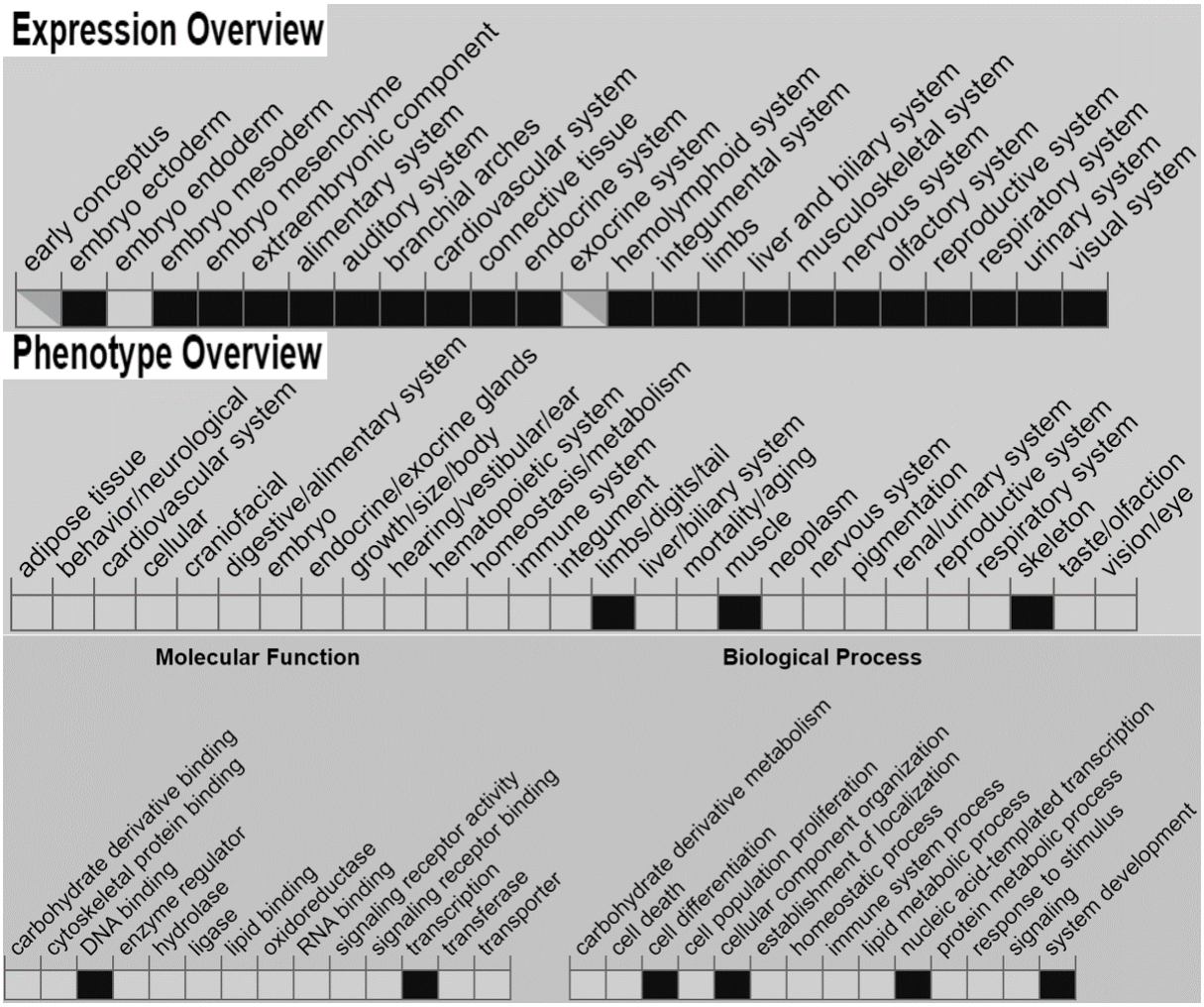

Figure 10. The General Information of *Mohawk homeobox (Mkx)*.
The figure shows the expression profile of the Mohawk homeobox (Mkx) genes, the profile of their phenotype, and the profile of their molecular biology and function. The leading site of expression is in skeletal muscle tissue; it plays a vital role in tendon development and the maintenance of homeostasis. (Adapt from http://www.informatics.jax.org/marker/MGI:2687286, on 6th.May 2021)

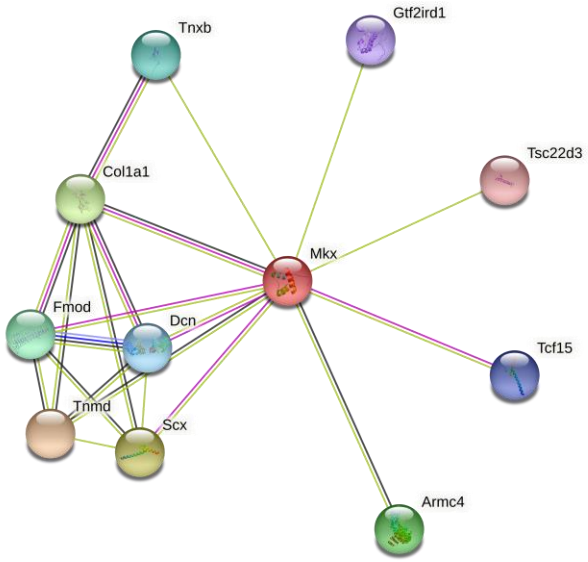

Figure 11. The protein-protein interaction network of *Mohawk homeobox (Mkx)*.
An overview of the protein-protein interaction network associated with Mkx. col1a1 is a crucial component of the tendon matrix, and Tnmd, Fmod, and Dcn are critical regulatory components of the tendon matrix. Scx and Mkx are key transcription factors in tendon development. (Adapt from https://string-db.org/network/10090.ENSMUSP00000078718, on 6th. May 2021)

1.3.3 Early growth response 1, 2 (Egr1 and Egr2)

In addition to the basic helix-loop-helix transcription factor Scx and the Mkx homology cassette genes, the early growth response transcription factors Egr1 and Egr2 are participate in vertebrate tendon development (Figure 12,13). They are involved in tendon formation through the regulation of Scx and the major extracellular matrix components of the tendon (such as Col1a1, Col3a1, Col5a1, Col12a1, and Col14a1). Deletions of Egr1 or Egr2 result in reduced expression of Col1a1 and reduced collagen fibrils in mutant mice (Lejard et al. 2011b).

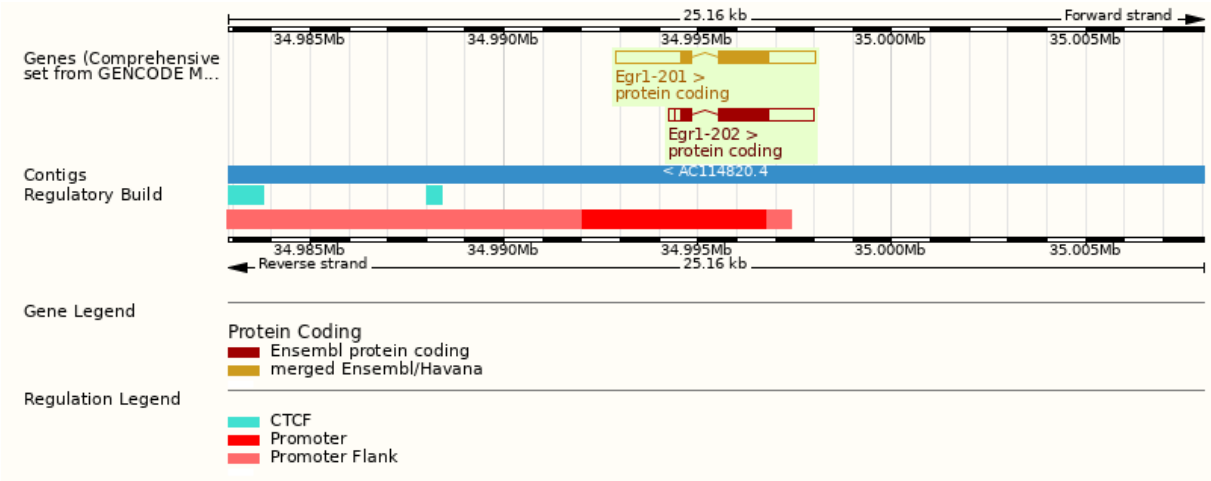

Figure 12. Schematic diagram of *Early growth response 1 (Egr1)*.

The early growth response gene is located on mouse chromosome 18, has two transcripts, and is associated with up to 103 phenotypes. Its absence has been shown to impair the expression of Scx and Col1a1, which are essential for repair after tendon injury. (Lejard et al. 2011a) (Adapt from http://www.ensembl.org/Mus_musculus/Gene/Summary?db=core;g=ENSMUSG00000038418;r=18:34992876-34998037, on 6th. May 2021)

Interestingly, the expression patterns of Egr1 and Egr2 are not identical. Although they are both expressed at the stressed tendon and the long tendon, the timing of their expression is not synchronous. The former is co-expressed with Scx in tendon tissue at day E12.5; the latter is not detected until E14.5 and does not continue to be expressed in limb tendons until around day E18 (Lejard et al. 2011a).

These findings may imply that their regulation of tendon varies according to cellular status. The emergence of different states of cells in the transient reprogramming of tendon cells needs to be considered in conjunction with a possible complementary combination of transcription factors (such as Egr1 and Egr2).

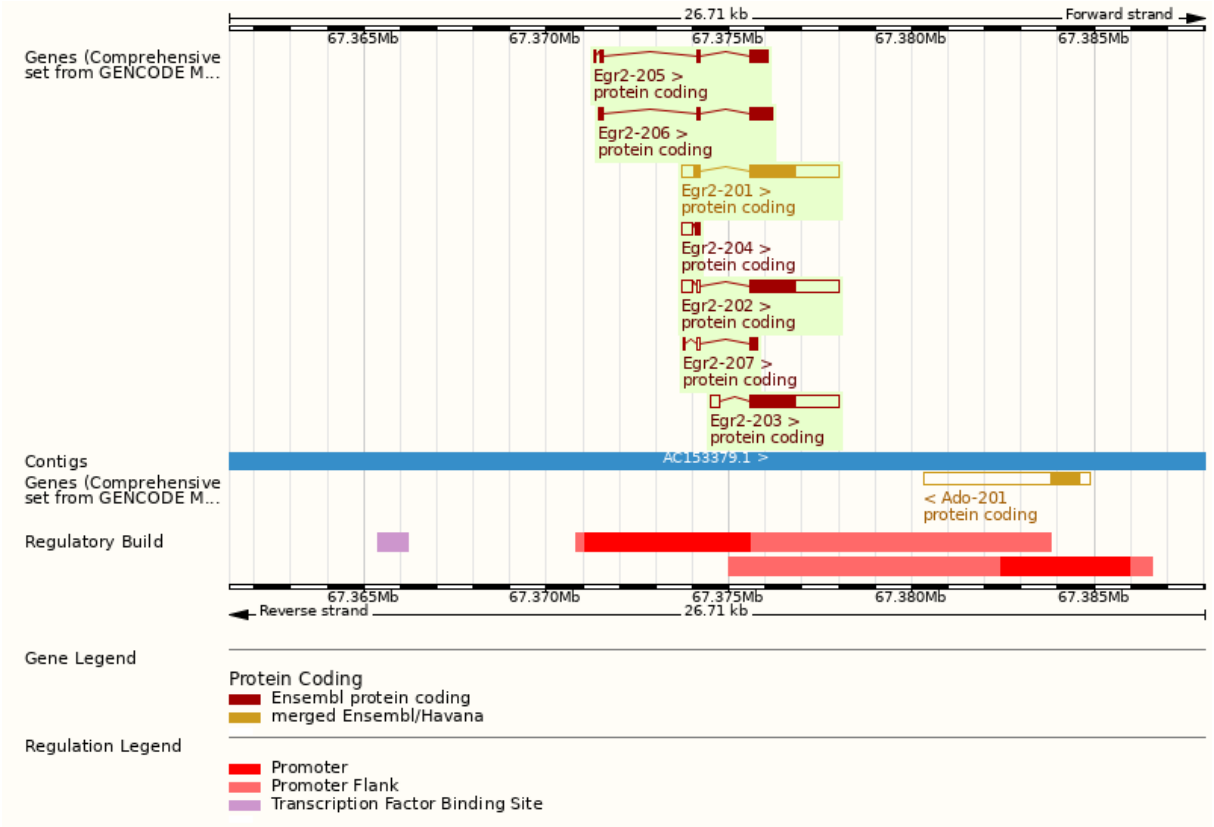

Figure 13. Schematic diagram of *Early growth response 2 (Egr2)*.
The early growth response 2 gene is located on mouse chromosome 10, has seven transcripts and is associated with 70 phenotypes. Some evidence shows that its expression pattern is different from that of Egr1, but both are involved in tendon development. It regulates the post-injury repair process of the tendon through the regulation of extracellular matrix homeostasis and Scx expression. (Adapt from http://www.ensembl.org/Mus_musculus/Gene/Summary?db=core;g=ENSMUSG00000037868;r=10:67371305-67378018, on 6th. May 2021)

Following tendon injury, EGR1 can activate Col1a1 and Col2a1 expression via the BMP12 / Smad1/5/8 pathway and regulate Scx transcript levels via TGF-β2 signaling (Guerquin et al. 2013a; Guerquin et al. 2013b; Tao et al. 2015). In Egr1-deficient adult mutant mice, the critical tendon transcription factor (Scx) and tendon's major extracellular matrix components (Col1a1 and Col1a2) are restricted but not completely absent. This result suggests, on the one hand, an essential role for normal tendon homeostasis and, on the other hand, an important secondary part for Scx, Mkx during tendon remodeling. Egr1 forced overexpression promotes MSC differentiation towards the tendon lineage and increases the repair process after Achilles tendon injury in vivo (Guerquin et al. 2013a).
In summary, Egr1 and Egr2 can better assist Scx and Mkx and complement the post-injury regenerative role of partially reprogrammed state tendon cells.

1.4 Cross-talks between two classes of transcription factors

The tendon is a rapidly ageing and degenerating motor organ, mainly associated with a decrease in tendon homeostasis due to ageing of the TSPC in situ. With age, TSPC in senescent tendons has an impaired capacity for self-renewal and proliferation. Although they can still differentiate into tendon cells, the remaining plasticity is insufficient to reverse the repair deficit in adult tendons. As TSPC ages, the senescence-associated marker p16 ^{INK4A} is upregulated. Age also causes poor adhesion and migration of TSPC cells, which manifests as delayed wound repair, and increased actin expression (smooth muscle actin-alpha: alpha-SMA), an actual cause of scarring (Kohler et al. 2013).

By transient overexpression of OSKM in prematurely aged mice, it has been shown partial reprogramming removes the ageing-induced molecular phenotype through epigenetic remodeling and promotes tissue reconstruction after injury (Lu et al. 2020; Ocampo et al. 2016). This finding suggests the potential for partial reprogramming to alter age-related repair malfunction. However, cellular rejuvenation and de-differentiation with oncogenic risk require capturing the fit cellular states, especially in vivo. The introduction of genealogically anchored transcription factors is, therefore, a potentially safe and reliable strategy (Lu et al. 2020; Sarkar et al. 2020; Wang et al. 2021). The reprogramming transcription factors would improve the cellular state and

create the conditions for the latter to play a typical regulatory role. On the other hand, the tendon transcription factors might prevent the oncogenic risk associated with the overexpression of the reprogramming transcription factors.

1.4.1 Enhanced restorative capacity

Fetal tendon injuries can be remodelled without scarring. Still, this healing capacity gradually decreases with age, so how to awaken cells' healing ability is a significant part of tendon regeneration. The decline in healing capacity due to ageing is mainly due to epigenetic changes that alter gene expression patterns. As a reliable tool for epigenetic remodelling, OSKM can remove senescence-associated phenotypes such as $p16^{INK4A}$ in TSPC, and this alteration helps restore cell adhesion, migration, and proliferation capacity (Chen et al. 2017).
Further studies have shown that retinal ganglial cell regeneration in aged mice can be restored by altering the TET1 and TET2 genes through OSK expression alone, restoring vision in mice with optic nerve damage (Lu et al. 2020). More interestingly, reprogramming after the injury occurs preferentially in stem/progenitor cells, which increases the efficiency and feasibility of the reprogramming strategy (Chiche et al. 2017).

In 2011, the expression of reprogramming factors (c-Myc and Klf4) and chondrogenic critical factor Sox9 successfully transformed fibroblasts into hyaline chondrocytes (Hiramatsu et al. 2011). Therefore, in addition to the reprogramming effect of OSKM, the direct transformation of reprogramming factors in combination with tissue-specific key transcription factors is also an essential mechanism for tendon remodeling.

Further studies have shown that expression of reprogramming factors (KLF4 and c-MYC) alone in combination with cartilage inducible medium (mTeSR: containing Fgf, Tgf-β) is sufficient for direct transformation of skin fibroblasts into cartilage lineage cells (Wang et al. 2017). This finding is of even greater relevance in vivo with an inducible microenvironment. In contrast, the tendon key transcription factors (Egr1, Mkx) for promoting the tendon repair growth factor (Tgf-β) is likely to concert with reprogramming factors (Guerquin et al. 2013a; Liu et al. 2015).

1.4.2 Reduce scar formation

The impaired regeneration accompanying ageing coincides with enhanced scarring (fibrosis) associated with ageing altering the crosstalk between hematopoietic and vascular cells. In senescent organs, Neuropilin-1/hypoxia-inducible-factor 2α (HIF2α) inhibits the endothelial protein C receptor (EPCR) pathway, which leads to the recruitment of pro-fibrotic TIMP1 high expressing macrophages (Chen et al. 2021b). Interestingly, fibrosis and scar formation were significantly reduced by transient reprogramming of OSKM in vivo (Doeser et al. 2018b).

Expression of OSKM results in reduced α-SMA expression in post-injury fibroblasts, which leads to a reduction in myofibroblasts and contributes to a decrease in scar formation. Further studies found that reprogramming transcription factors reduced scar repair by decreasing TGFß1 and vascular endothelial growth factor (VEGF) expression. This result implies that reprogramming factors can promote scar-free repair in a partially reprogrammed state without teratoma formation (Doeser et al. 2018b).

1.5 Purpose of the Study

Accordingly, I intend to activate the Td4F-OSKM transcription factor in this mutant after experimental tendon injury. The aim is to induce OSKM factors in tendon lesions to reduce scar tissue formation and Td4F factors to differentiate peri-wound cells into new tenocytes (tendon cells). I will also examine the extent to which OSKM-mediated partial reprogramming improves the cellular state by the reporter protein Scarlet and the role of eight transcription factors in reducing in situ fibrosis by BFP, thus creating a basis that may allow tissue healing. To this end, histological follow-up controls are performed to assess the pathology of the damaged tendon tissue. These are achieved by studying the expression profile of fibrosis and embryonic tendon development.

To achieve the above experimental goals, the understanding of to what extent OSKM-mediated in situ partial reprogramming can reduce fibrosis is necessary. Furthermore, it is essential to understand whether temporary and locally limited

pluripotent state tendon cells can differentiate into mature tendon cells by overexpressing key transcription factors. I have established inducible transgenic mouse lines that specifically overexpress two classes of (dedifferentiated and re-differentiated) transcription factors and fluorescent reporter transgenic mice that report tendon cell status fluorescent proteins. Through the above work, I established the animal models and experimental platform for the phase II experiments.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents

2.1.1.1 Chemicals

- Absolute Ethanol (Sigma, #51976)
- Agarose (GeneON, #604001)
- Amphotericin B solution (Sigma, #A2942)
- Antarctic Phosphatase (NEB #M0289)
- Boric acid (Sigma, #B7901)
- DAPI for nucleic acid staining (Sigma, #D9542)
- Double Distilled Water (Mili Q water)
- Dimethyl Sulfoxide (DMSO) (Sigma, #D2650)
- Dulbecco's Modified Eagle Medium (Gibco, #41965062)
- Doxorubicin hydrochloride (Sigma, #D1515)
- deoxyribonucleotide triphosphate (Fisher Scientific, #R0193)
- Fetal Bovine Serum (Fisher Scientific, #A4736401)
- Gelatin from bovine skin (Sigma, #G1890)
- Glycerol (Sigma, #G5516)
- Go Taq DNA Polymerase Kit (Promega, #M3001)
- Isoflurane (Baxter, #FDG9623)
- Isopropanol (VWR, #NC9704454)
- Kanamycin (Roche, #T832.4)
- Liberase Blendzyme III (Roche)
- Lithium acetate dihydrate (Sigma, #L6883)
- Magnesium Chloride 6-hydrate for analysis ($MgCl_2$, AppliChem #131396)
- Mowiol 4-88 (Roche, #0718)
- NEBuilder HiFi DNA Assembly Master Mix (NEB #E2621)
- Normal Goat Serum (Cell Signaling Technologies, #5425)
- NP-40 Surface-Amps Detergent Solution (Fisher Scientific, #R85124)
- Phosphate buffered saline (PBS, Gibco #10010-015)

- Penicillin-Streptomycin (Gibco, #15140122)
- peqGREEN DNA/RNA dye (VWR, #732-2960)
- Paraformaldehyde (Sigma, #158127)
- Poly-L-lysine solution (Sigma, #P8920)
- Potassium Chloride (Merck, #7447-40-7)
- Proteinase K (Roche, #3115801001)
- Quick Ligation Kit (NEB #M2200)
- Q5 High-Fidelity 2X Master Mix (NEB #M0492)
- Ribonuclease (Roche, #10109169001)
- Sterile endotoxin-free ultra-pure water (Sigma, #W1503)
- Sucrose (Sigma, #S0389)
- Tissue-Tek O.C.T. Compound (A. Hartenstein)
- Tris-Sodium citrate dihydrate (Tris-HCl, Sigma #T2663)
- Trypsin-EDTA (Gibco, #25300054)
- Triton-X-100 (Sigma, #T8787)
- TWEEN- 20 (Sigma, #9005-64-5)
- UltraPure™ Phenol:Chloroform: Isoamyl Alcohol (Fisher Scientific, #R15593031)
- Xylene (VWR, #XX0020)
- 3M Sodium acetate (NaOAc) buffer solution (Sigma, #S7899)
- 37%Hydrochloric acid (HCl, Sigma# 320331)

2.1.1.2 Enzymes

Restriction Endonucleases (New England Biolabs):

Table 1. Restriction Endonucleases List

Restriction Endonucleases	Catalog #	Amount(units)	Concentration(units/ml)
Afe Ⅰ	R0652S	200 units	10,000 units/ml
AflⅡ	R0520S	2,000 units	20,000 units/ml
Age Ⅰ	R0552S	300 units	5,000 units/ml
Asc Ⅰ	R0558S	500 units	10,000 units/ml
AtaⅡ	R0117S	500 units	20,000 units/ml
Bamh Ⅰ	R0136S	10,000 units	20,000 units/ml
Bgl Ⅰ	R0143S	2,000 units	10,000 units/ml
Dpn Ⅰ	R0176S	1,000 units	20,000 units/ml
Eag Ⅰ	R3505S	500 units	20,000 units/ml
EcoR Ⅰ	R0101S	10,000 units	20,000 units/ml
EcoR Ⅴ	R0195S	4,000 units	20,000 units/ml
Fse Ⅰ	R0588S	100 units	2,000 units/ml
HindⅢ	R0104S	10,000 units	20,000 units/ml
Kpn Ⅰ	R0142S	4,000 units	10,000 units/ml
Mlu Ⅰ	R0198S	1,000 units	10,000 units/ml
Nco Ⅰ	R0193S	1,000 units	10,000 units/ml
Nhe Ⅰ	R3131S	1,000 units	20,000 units/ml
Not Ⅰ	R0189S	500 units	10,000 units/ml
Pst Ⅰ	R0140S	10,000 units	20,000 units/ml
Sal Ⅰ	R0138S	2,000 units	20,000 units/ml
Sma Ⅰ	R0141S	2,000 units	20,000 units/ml
Spe Ⅰ	R0133S	500 units	10,000 units/ml
Sph Ⅰ	R0182S	500 units	10,000 units/ml
Swa Ⅰ	R0604S	2,000 units	10,000 units/ml
Xba Ⅰ	R0145S	3,000 units	20,000 units/ml
Xho Ⅰ	R0146S	5,000 units	20,000 units/ml
Xma Ⅰ	R0180S	500 units	10,000 units/ml

2.1.1.3 Plasmids

Donor Plasmid (Addgene; Origene; BACPAC):

Table 2. Donor Plasmids List

NO.	Plasmid Name	Source	Catalog#
14a. pXL117-Insulators-TetO-4TF-EmGFP			
1	pXL117(pSL1180-ß+ASA+FSP)	Our Lab	
2	Ai62(TITL-tdT)Flp-in replacement vector	Addgene	61576
3	mCol.4F 2A targeting construct	Addgene	25794
4	TetO-FUW-OSKM	Addgene	20321
5	pBluescript II KS (+)	Our Lab	
6	pCMV6-mMkx-DDK-tag	Origene	225168
7	pCMV6-mScx-DDK-tag	Origene	202202
8	pCMV6-mEgr1-DDK-tag	Origene	227136
9	pcDNA 3.1-mouse Egr2	Addgene	107997
10	pLenti6.3/TO/CD9-GFP	Addgene	104402
11	pBS-T2A-EGFP	Our Lab	
15b. pXL117-Insulators-pCol1a1-TagBFP-HA-Insulators			
12	BAC CLONE Col1a1	BACPAC	23-10B22
13	Fuw-dCas9-Tet1CD-P2A-BFP	Addgene	108245
19. pXL117-Scx gDNA-Scarlet-V5-T2A-rtTAV16			
14	BAC CLONE SCX	BACPAC	23-208P14
15	AAVp CAG-FLEX-mScarlet WPRE	Addgene	99280
16	AAV-hSynl-rtTAV16	Addgene	102367

2.1.2 Equipment

- Autoclave (Fedegari FNR 9248 E and NF6276 B)
- Anotop 10 Syringe Filter, 0.22um (Whatman, #6809-1002)
- Bacteria Cooled Incubator (BINDER KB 53)
- Bacterial Lab Stackable Incubation Shaker (Sartorius CERTOMAT BS-1)
- Binocular Stereomicroscope (Carl Zeiss Semi 2000-CS)
- Cell Culture Incubator (HERA Cell 150)
- Confocal Microscope (Carl Zeiss LSM900)
- Cryostat microtome (Leica CM3050S)
- Cryotubes (Thermo Scientific)
- Dialysis Tubes (Millipore Sigma, #D9652-100FT)
- Eppendorf Thermomixer (Eppendorf, #comfort 5355)
- Falcon tubes 15ml, 50ml (Fisher Brand)
- Gel imaging system (INTAS GEL IX IMAGER)
- GenElute™ HP Plasmid Miniprep Kit (Millipore Sigma, #NA0150)
- Gene Pulser/MicroPulser Electroporation Cuvettes (Bio Rad, #1652089)
- Glass Wool (Thermo Scientific)
- Light Microscope (Leica)
- Microtome (Microtech)
- MicroPulser Electroporator (Bio Rad, #1652100)
- Mr Frosty ™ Freezer Container (Thermo Scientific, #5100-0001)
- Nanodrop Spectrophotometers (Thermo Scientific)
- Petri plate (Falcon)
- Refrigerated Benchtop Centrifuge (Eppendorf, #5415r)
- Research plus (IVD) single-channel pipettes (Eppendorf,10 - 1000 µL)
- Serological Pippettes (Cellstar, 2ml- 50ml)
- Slides Superfrost plus (Thermo Scientific)
- Spectrophotometers (Eppendorf, #6131)
- Sorvall Centrifuge (Sorvall RC 5B plus)
- Vortex (NeoLab)
- Water Bath (Julabo)

- ZymoPURE II Plasmid Maxiprep (Zymo Research, #D4203)
- 1.5ml Wechselblock Top Shaker Thermo Mixer (Eppendorf)

2.2 Methods

2.2.1 Molecular clone

We are designing target constructions and developing molecular clone strategies. The molecular cloning of the target DNA is then carried out as outlined below (Table 1,2)：

2.2.1.1 pXL117-Insulator-TetO-4TF-EmGFP (# 14a.)

1.The pBluescript II KS (+) and TetO-FUW-OSKM plasmids were cleaved by EcoR Ⅰ, the former as vector and the latter carrying the OSKM fragment as insertion. The insertion was treated with alkaline phosphatase to remove the phosphate group at the 5' end to prevent DNA self-association (5' end linked to the 3' end), thus making the insertion can only be ligated to the vector to produce a pBS-OSKM recombinant plasmid with OSKM gene.

2.The PBS-OSKM plasmid harvested in the previous step was double digested by Kpn Ⅰ+Sal Ⅰ. The plasmid was re-ligated with Klenow catalysis in the presence of supplemental dNTPs to remove the Xho Ⅰ digestion site. The purpose is to prepare for further molecular cloning. (Obtain the reconstruction vector named pBS-OSKMv)

3.Amplification of the Mkx gene in the pCMV6-mMkx-DDK-tag plasmid under the Q5 High-Fidelity 2X Master Mix. The resulting PCR product was digested with the DpnI enzyme to remove the template DNA. pBS-OSKMv was cleaved by the SphI enzyme to remove the Sox2 gene and form the vector, which was ligated to the Mkx fragment under the NEBuilder HiFi DNA Assembly Master Mix. (to construct pBS-Mkx-OKMv recombinant plasmid)

4.The Egr1 gene in the pCMV6-mEgr1-DDK-tag plasmid was amplified in the Q5 High-Fidelity 2X Master Mix as described in the previous step. The resulting PCR product was digested with the DpnI enzyme to remove the template DNA. pBS-Mkx-OKMv was cleaved by the XhoI enzyme to remove the Klf4 gene and form a vector, which was ligated to the Egr1 fragment under the NEBuilder HiFi DNA Assembly

Master Mix. (to construct pBS-Mkx-Egr1-OMv recombinant plasmid)

5.The pBS-Mkx-Egr1-OMv plasmid was partially cleaved with EcoR Ⅰ followed by complete cleavage with Nhe Ⅰ to remove the Oct4 gene and form a vector. The Scx gene in the pCMV6-mScx-DDK-tag plasmid was amplified under the Q5 High-Fidelity 2X Master Mix. The obtained PCR product was digested with the DpnI enzyme to remove the template DNA. The received vector was ligated to the Scx gene fragment under the NEBuilder HiFi DNA Assembly Master Mix. (to form pBS-Scx-Mkx-Egr1-Mv recombinant plasmid)

6.The pBS-Scx-Mkx-Egr1-Mv plasmid was partially cleaved with EcoR Ⅰ followed by complete cleavage with Nhe Ⅰ to remove the Myc gene and form a vector. The EGFP gene in the pLenti6.3/TO/CD9-GFP plasmid was amplified in Q5 High-Fidelity 2X Master Mix. The obtained PCR product was digested with the DpnI enzyme to remove the stencil DNA. The received vector was ligated to the EGFP gene fragment under the NEBuilder HiFi DNA Assembly Master Mix (Figure 14). (to form pBS-Scx-Mkx-Egr1-EGFP recombinant plasmid)

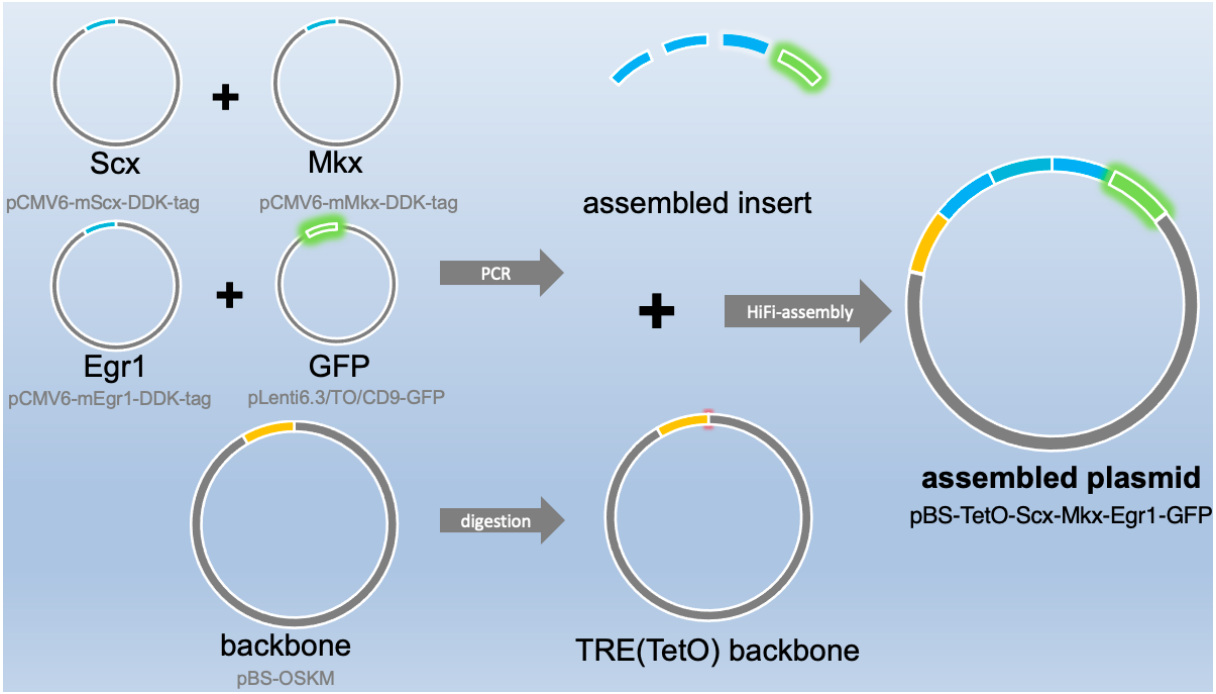

Figure 14. Td4F plasmid I – Scx, Mkx, Egr1, GFP & TetO.
The diagram above shows the PCR insertion of Scx, Mkx, Egr1, GFP to generate the recombinant plasmid pBS-TetO-Scx-Mkx-Egr1-GFP in steps 1-6.

7.To introduce T2A into the pBS-TetO-Scx-Mkx-Egr1-EGFP plasmid, the EGFP was removed by Asc Ⅰ digestion and then re-ligated to obtain pBS-TetO-Scx-Mkx-Egr1-E2A.The pBS-TetO-Scx-Mkx-Egr1-E2A plasmid was double digested with BamH Ⅰ and Not Ⅰ to form the vector. The T2A-EGFP gene in the pBS-T2A-EGFP plasmid was amplified under Q5 High-Fidelity 2X Master Mix. The resulting PCR product was digested with the Dpn I enzyme to remove the stencil DNA. The vector pBS-TetO-Scx-Mkx-Egr1-E2A was ligated with the T2A-EGFP gene fragment under NEBuilder HiFi DNA Assembly Master Mix to form pBS-TetO-Scx-Mkx-Egr1-d (double 2A elements)-EGFP recombinant plasmid (Figure 15).

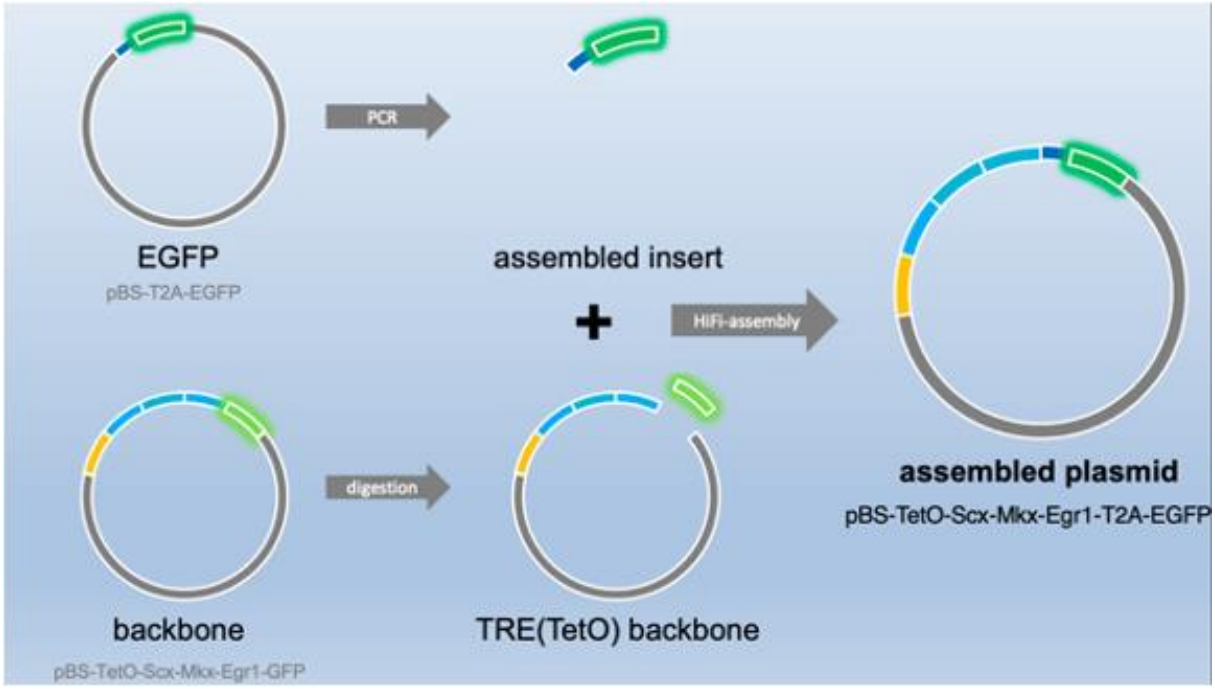

Figure 15. Td4F plasmid II – T2A & EmGFP.
The diagram above shows the PCR insertion of T2A and EmGFP to generate the recombinant plasmid pBS-TetO-Scx-Mkx-Egr1-T2A-EGFP in steps 7.

8.pBS-TetO-Scx-Mkx-Egr1-d-EGFP was subjected to Asc I digestion to form the vector. The Egr2 gene in the pcDNA 3.1-mouse Egr2 plasmid was amplified under Q5 High-Fidelity 2X Master Mix. The resulting PCR product was digested with the Dpn I enzyme to remove the stencil DNA. The vector was ligated with the Egr2 gene fragment under NEBuilder HiFi DNA Assembly Master Mix to form the pBS-Scx-Mkx-Egr1-Egr2-EGFP recombinant plasmid (Figure 16).

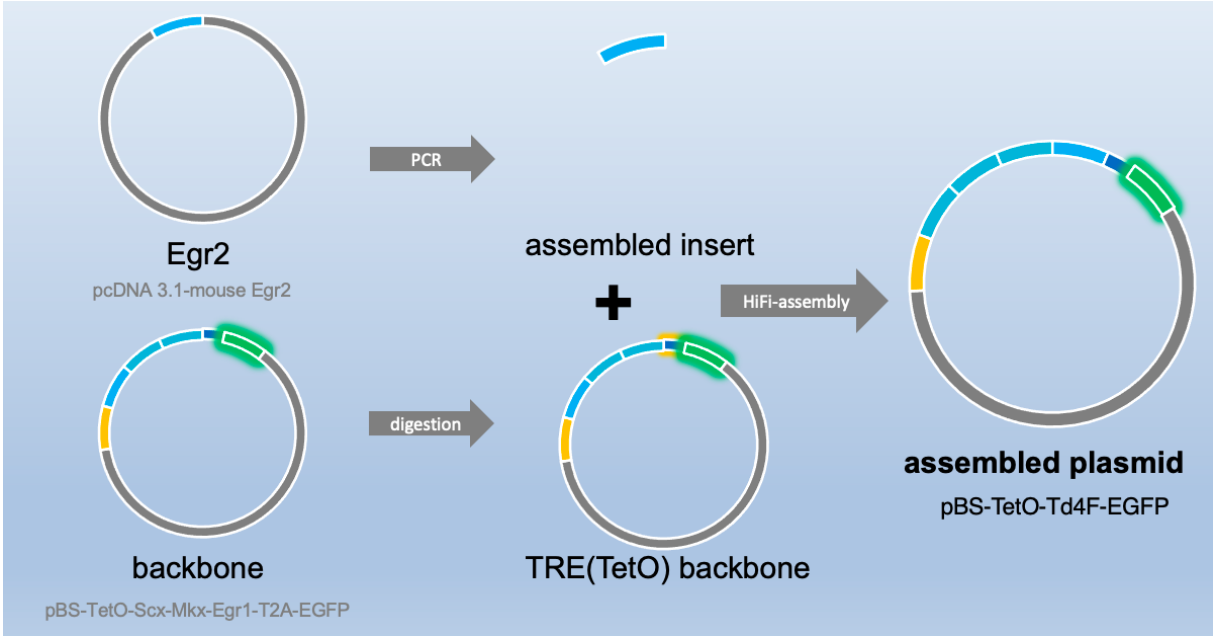

Figure 16. Td4F plasmid III – Egr2.
The diagram above shows the PCR insertion of Egr2 to generate the recombinant plasmid pBS-TetO-Td4F-EGFP in steps 8.

9.The pXL117 (pSL1180-ß+ASA+FSP) vector was modified for the next cloning step. The pXL117 was double digested with EcoRI and NsiI, then blunt the ends, and the pXL117m1 recombinant vector was obtained by re-ligation.

10.pXL117m1 was double digested with the Fse I and Asc I as a vector, and Ai62(TITL-tdT)Flp-in replacement vector was double digested with the Fse I and Asc I to obtain Insulator-TetO-tdTomato insert fragment. The pXL117m1-Insulator-TetO-tdTomato plasmid was obtained after enzyme ligation.

11.To introduce the NotI digestion site for the next cloning step, the pXL117m1-Insulator-TetO-tdTomato plasmid was double digested with SwaI and MluI as a vector. The vector was linked to the designed Linker oligos annealing enzyme to obtain the pXL117m1-Insulator-TetO-Linker plasmid.

12.Using the NotI cut site introduced in the previous step, the pXL117m1-Insulator-TetO-Linker plasmid was double cleaved with SwaI and NotI and used as a vector. The pBS-Scx-Mkx-Egr1-Egr2-EmGFP was double digested with EcoRV and NotI to obtain the Scx-Mkx-Egr1-Egr2-EmGFP insert (Figure 17). The pXL117-Insulator-TetO-4TF-EmGFP (# 14a.) plasmid was obtained by enzyme ligation of both. (# 14a.

linearized with EcoRV + SalI)

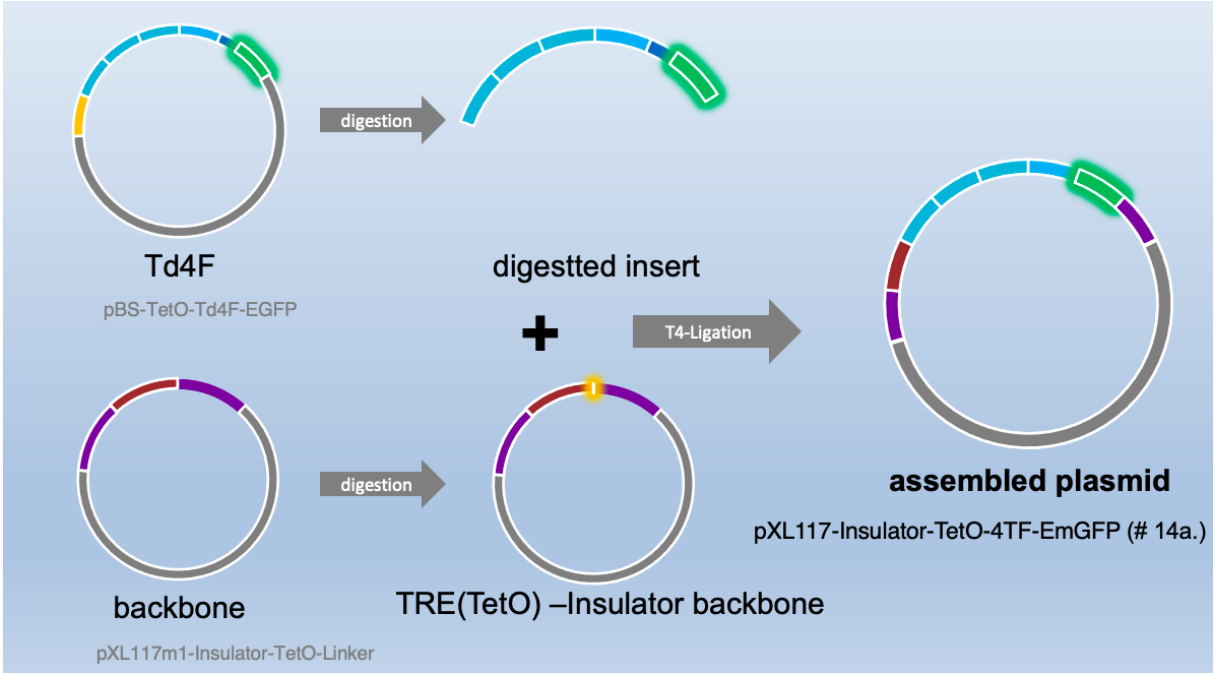

Figure 17. Td4F plasmid IV – TetO & Insulator.
The diagram above shows the insertion of Td4F-EGFP into the pXL117m1-Insulator-TetO-Linker backbone in an enzymatic cleavage and enzymatic linkage approach to generate the recombinant plasmid pXL117-Insulator-TetO-4TF-EmGFP (# 14a.) of step 12.

2.2.1.2 pXL117-Insulators-pCol1a1-TagBFP-HA (# 15b.)

13. The pXL117m1-Insulator-TetO-tdTomato obtained in step 11 was subjected to AgeI and MluI double digestion to remove TetO-tdTomato and then used as a vector. The Col1a1 proximal promoter DNA from BAC CLONE Col1a1 was amplified in Q5 High-Fidelity 2X Master Mix, and the two were ligated in NEBuilder HiFi DNA Assembly Master Mix to obtain pXL117m1-Insulator-pCol1a1L recombinant plasmid (Figure 18).

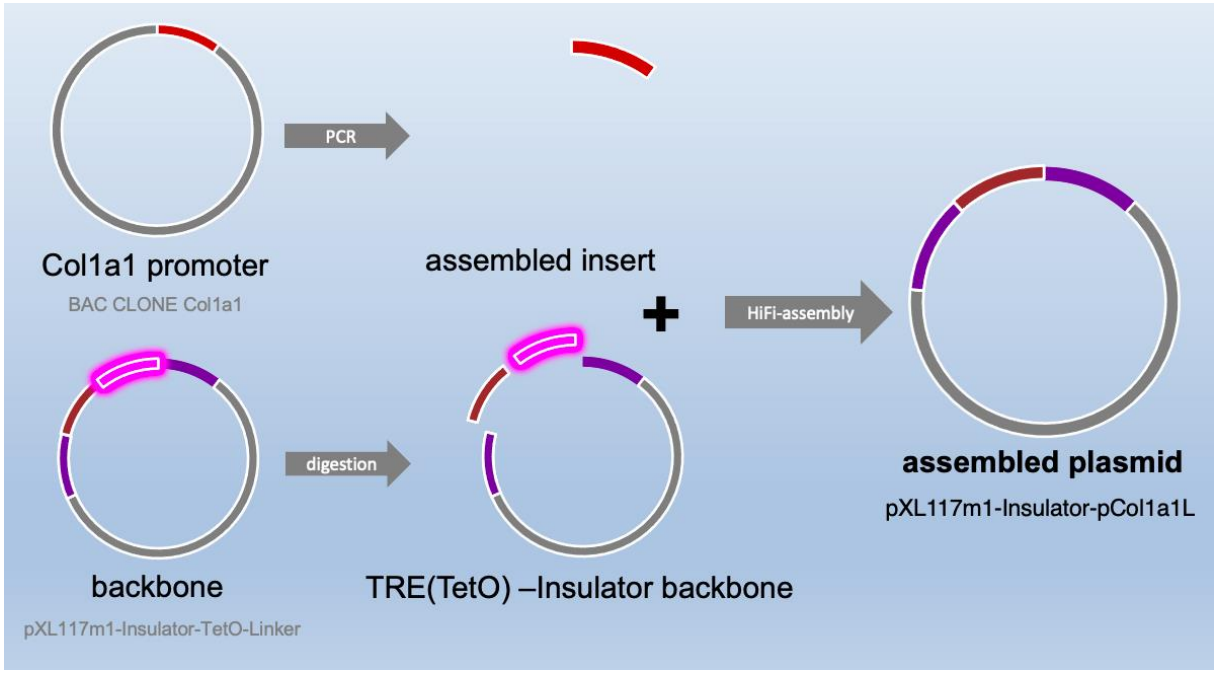

Figure 18. Col1a1-BFP plasmid I – Col1a1 promoter.
The diagram above shows the PCR insertion of Col1a1 proximal promoter to generate the recombinant plasmid pXL117m1-Insulator-pCol1a1L in steps 13.

14. pXL117m1-Insulator-pCol1a1L was subjected to NheI digestion as a vector. A BFP gene fragment from Fuw-dCas9-Tet1CD-P2A-BFP was amplified under Q5 High-Fidelity 2X Master Mix as an insert. The two were ligated under NEBuilder HiFi DNA Assembly Master Mix to obtain the pXL117m1-Insulator-pCol1a1L-TagBFP recombinant plasmid (Figure 19).

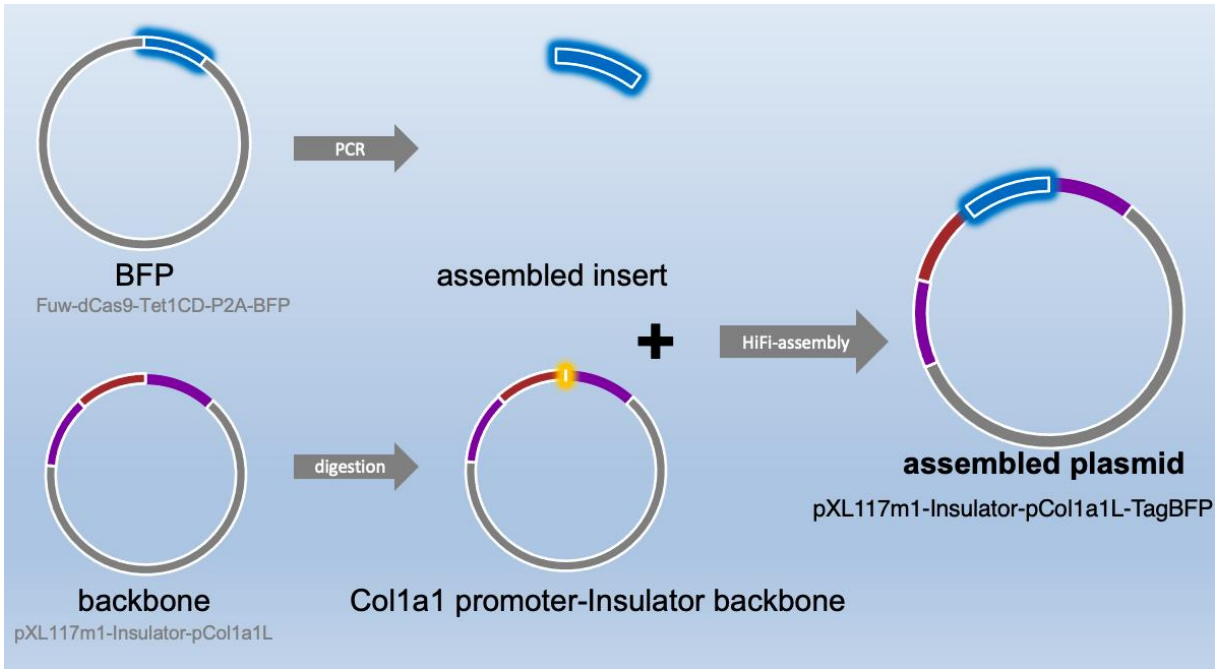

Figure 19. Col1a1-BFP plasmid II – BFP.
The diagram above shows the PCR insertion of TagBFP to generate the recombinant plasmid pXL117m1-Insulator-pCol1a1L-TagBFP in steps 14.

15. The pXL117m1-Insulator-pCol1a1L-TagBFP was subjected to SwaI and NheI double digestion as a vector. It was ligated with HA oligos annealing to obtain a pXL117-Insulators-pCol1a1-TagBFP-HA-Insulators (# 15b.) recombinant plasmid containing HA-tag (Figure 20). (# 15b. linearized with EcoRV + SalI)

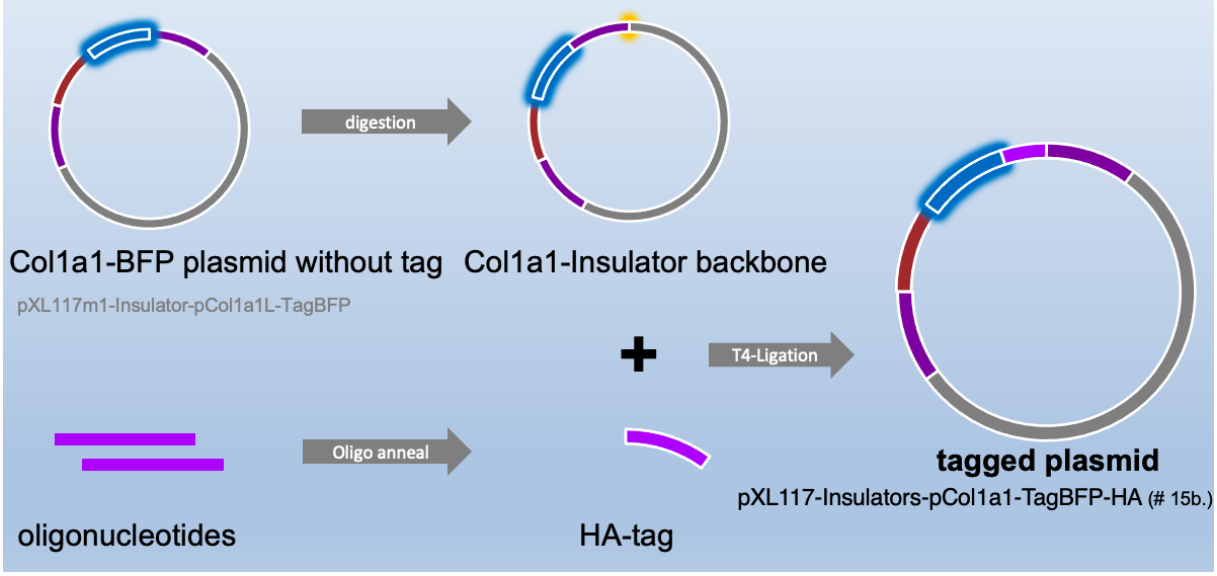

Figure 20. Col1a1-BFP plasmid II – HA-tag.
The diagram above shows the oligo anneal and Ligation of HA-tag to generate the recombinant plasmid pXL117-Insulators-pCol1a1-TagBFP-HA (# 15b.) in steps 15.

2.2.1.3 pXL117-pScx-Scarlet-V5-T2A-rtTAV16 (# 19)

16. For the next cloning step, the designed oligos annealing needs to be introduced into pXL117m1. pXL117m1 was double digested with MluI and XhoI and then ligated with oligos annealing to obtain the pXL117m2 recombinant vector.

17. Referring to step 13, pXL117m2 was subjected to XhoI digestion as a vector. The Scx promoter DNA in BAC CLONE SCX was amplified in Q5 High Fidelity 2X Master Mix. Both were ligated in NEBuilder HiFi DNA Assembly Master Mix to obtain the pXL117m2-Scx gDNA recombinant plasmid (Figure 21).

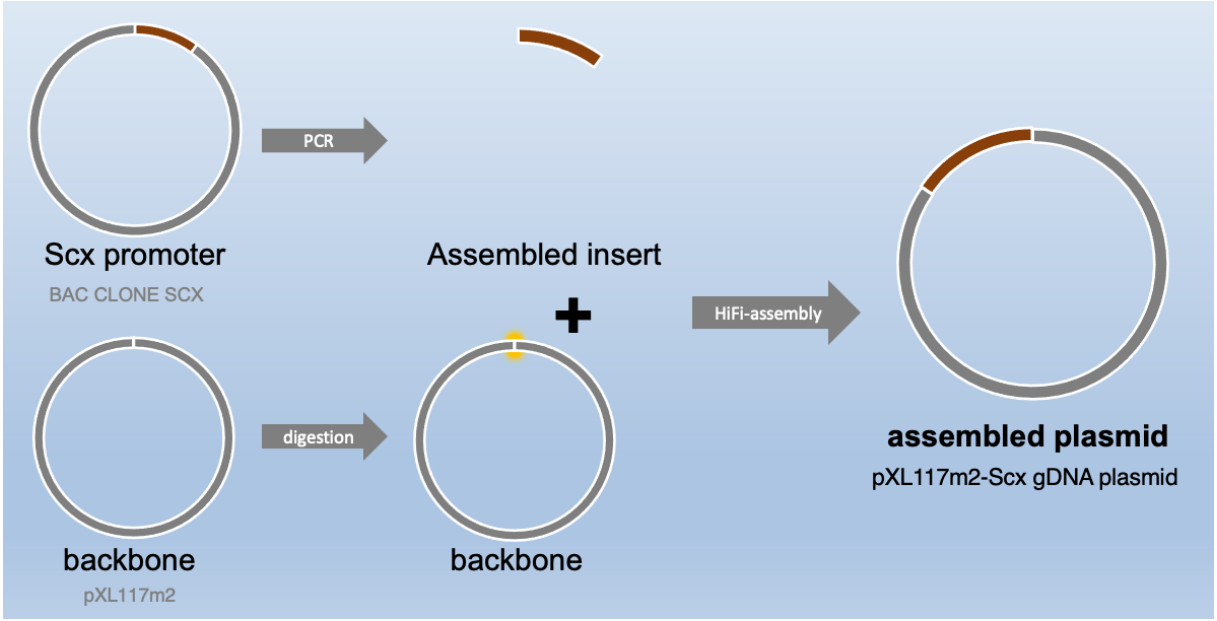

Figure 21. Scx-Scarlet plasmid I – Scx promoter.
The diagram above shows the PCR insertion of Scx promoter to generate the recombinant plasmid pXL117m2-Scx gDNA in steps 17.

18. The pBS-T2A-EmGFP was subjected to NcoI digestion to remove EmGFP as a vector. The rtTAV16 of AAV-hSynI-rtTAV16 was amplified in Q5 High Fidelity 2X Master Mix, and both were ligated in NEBuilder HiFi DNA Assembly Master Mix to obtain pBS-T2A-rtTAV16 recombinant plasmid.

19. The pBS-T2A-rtTAV16 was subjected to BamHI digestion as a vector. The mScarlet of AAV pCAG-FLEX-mScarlet WPRE was amplified in Q5 High Fidelity 2X Master Mix, and both were ligated in NEBuilder HiFi DNA Assembly Master Mix to obtain pBS-Scarlet-T2A-rtTAV16 recombinant plasmid (Figure 22).

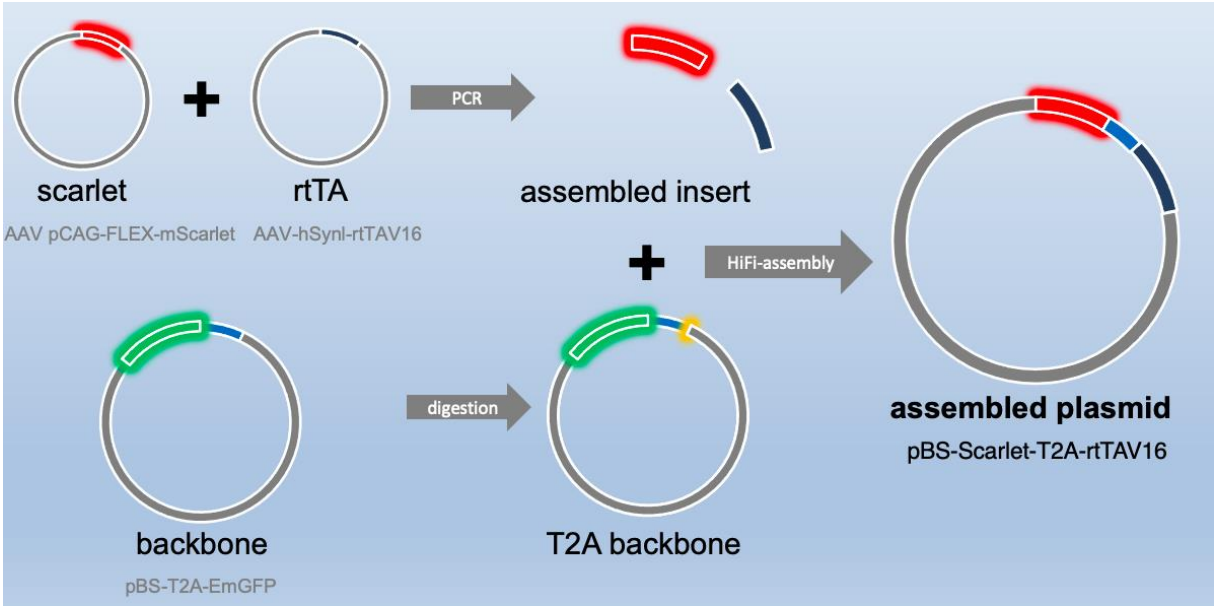

Figure 22. Scx-Scarlet plasmid II – Scarlet, T2A & rtTA.
The diagram above shows the PCR insertion of Scarlet and rtTA to generate the recombinant plasmid pBS-Scarlet-T2A-rtTAV16 in steps 18,19.

20. The pBS-Scarlet-T2A-rtTAV16 was subjected to BglII and MluI double digestion as a vector. The vector was ligated with V5 oligos annealing to obtain the pBS-Scarlet-V5-T2A-rtTAV16 recombinant plasmid containing the V5-tag (Figure 23).

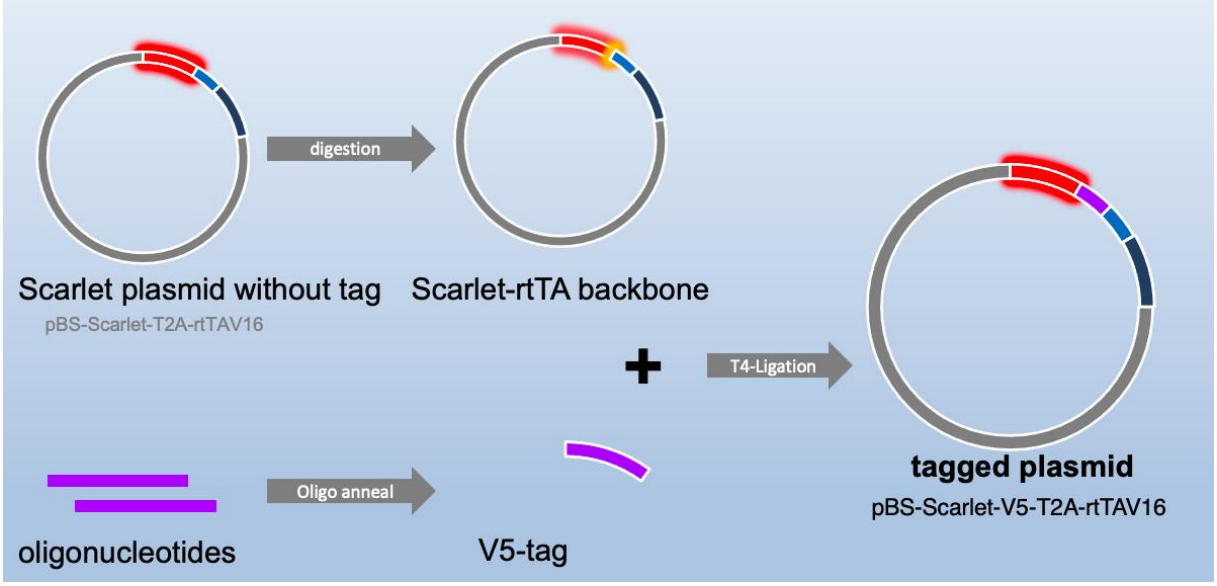

Figure 23. Scx-Scarlet plasmid III – V5-tag.
The diagram above shows the oligo anneal and Ligation of V5-tag to generate the recombinant plasmid pBS-Scarlet-V5-T2A-rtTAV16 in steps 20.

21. The pXL117m2-Scx gDNA was subjected to EcoRV and XhoI double digestion as a vector. The pBS-Scarlet-V5-T2A-rtTAV16 was subjected to EcoRV and XhoI double digestion as a insert. The vector was ligated with insert to obtain the pXL117-Scx gDNA-Scarlet-V5-T2A-rtTAV16 (# 19) recombinant plasmid (Figure 24). (# 19. linearized with AscI + FseI)

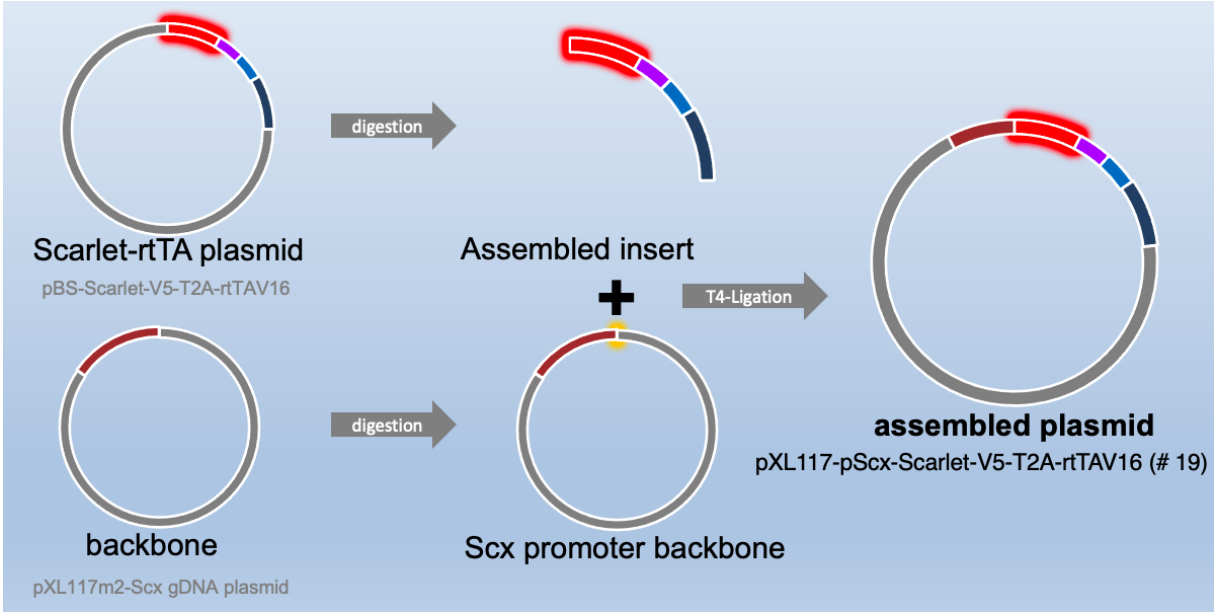

Figure 24. Scx-Scarlet plasmid IV – Scx backbone.
The diagram above shows the insertion of Scarlet-V5-T2A-rtTAV16 into the pXL117m2-Scx gDNA backbone in an enzymatic cleavage and enzymatic linkage approach to generate the recombinant plasmid pXL117-pScx-Scarlet-V5-T2A-rtTAV16 (# 19) of step 21.

The target fragment was cut from the donor plasmid using restriction enzymes (Table 3). Bands were separated on the gel by agarose gel electrophoresis. The target fragment is recovered from the agarose gel by using glass wool as a filter. The recovered fragments are cleaned and enriched by phenol-chloroform aspiration precipitation. The target fragment was linked to the backbone vector using DNA ligase (Table 4).

Table 3. Restriction Endonucleases Reaction System

COMPONENT	10 µl REACTION	50 µl REACTION
Restriction enzyme	1 µl	5 µl
10X Buffer	1 µl	5 µl
DNA	Variable	Variable
Double-distilled Water (ddH2O)	Up to 10 µl	Up to 50 µl

Table 4. DNA Ligase Reaction System

COMPONENT	10 µl REACTION
Quick Ligase	1 µl
2X Buffer	5 µl
Vector	Vector : Insert=1: 3
Insertion	Total≤100ng
double-distilled water (ddH2O)	Up to 10 µl

The donor plasmid was used as a template to amplify the target fragment with high fidelity using Q5 high fidelity DNA polymerase (Table 5). The target fragment was separated on the gel by agarose gel electrophoresis. The cut gels were loaded into dialysis, and the target fragments were recovered by electrophoresis. The recovered fragments were purified by phenol aspiration and alcohol precipitation. The recovered DNA fragments are assembled onto the backbone vector by NEBuilder HiFi DNA assembly master mix to obtain a recombinant plasmid containing the target gene (Table 6).

Table 5. Q5® High-Fidelity 2X Master Mix Reaction System

COMPONENT	20 µl REACTION	CONCENTRATION
Q5 High-Fidelity 2X Master Mix	10 µl	1X
10 µM Forward Primer	1 µl	0.5 µM
10 µM Reverse Primer	1 µl	0.5 µM
Template DNA	5ng	0.2 ng/µl
High GC Enhancer (for Scx Bac Clone)	(4 µl)	(1X)
Double-Distilled Water (ddH2O)	to 20 µl	

Q5 PCR:

	Initial Denaturation	98 °C	30 sec
30 cycles	Denaturation	98 °C	10 sec
	Annealing	Variable (50-72)	20 sec
	Elongation	72 °C	25 sec
	Final Extension	72 °C	2 min

Hold	8 °C	Hold

Table 6. NEBuilder® HiFi DNA Assembly Master Mix Reaction System

COMPONENT	20 µl REACTION	FINAL CONCENTRATION
Vector	vector:insert = 1:2	Total Amount of Fragments:
Insertion	Total≤100ng	0.03–0.2 pmols
NEBuilder	10 µl	1X
Double-Distilled Water	Up to 20 µl	

The recombinant plasmid is transferred into COMPETENT E. coli by MicroPulser Electroporator (Bio-Rad, #1652100) (transformation). or transformation by heat shock. The transformed bacteria were recovered in 1.5 ml Wechselblock Top Shaker Thermo Mixer (Eppendorf). The recovered bacteria are coated in high and low concentrations on agar plates with the appropriate antibiotics.

After spreading the plates, place them upside down in an incubator (BINDER KB 53) and incubate overnight at 37°C. The monoclonal clones are picked and left overnight on an incubation shaker (Sartorius CERTOMAT BS-1). Extract recombinant plasmids with target fragments using the GenElute™ HP Plasmid Miniprep Kit (Millipore Sigma, #NA0150). The DNA pattern was verified by restriction endonuclease digestion, and those with the correct bands were checked by sequencing (Eurofins Genomics TubeSeq Service). The valid clone will be made into a glycerol bacterium to preserve the strain.

Target plasmids were prepared via the ZymoPURE II Plasmid Maxiprep (Zymo Research, #D4203). On LB agar plates with the appropriate antibiotics, glycerol bacteria were dabbed and scratched on plates. Single clones were picked after incubating at 37°C overnight. Maxiprep was performed using 300 ml LB medium. After that, a portion of the bacterium was dispensed at the end of the shaking. Plasmids were recovered by GenElute™ HP Plasmid Miniprep Kit (Millipore Sigma, #NA0150) and identified by enzymatic digestion to exclude the possibility of recombination.

After eluting the DNA from the column provided by kit with TE buffer, 1/10 volume of 3M NaOAc (pH 5.2) and double the volume of isopropanol were added. Precipitate at -20°C for 30 minutes. After 10 minutes of centrifugation at >12,000 rpm, the pellet was washed with 70% ethanol to remove excess salts. The DNA pellets were stored in 70% ethanol in the precipitated state at -20°C.

2.2.2 DNA fragment isolation and purification for Pronuclear injection

Prepare a 5mM Tris, 0.1mM EDTA solution with 1M Tris-HCl stock (Sigma T2663) and 0.5 M EDTA stock (Sigma E7889) and ultrapure water (Simga W1503).
For 50 ml microinjection buffer:

1M Tris-HCl	250 µl
500 mM EDTA	10 µl

Adjust the pH to 7.4 with 1M HCl, filter buffer through a pre-washed 0.02 µM Anotop syringe filter.

Digest 20–60 µg of freshly prepared plasmid DNA (100-200 µl reaction) for 2-3 hours using appropriate restriction enzymes (1 µg DNA / 5 u restriction enzyme). The exact amount of plasmid varies depending on the size of the insert relative to the vector backbone. Ideally, 15-40 µg of transgene insert should be released from the vector. Extended incubation at 37 °C can increase DNA nicking. Completing digestion can be checked by running an aliquot of the digestion mixture (0.2 µg DNA) on an agarose gel. Heat-inactivate the restriction enzymes.

Load the digested DNA sample into a wide slot (formed by taping multiple teeth of the comb together) in an 0.8% agarose gel in TAE. Run the gel at a relatively low voltage of 2–4 V/cm (O/N). Low voltages can increase the gel's resolution. (Add DNA-green dye in the gel for staining)

View the gel with a blue light transillumination table and then excise the gel fragment containing the transgenic insert with a clean scalpel.

Place the gel fragment in a dialysis tube, fill the tube with TEA buffer and place it in a conventional electrophoresis chamber. Electroelute the DNA in TAE buffer for 3 hours at 3-4V/cm.

Perform two times phenol-chloroform extractions with equal volume (about 400 µl) of phenol-chloroform isoamyl (phenol: chloroform: isoamyl = 25:24:1) alcohol extraction at RT. Then two fresh chloroform extraction and two Ether extraction (discard the upper phase). Open the lid of the tube, let ether evaporate under the hood.

After the final extraction, carefully transfer the aqueous phase into a rinsed microcentrifuge tube using a rinsed pipette tip. Both the tube and tip are rinsed with filtered (0.22 µm) TE buffer to eliminate any particles. In all subsequent steps, tips and tubes should be rinsed with filtered TE, and the buffers or water used to dissolve or dilute the DNA should also be filtered through 0.22 µm filters.

Add slightly more than 1/10 volume of 3.0 M sodium acetate (pH 5.2), e.g., if you have 400 µl of the solution, add 50 µl. Then add two volumes of 100% EtOH, mix well, let sit at -80°C for 15 min, and microcentrifuge at four °C for 30 min to recover DNA. Rinse the pellet with 500 µl 70% EtOH (RT) two times, dry pellets, and resuspend 400 µl of TE.

Reprecipitate DNA again by adding "1/10 volume" 3.0 M sodium acetate (pH 5.2) and at least two volumes of 100% EtOH. Precipitate the DNA either on dry ice for 15 min or O/N at -20°C, and recover by centrifugation - full speed microfuge for 30 min. Rinse pellet with 70% EtOH (RT), spin for 5 min, aspirate S/N, wash again with 70% EtOH. Spin, aspirate, dry. Re-dissolve in 100 µl of injection buffer (5 mM Tris pH 7.4, 0.1 mM EDTA).

Centrifuge the DNA solution at four °C, top speed for 30 minutes, discard the bottom 10% of the solution. Repeat this step one more time (discard another 10 %). Determine OD_{260}. Run an aliquot 0.5 µl; 1 µl and 2 µl on a gel adjacent to size markers to determine DNA quality; photograph gel. Keep the purified DNA at -20°C and bring it to the transgenic mouse facility. Provide a Polaroid gel photo showing

about 40ng EtdBr-stained DNA (after the last purification) next to a molecular weight standard.

Check the concentration and read the 260/280 nm on a NanoDrop. The A260/280 ratio should be 1.8 for pure DNA. Anything lower may indicate contaminating chemicals such as agarose or ethidium bromide, which will be fatal to the injected embryos.

Run the agarose gel electrophoresis with molecular weight markers of known concentration. Ensure that your transgene DNA is intact, of the right size, and has no smear of sheared DNA.

Adjust the final concentration to the desired 50+ng/ul with filtered microinjection buffer. (Wash tubes and tips with microinjection buffer or filtered water before use)

2.2.3 Pronuclear (PN) Microinjection

All mouse lines were constructed and maintained by the IBF following the German Animal Welfare Regulations (TierSchG) and the Council of the European Communities Directive. Karlsruhe, Baden-Württemberg, authorized the construction of the mouse strains and the acquisition and processing of animal samples. Animal experiments were performed at the Department of Neuroanatomy of the University of Heidelberg, the Institute for Intercultural Biomedicine of the University of Heidelberg, and The Cluster of Excellence Cell Networks.

Female C57BL/6 mice are hormonally ovulated and mated overnight with males, and single-cell stage fertilized eggs are collected approximately 20 hours later. Linearised exogenous DNA was transferred by micro-injection into the pronucleus during a brief phase with two anterior nuclei. The surviving fertilized eggs are embryonically transferred into the oviducts of pseudo-pregnant females within approximately two hours. Typically, about 10 to 20% of the offspring will carry exogenous DNA in their genome (Fielder et al. 2010) (i.e., transgenic animals). The linearised DNA is randomly integrated into the genomic DNA of the mouse. Transgenic mice inserted

into transcriptionally inactive loci have exogenous genes that are not expressed. The level of expression of successfully expressed zygotes may vary (Figure 25).

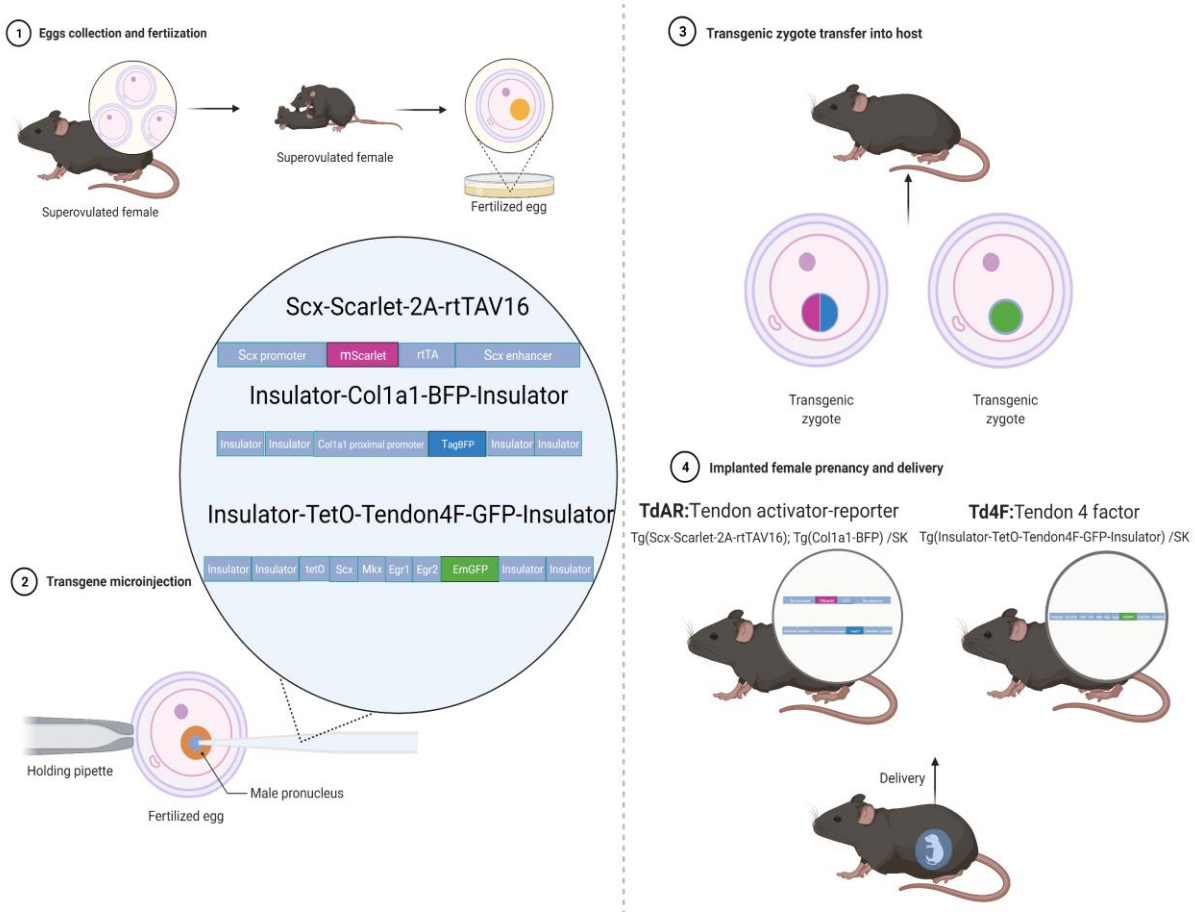

Figure 25. Schematic of Pronuclear (PN) Microinjection.
The constructed recombinant plasmids were linearized and purified. Scx-Scarlet-2A-rtTAV16 and Col1a1-BFP were mixed at equimolar numbers and microinjected. A second set of microinjections was performed to obtain mouse Tg(Scx-Scarlet-2A-rtTAV16) Tg(Col1a1-BFP) /SK for the tendon activator-reporter transgenic mouse (TdAR). insulator-TetO-Tendon4F-GFP-Insulator was microinjected to obtain mouse Tg(Insulator-TetO-Tendon4F-GFP-Insulator) /SK for the Tendon 4 factors transgenic mouse (Td4F).

Following the acquisition of progeny, the founding mice with the insertion gene need to be identified using PCR. Specific primer sets that can amplify the target gene are designed to identify the target gene of the insertion genome. Two primer sets per gene set are designed and mixed with plasmids in tail lysate products of a wild-type mouse. Genotyping was performed using the above mixture as a template to test the amplification efficiency of each set of primers. Genotyping PCR was performed using the same protocol, and PCR templates were obtained from mouse tail lysates (Table 7-9).

Table 7. Transgenic Mouse Lines

Name	Description	Origin
Gt(ROSA)26 Sor$^{tm1(rtTA*M2)Jae}$ Col1a1$^{tm3(tetO-Pou5f1,-Sox2,-Klf4,-Myc)Jae}$/J	Tetracyclin inducible four reprogramming transcription factors (Oct4, Sox2, Klf4, and cMyc) were targeted into the 3' end of the Col1a1 gene. The endogenous Col1a1's function is intact. Without tetracyclin induction, the four reprogramming transcription factors are not expressed.	Jackson Laboratory #011004
Tg(Scx-Scarlet-2A-rtTAV16); Tg(Col1a1-BFP) /SK	The reversed tetracyclin trans-activator (rtTA) and mScarlet red fluorescent protein are expressed under the control of the Scx promoter. The Scx promoter is expressed in the tendon stem/progenitor cells. When the Tendon stem/progenitor cells appear during the differentiation or regeneration process, the mScarlet red fluorescent reporter will be switched on. The reversed tetracyclin trans-activator will be expressed. The mScarlet reports Scx activity, and the rtTA activates tetracyclin inducible promoter. The blue fluorescent protein reporter is expressed under the control of 3.6 kb Col1a1 proximal promoter. This region contains bone and tendon tissue-specific elements. When the tenocytes are mature from the differentiation of the tendon stem/progenitor cells, the blue fluorescent protein will be turned on.	This article
Tg(Insulator-TetO-Tendon4F-GFP-Insulator)/SK	This construct consists of a tetracycline-inducible promoter and four tendon transcription factors (Scx, Mkx, Egr1, and Egr2) and GFP (Green fluorescent protein). When this line is crossed with a tendon promoter-driven reversed tetracycline trans-activator (rtTA) mouse line, the double-positive transgenic mice will express tendon transcription factors and reporter GFP when doxycycline is provided in drinking water.	This article

2.2.4 Genotyping

2.2.4.1 Tissue Lysis

Prepare 1L PBND tissue lysate as the following recipe (adjust pH to 8.3 by 37% HCl, then volume to 1L):

3.72g 50mM Potassium Chloride (Merck, #7447-40-7)
1.20g 10mM Tris-Sodium citrate dihydrate (Tris-HCl, Sigma #T2663)
0.10g 0.1 mg/mL Magnesium Chloride 6-hydrate ($MgCl_2$, AppliChem #131396)
0.10g 0.1 mg/mL Gelatin from bovine skin (Sigma, #9000-70-8)
202.5µl 10%NP-40 Surfact-Amps™ Detergent Solution (Fisher Scientific, #R 85124)
202.5µl 10%TWEEN® 20 (Sigma, #9005-64-5)

Tissue from transgenic mice (typically mouse tail or embryonic, fetal membranes) is centrifuged and allowed to collect at the bottom of the tube. Add 98uL PBND tissue lysate with 2ul 20mg/mL protease and check for complete tissue immiscibility. Place the tubes in an Eppendorf Thermomixer (Eppendorf, #comfort 5355).
Incubate overnight at 55°C with shaking at 550rpm. Stop shaking the next day and incubate at 85°C for 60 minutes to inactivate the enzyme. Phenol-chloroform extraction was carried out to remove impurities and inactivate the protein. DNA was precipitated using alcohol, then diluted in 100 ul TE (pH=7.5), stored at 4°C.

2.2.4.2 PCR

The primers were designed to perform amplification of the exogenous DNA fragment by polymerase chain reaction (PCR).

Table 8. Genotyping Primers

Gene	Rosa26 M2rtTA		Product Size(bp)	Tm(°C)
Primers:	Rosa.rtTA-F	AGTAAGGGAGCTGCAGTGGAGTAG	505	61
	rtTA-R	CTTGTTCTTCACGTGCCAGTACAG		59
Gene	OSKM		Product Size(bp)	Tm(°C)
Primers:	Col.4F-F2	AGGCAAAGGAATTGCTCTAGAGCGG	383	63
	Col.4F-R2	GTACCGAGCCAGCATGCTATCGTC		64
Gene	Td4F		Product Size(bp)	Tm(°C)
	Td4F-F2	CCCAAACAGATCTGCACCTTCTGC	506	62
	Td4F-R2	TTTGACACCTGCACTAGCGTCATC		62
Gene	TdAR		Product Size(bp)	Tm(°C)
	ScxRTA-F2	AGCTGTACAAGATCTCGGGTAAGC	284	61
	rtTA-R	CTTGTTCTTCACGTGCCAGTACAG		59
	ColB-F1	CTCCCAGCTCTCCATCAAGATGG	353	61
	ColB-R1	AAGCTAGTAGCCAGGATGTCGAAG		60

Prepare the PCR master mix in the following proportions and set up the PCR program:

Table 9. PCR Master Mix

PCR master mix	Portion
5 x PCR buffer	40
25 mM $MgCl_2$	16
10 mM dNTPs	4
100 mM forward primer	1
100 mM forward primer	1
5 U/µl Taq DNA polymerases	1
Genomic DNA	8
Double-Distilled Water	89

Genotyping PCR:

	Initial Denaturation	94 °C	5 min
35 cycles	Denaturation	94 °C	20 sec
	Annealing	64 °C	30 sec
	Elongation	72 °C	40 sec
	Final Extension	72 °C	10 min
	Hold	8 °C	Hold

2.2.4.3 Electrophoresis

After completing the PCR, a 1.5% agarose gel was prepared using 1x Lithium Acetate Borate (LAB) buffer (3 µl of peq-GREEN DNA/RNA dye (VWR, #732-2960) was added per 100 ml). After sampling, agarose gel electrophoresis was carried out at 150 V for approximately 30 min. Bands were analyzed under a UV imaging system, and images were saved.

2.2.5 Transgene expression validation

Firstly, I repeated the in vitro induction assay by reprogramming cells derived from the mouse line Gt (ROSA)26Sor$^{tm1(rtTA*M2)Jae}$ Col1a1$^{tm3(tetO-Pou5f1,-Sox2,-Klf4,-Myc)}$/J to

determine whether the tetO promoter and the transcription factors OSKM would work properly.

The specific test was performed by extracting mouse fibroblasts. The cells were exposed to doxycycline for 12 days for induction. Cellular immunofluorescence staining was performed using Nanog antibody(ab80892) (Carey et al. 2010). After determining their work, the novel mouse lines constructed herein were further characterized for gene expression function.

2.2.5.1 Tg (Scx-Scarlet-2A-rtTAV16); Tg (Col1a1-BFP) /SK

The Insulators-pCol1a1-TagBFP-HA-Insulators and the Scx gDNA-Scarlet-V5-T2A-rtTAV16 linearised fragments were subjected to pre-nuclear microinjection in equal proportions. Due to the random nature of their insertion into the DNA sites of the mouse genome, there were cases where the two genes separated or where the gene was successfully inserted but not expressed due to the insertion site.

After identification by several generations of genotyping, all two-gene separated mouse founders were removed. Transgenic mice with successful insertion of the target fragment and without separation were screened. However, the functions of rtTAV16 and the reporter genes Scarlet and BFP are still unknown. Thus, an identification strategy was developed to characterize whether the reporter gene worked, whether rtTAV16 worked, and whether all three were expressed explicitly in tendon tissue.

Specifically, this was divided into three parts.
In the first step, tendon tissue was collected from 4-6 weeks old transgenic mouse offspring, and the expression of the insert was identified based on whether fresh tendon cells expressed Scarlet and BFP

In the second step, zygotic embryos screened for successful expression of the insert founders identified in the previous section were collected. The two fluorescent expression ranges were observed under a binocular microscope to faithfully determine whether the inserted gene followed its promoter expression specificity.

In a third step, Tg (Insulator-TetO-Tendon4F-GFP-Insulator)/SK(Td4F) was crossed with Tg (Scx-Scarlet-2A-rtTAV16); Tg (Col1a1-BFP) /SK(TdAR) crosses to obtain progeny TdAR-Td4F with all three construct insertions.

TdAR-Td4F mice were assayed by activation of rtTAV16 after exposure to doxycycline. The rationale is that rtTA activates the tetO promoter in the Insulator-TetO-Tendon4F-GFP-Insulator insertion fragment. EGFP will be expressed in transgenic mice that can express both the tendon transcription factor and EGFP. Thus, when E12.5 days TdAR-Td4F mouse embryonic fibroblasts are extracted and cultured in a doxycycline-containing medium, the function of rtTAV16 can be detected based on the expression of green fluorescence (Figure 26). (The expression function of the Insulator-TetO-Tendon4F-GFP-Insulator insert has been tested for faithful expression before this, see below for detailed methods)

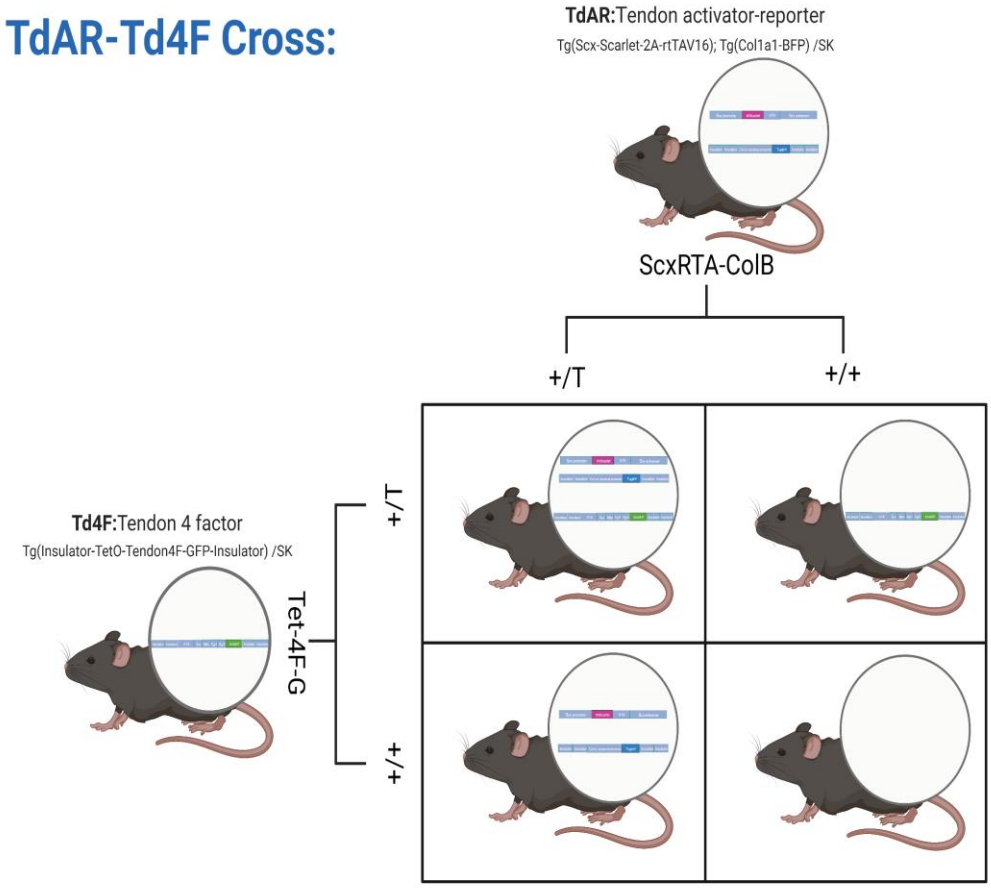

Figure 26. Schematic of TdAR-Td4F Cross.
Insulators-pCol1a1-TagBFP-HA-Insulators as well as the Scx gDNA-Scarlet-V5-T2A-rtTAV16 linearised fragments were microinjected prior to nucleation and genotypically identified by transgenic mice Tg(Scx-Scarlet-2A-rtTAV16); Tg(Col1a1-BFP) /SK(TdAR) were crossed with the mouse strain Tg(Insulator-TetO-Tendon4F-GFP-Insulator)/SK(Td4F). The progeny of mice with TdAR-Td4F were obtained and used to test the expression function of the genes mentioned above.

1. Prepare PBS buffer pre-cooled to 4°C and place on ice and dissect 4-6 weeks old mice to separate the Achilles tendon and patellar tendon.

2. Prepare a PBS mixture of 3 mg/ml to type I collagenase (Worthington) and 4 mg digest/ml dispersion enzyme (Roche) and mix in partial digestion (incubate at 37°C for 0.5 h). (Bi et al. 2007)

3. Wash the samples three times in PBS and examine the fluorescent reporter cells exposed under the tenaculum under a Confocal Microscope (Carl Zeiss LSM900).

4. Samples to be preserved can be fixed in freshly prepared 4% PFA in PBS solution overnight at 4°C.

5. Wash samples in PBS at four °C for ten minutes at a time.

6. Protected by precipitation in a 10% to 30% sucrose solution.

7. Embed the Achilles tendon in OCT and placed it on dry ice to solidify long-term storage in a -80°C refrigerator.

2.2.5.2 Tg(Insulator-TetO-Tendon4F-GFP-Insulator) /SK

The Insulator-TetO-Tendon4F-GFP-Insulator linearised fragment was subjected to pre-nuclear microinjection. Because of its random insertion into mouse genomic DNA, there is a phenomenon that the gene is successfully inserted but not expressed due to the insertion site.

After identification by genotyping, all transgenic mice with successful insertion of the target fragment will be selected. However, the expression function of tetO and the

tendon transcription factor is still unknown. Therefore, an identification strategy is needed to detect the expression of the inserted gene by the expression of EGFP. Specifically, Tg(Insulator-TetO-Tendon4F-GFP-Insulator) /SK (Td4F) was crossed with the mouse line Gt(ROSA)26Sor$^{tm1(rtTA*M2)Jae}$Col1a1$^{tm3(tetO-Pou5f1,-Sox2,-Klf4,-Myc)Jae}$/J from Jackson Laboratory to obtain progeny with the construct insert, RosaM2rtTA-Td4F-OSKM.

RosaM2rtTA-Td4F-OSKM mice screened by genotyping for M2rtTA function at the Rosa26 locus after exposure to doxycycline. rtTA activates the tetO promoter in the Insulator-TetO-Tendon4F-GFP-Insulator insertion fragment and can express the tendon transcription factor, and EGFP in transgenic mice will be initiated. Thus extracted RosaM2rtTA-Td4F-OSKM fibroblasts can be exposed to doxycycline in culture, and the function of Insulator-TetO-Tendon4F-GFP-Insulator insert gene expression can be assayed based on the expression of green fluorescence (Figure 27).

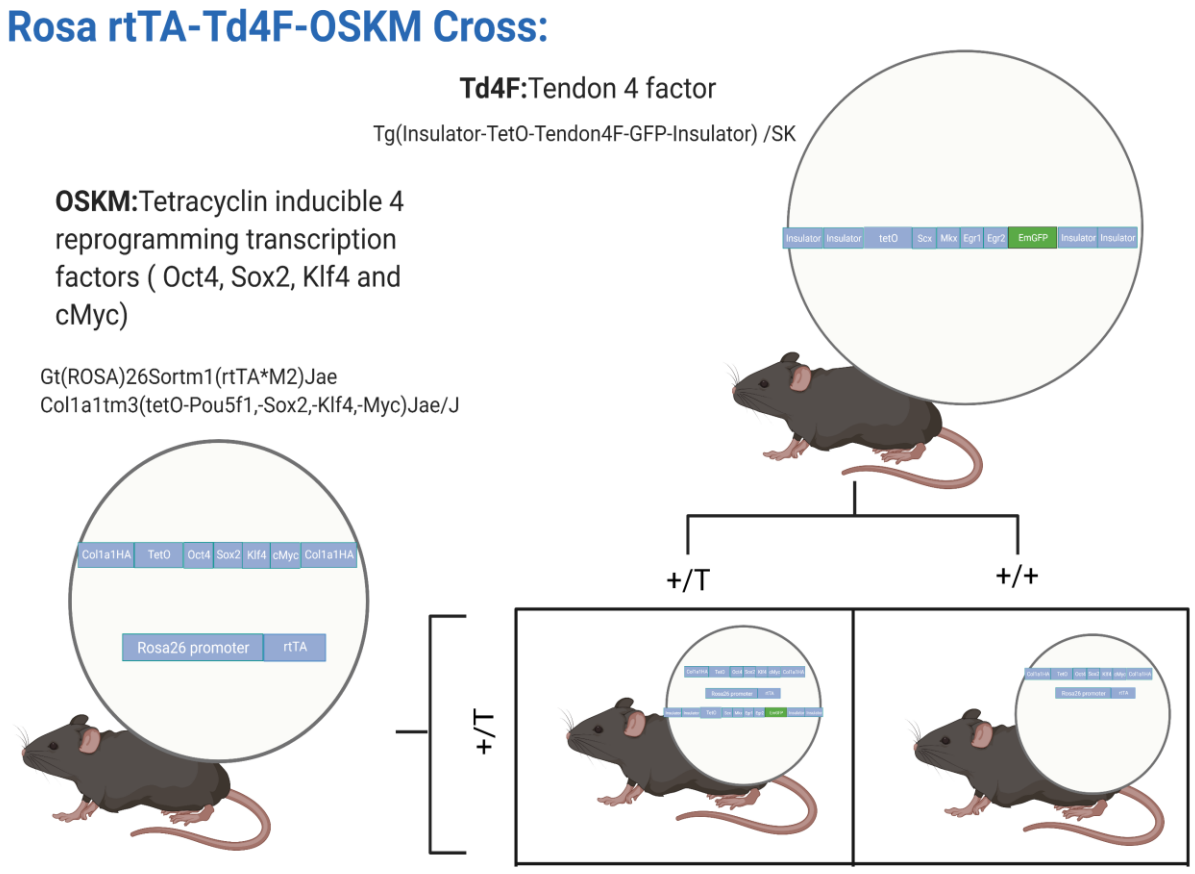

Figure 27. Schematic of Rosa rtTA-Td4F-OSKM Cross.
The Insulator-TetO-Tendon4F-GFP-Insulator linearized fragment was micro-injected before nucleation. The transgenic mouse Tg(Insulator-TetO-Tendon4F-GFP-Insulator) /SK(Td4F) identified by genotyping was compared to the transgenic mouse Tg(Insulator-TetO-Tendon4F-GFP-Insulator) from Jackson Laboratory mouse line Gt(ROSA)26Sortm1(rtTA*M2)JaeCol1a1tm3(tetO-Pou5f1,-Sox2,-Klf4,-Myc)Jae/J were crossed to obtain mice with RosaM2rtTA-Td4F-OSKM offspring for detection of the function of the former gene expression.

Primary cells were isolated according to the following protocol(Seluanov et al. 2010) and induced by adding doxycycline on the second day.

1. The mouse lungs and tail were minced (for the tail: the skin was removed from the tail, and the skin was cut into small pieces).

2. Transfer the tissue fragments into 2 ml of medium (DMEM, supplemented with 20% FBS, penicillin/streptomycin, and Amphotericin B).

3. Add 200 µL of Liberase Blendzyme III (Roch) stock solution (10 mg/mL) to the medium and incubated overnight in a cell incubator.

4. Transfer the medium with the tissue debris to a 10 ml Falcon tube.

5. Centrifuge the cells and tissue debris at 1200 rpm for 5 minutes to precipitate the cells and tissue debris.

6. Discard the supernatant and transfer the mixture, including the tissue debris, into 2 mL of medium (DMEM, supplemented with 20% FBS, penicillin/streptomycin, Amphotericin B and Doxycycline).

7. Replace the medium with fresh medium every day until all tissue debris has been washed away.

8. After approximately four days, replace the medium without Amphotericin B.

9. Examine the fluorescence expression of fibroblasts after induction under a confocal microscope (Carl Zeiss LSM900).

Amphotericin B solution (Sigma, #A2942): final concentration 25 µg/ml
Liberase Blendzyme III (Roch): prepare storage solution by dissolving one vial (100 mg) in 10 ml of PBS.

2.2.6 Collection of Tissue Samples

The 4-6 weeks old mice were anaesthetized with isoflurane and executed by cervical dissection. The carcasses were disinfected with alcohol, and the skin of the Achilles tendon area of the mice was incised to expose the Achilles tendon. The Achilles tendon was cut after it was peeled out. The tissue was processed as described later.

Set the mice mating and check the time of emergence of the plug to calculate the age of the embryo. Pregnant mice are anaesthetized with isoflurane and sacrificed by cervical dissection. Embryos of E10.5, E12.5, and E14.5 in utero were detached on ice, placed in pre-chilled PBS, and the fetal membranes were reserved for genotyping. All operations were performed after completing a training course and obtaining permission, and all steps complied with the requirements of the Animal Welfare Act (Tierschutzgesetz).

2.2.6.1 Fixed Frozen Embryo Sections

1. Embryos washed in PBS were fixed overnight at four °C in freshly prepared 4% PFA solution.

2. Wash the samples well three times in PBS at four °C for one h each.

3. Photograph whole-mount embryos by a binocular microscope.

4. Immersion in 10%, 20%, and 30% sucrose solutions for pre-embedding protection of cryosections.

5. Embryos are embedded in OCT and placed on dry ice to solidify long-term storage in a -80°C refrigerator.

6. The tissue blocks were left overnight in the -20°C refrigerator for sectioning the next day.

7. Adjust the temperature of the Cryostat microtome (Leica CM3050S) and trim the tissue block. Continuous sectioning of embryos at 10 μm thickness.

8. Mount the cut tissue pieces on SuperFrost slides and perform Confocal Microscope (Carl Zeiss LSM900) for photography and store at -20°C away from light.

. **2.2.6.2 Fixed Frozen Tendon Sections**

1. Fix the Achilles tendon overnight in 4% PFA at 4°C. (The volume of fixative is more than ten times the size of the Achilles tendon)

2. Wash samples three times in PBS at four °C for 1 hour each.

3. Submerge the Achilles tendon in a 10%-30% sucrose PBS solution for glycol-precipitation protection

4. Mount the Achilles tendon in OCT-embedded compound and solidify at -80°C.

5. Overnight at -20°C before preparing the slides.

6. Prepare frozen sections using Cryostat microtome (Leica CM3050S) in10 μm thickness.

7. Mount the sections on SuperFrost slides and take photographs by Confocal Microscope (Carl Zeiss LSM900).

8. Store the sections at -20°C away from light.

2.2.7 Doxycycline Induction

For MEF cells derived from the E12.5 embryos of Gt(ROSA)26Sor$^{tm1(rtTA*M2)Jae}$ Col1a1$^{tm3(tetO-Pou5f1,-Sox2,-Klf4,-Myc)Jae}$/J mouse line, cultured in MEF medium containing doxycycline(2 μgml $^{-1}$) to induce OSKM expression. The aim was to test the working of OSKM in this mouse line.

For fibroblasts isolated from the lung tissue of 4-6 weeks old RosaM2rtTA-Td4F-OSKM transgenic mice. They were cultured in a fibroblast medium containing doxycycline (2 μgml $^{-1}$) to induce EGFP expression. The aim was to detect the functioning of this mouse line TetO as well as Scx, Mkx, Egr1, Egr2.

MEF cells derived from embryos of the E12.5 TdAR-Td4F transgenic mouse line were cultured in a MEF medium containing doxycycline (2 μgml $^{-1}$) to induce the expression of EGFP. The aim was to test the working of rtTA in this mouse line.

2.2.8 Cell Culture and Immunocytochemistry (ICC)

Tissue from the embryo is used to extract embryonic fibroblasts. (Jozefczuk et al. 2012) All the instruments and materials were sterilized. The detailed protocols are following:

2.2.8.1 Primary Cell Culture

1. Submerge coverslips in concentrated sulphuric acid overnight. Rinse well in VE water and submerge overnight in anhydrous wine varnish. Rinse well in double-distilled water. Autoclave and bake dry and set aside.

2. Place the cell crawls in a 24-well plate and UV sterilize on a laminar flow carbinet.

3. Pregnant mice are anaesthetized with isoflurane and subjected to cervical dissection and execution. The E12.5 embryos were dissected out after alcohol sterilization.

4. Remove viscera and heads in pre-cooled PBS. After washing and removal of PBS, the remaining tissue was minced. (This step is performed on ice)

5. Digest tissue fragments in the addition of Trypsin-EDTA (Gibco, #25300054) in a 37°C cell incubator (5% CO2) for 30 minutes.

6. Stop digestion by adding DMEM containing 10% FBS. Centrifuge at 1200 rpm for 5 minutes, then discard the supernatant.

7. Resuspend the cells in MEF medium (10% FBS, 1x penicillin-streptomycin in high sugar-DMEM) and seed the cells in six-well plates at a density of 3 x 105 cells/ml.

8. Change the medium containing doxycycline every two days and pass it on to the 24-well plate in which the cell crawls were placed after 12 days of induction.

2.2.8.2 Immunocytochemistry (ICC)

1. Wash the cells on the crawl sheet three times with pre-warmed PBS to remove impurities such as cell debris.

2. Fix the cells with 4% PFA for 30 minutes at room temperature.

3. Wash the cells three times with PBS for 10 minutes each.

4. Permeabilise the cells in PBS containing 0.1% Triton X-100 for 15 minutes to permeabilize the cell membrane.

5. Block endogenous cellular antigens with 10% BSA for 1 hour at room temperature.

6. Cells were submerged in diluted primary antibody and incubated overnight at 4°C.

7. Wash the cells three times with PBS for 10 minutes each.

8. Cells are submerged in diluted secondary antibodies and incubated for 1 hour at room temperature.

9. Wash the cells three times with PBS for 10 minutes each time.

10. Add DAPI and stain for 10 minutes for nuclear staining.

11. Wash cells three times with PBS for 10 minutes each.

12. After flow-through rinsing, Coverslip slides with Mowiol 4-88 (Roche, #0718).

2.2.9 Microscope Imaging

All specimens to be fluorescently visualized were first observed in a fluorescence microscope (Zeiss Axiover 200M) to determine the fluorescence intensity, general location and to make preliminary observations. After the initial observation, a confocal laser scanning microscope (Zeiss LSM 510) was used for further documentation, and photographs were taken and stored.

2.2.10 Statistical Analysis

All schematic diagrams in this paper were created with biorender.com, and statistical plots were produced using Prism 5.01 software (GraphPad Software, San Diego, CA, USA). Preliminary statistical analysis of the data was performed. P-values ≤ 0.05 were considered to be statistically significant.

CHAPTER 3: RESULTS

3.1 Transgenic mouse construct validation

3.1.1 Restriction enzyme digestion

After obtaining the target plasmids, verified by enzymatic digestion (# 14a.pXL117-Insulator-TetO-4TF-EmGFP/EcoRV + SalI, # 15b.pXL117-Insulators-pCol1a1-TagBFP-HA-Insulators/ EcoRV + SalI, # 19. pXL117-Scx gDNA-Scarlet-V5-T2A-rtTAV16/ AscI + FseI) and after sequencing, the linearised gene fragment is shown below (Figure 28).

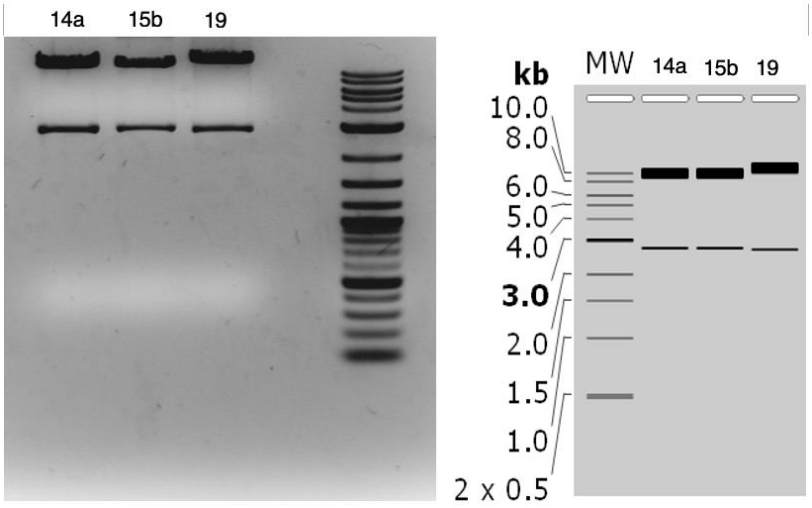

Figure 28. Restriction Endonuclease Digestion Verification.
The above is the agarose gel electrophoresis results after restriction endonuclease digestion of 14a, 15b, and 19 in that order.

MW: 1 Kb DNA Ladder
1: 14a. pXL117-Insulator-TetO-4TF-EmGFP
EcoRV + SalI
1. 11,732 bp
2. 2730 bp

2: 15b. pXL117-Insulators-pCol1a1-TagBFP-HA-Insulators
EcoRV + SalI
1. 11,694 bp
2. 2730 bp

3: 19. pXL117-Scx gDNA-Scarlet-V5-T2A-rtTAV16
AscI + FseI
1. 14,160 bp
2. 2672 bp

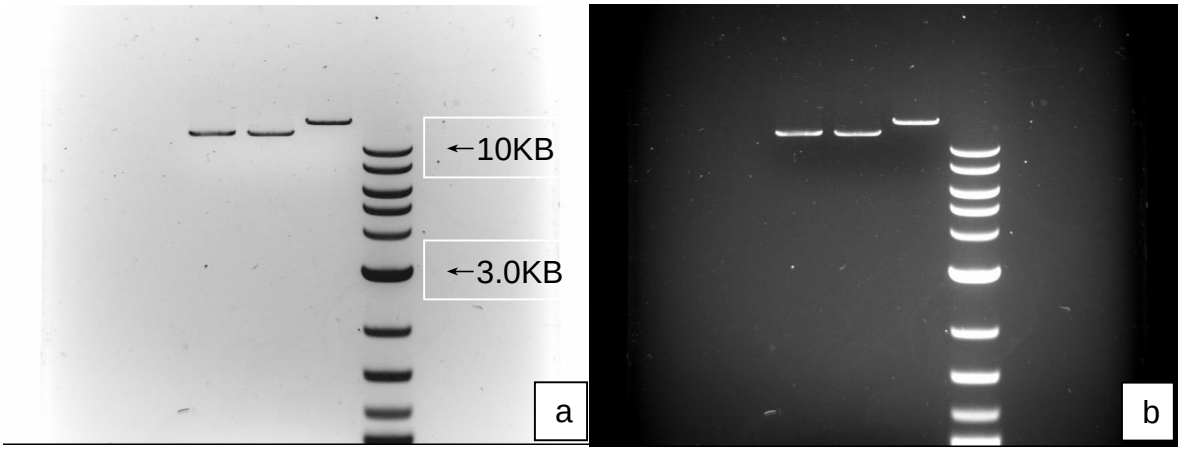

Figure 29. Linearized Re-constructed Plasmids.
Linearized reconstituted plasmids were generated in sanpgene to simulate gel electrophoresis images and corresponding fragment sizes after enzymatic digestion. a, b plots are consistent with the results of gel electrophoresis performed after enzymatic digestion.

After linearisation, the DNA was subjected to agarose gel electrophoresis, and fragments of the correct size were separated on the gel (Figure 29). The OD260/280 ratio was determined to be 1.8. after the purification process. 0.5 micro was taken for agarose gel electrophoresis, and the results are shown above, with molecular weight standards next to the bands.

3.1.2 Sequencing of three Constructions

I sequenced the gene fragments amplified by PCR and inserted them in the reconstituted plasmid using the Sanger method, and the sequencing results are shown below (Figure 30-36).

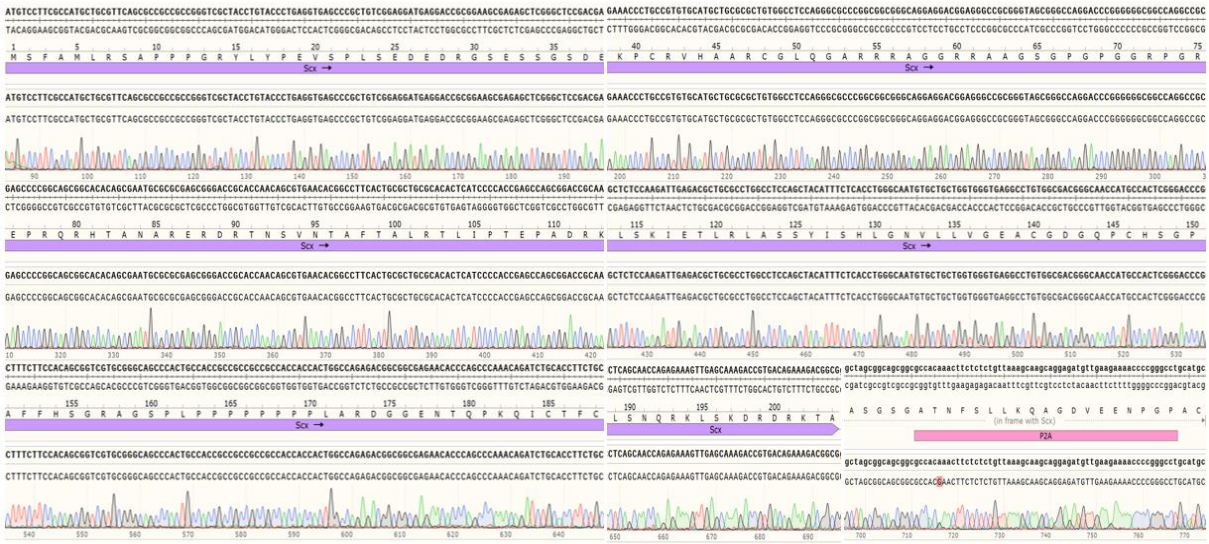

Figure 30. Sequencing results of Scx-P2A.
One of the deoxyribonucleotides in the P2A element is mutated from A to G. Still, this mutation changes the codon ACA to ACG, which encodes threonine, and the mutation is meaningless.

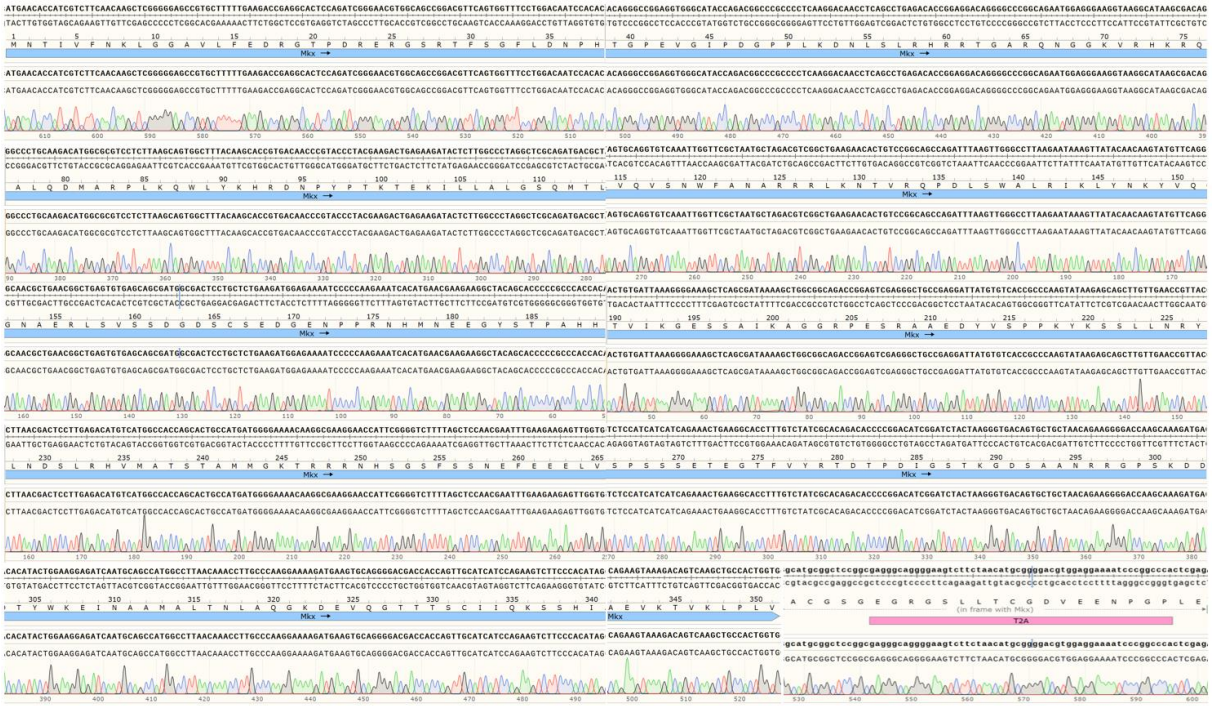

Figure 31. Sequencing results of Mkx-T2A.
No mutations occurred in this gene region.

Figure 32. Sequencing results of Egr1-E2A.
No mutations occur.

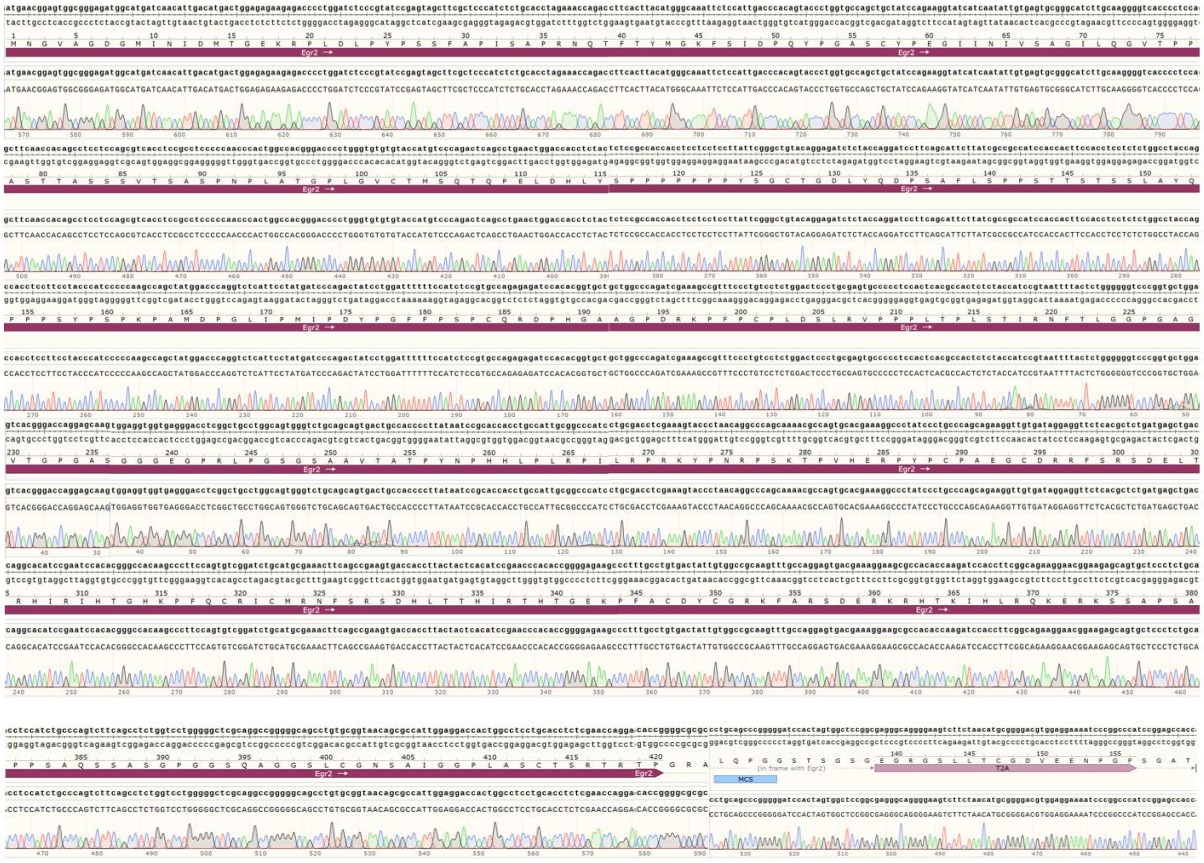

Figure 33. Sequencing results of Egr2-T2A.
Sequencing of Egr2 and T2A gene shows correct sequences.

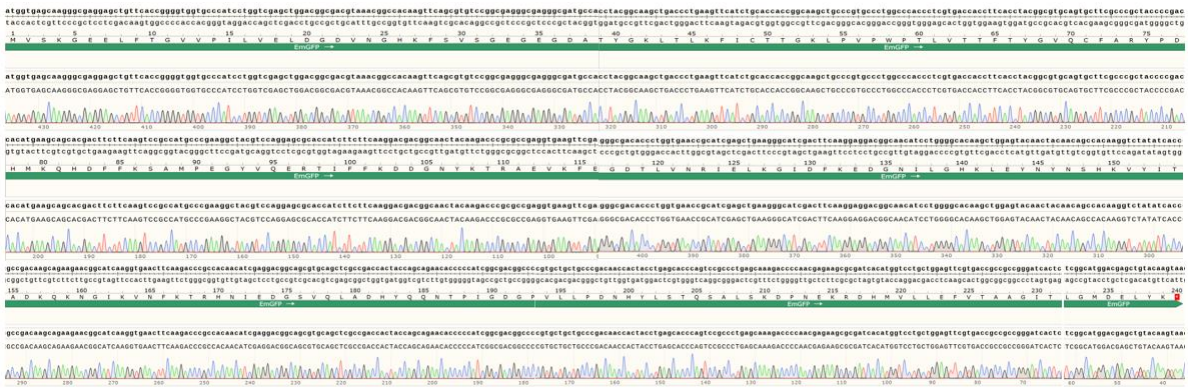

Figure 34. Sequencing results of EmGFP.
Sequencing of the fluorescent protein reporter gene EmGFP shows the correct sequence.

Figure 35. Sequencing results of TagBFP.
Sequencing of the fluorescent protein reporter gene TagBFP shows the disappearance of a deoxyribonucleotide G in the primer region of the ligand. In combination with the poor quality caused by the sequencing having reached the end and the three consecutive G's in front of it, it was judged to be a sequencing error here..

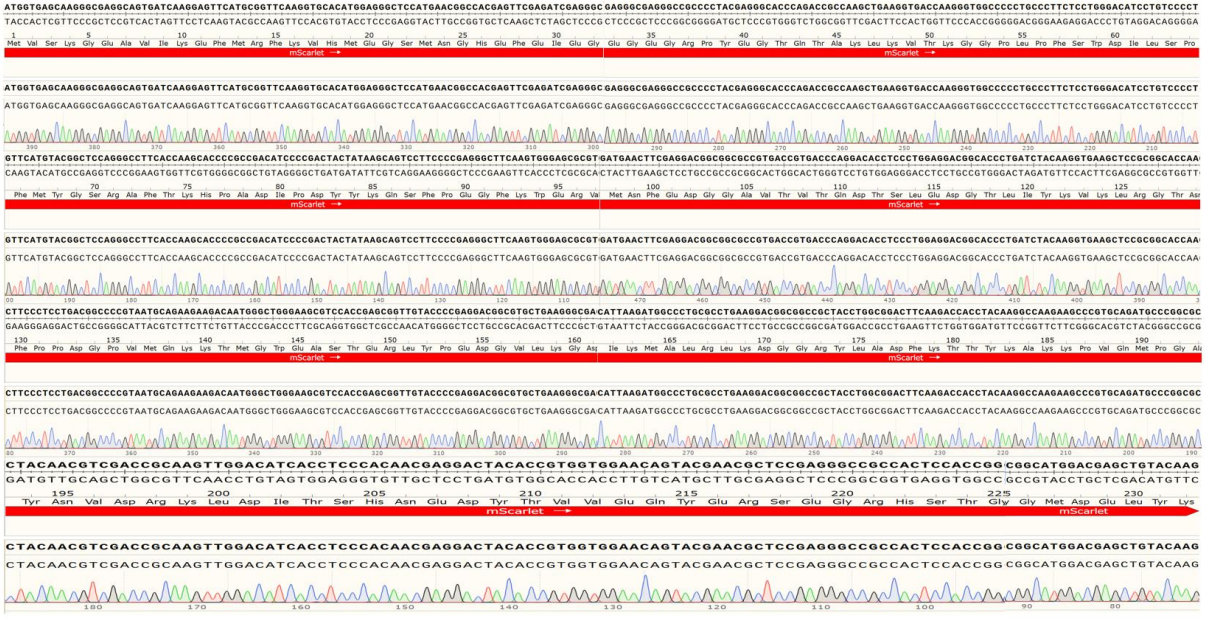

Figure 36. Sequencing results of mScarlet.
Sequencing of the fluorescent protein reporter gene mScarlet shows the correct sequence.

Insert sequences of reconstructed plasmids were obtained via Tube sequencing service (Eurofins Genomics). Snapgene was used to align multiple sequence analyses of the sequencing results. The reconstituted plasmids that were sequenced correctly were linearised and purified before pre-nuclear microinjection at

Interfakultäre Biomedizinische Forschungseinrichtung (IBF) of Heidelberg University.

3.2 Transgenic mouse analysis

3.2.1 Founder analysis

The 11,732 bp linearized gene fragment Insulator-TetO-4TF-EmGFP- Insulator was microinjected to obtain the Td4F (tendon four factors) mouse line. 11,694 bp linearized gene fragment Insulators-pCol1a1- TagBFP-HA-Insulators was microinjected with a 14,160 bp linearized gene fragment Scx gDNA-Scarlet-V5-T2A-rtTAV16 in equal proportions to obtain a TdAR (tendon activator reporter) mouse line with a double insertion gene.

After injection of linearized DNA into the donor congeners and transplantation into recipient foster mice, F0 was obtained as follows:

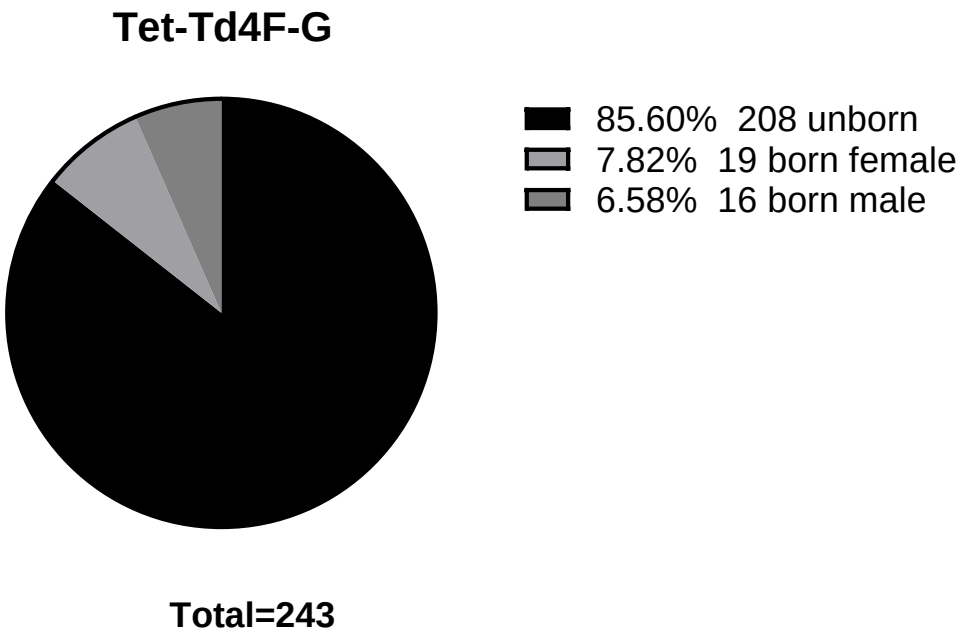

Figure 37. Microinjection results of TetO-Td4F-GFP.
TetO-Td4F-GFP DNA microinjection of 243 pro-nuclear fertilized eggs was performed, and 35 mice were born, of which 19 females and 16 males, and 2 died before reaching one month of age.

Td4F (tendon four factors) mice were injected with 243 zygotes, 35 mice were born, 14.4% birth rate, two mice died before one month of age, 33 mice remained (Figure 37).

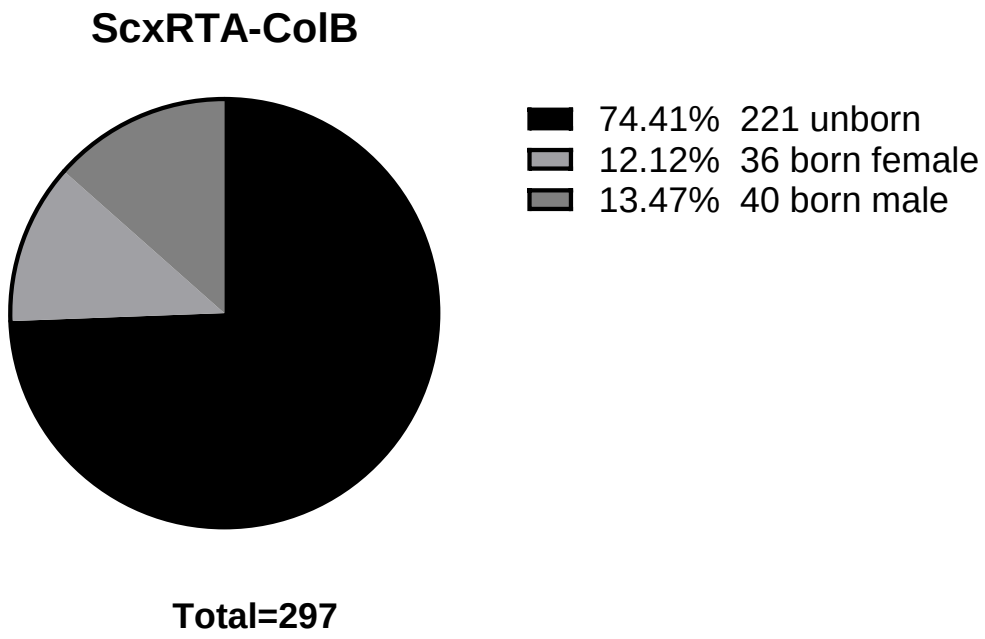

Figure 38. Microinjection results of ScxRTA-ColB.
ScxRTA-ColB DNA microinjection of 297 pro-nuclear fertilized eggs was performed, and 76 mice were born, 36 females and 40 males.

TdAR (tendon reporter activator) mice were injected with 297 zygotes, 76 mice were born, the birth rate was 25.59% (Figure 38).

3.4 Founder Generation and Genotyping

Three weeks after birth, ear specimens were taken for genotyping, and the results were as follows. 10 F0 mice in the Td4F mouse line were founders carrying the target gene, a positive rate of 31.3% (Figure 39,40). 20 F0 mice in the TdAR mouse line were founders carrying the target gene, a positive rate of 26.32%, of which 15 were double-gene positive founders (Figure 41,42).

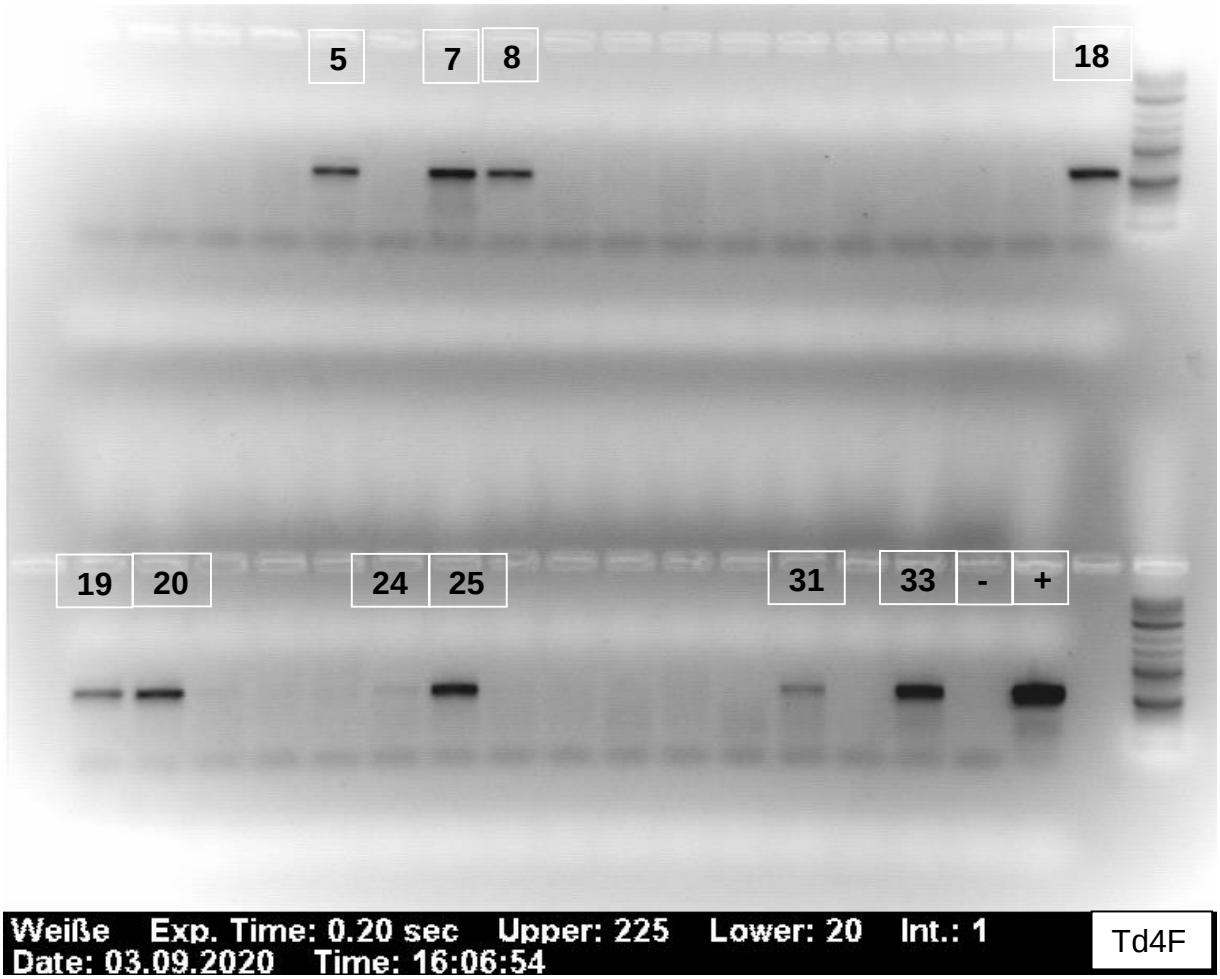

Figure 39. Genotyping results of TetO-Td4F-GFP.
Thirty-three F0 mice were genotyping with ear samples taken at three weeks, ten of which carried the target gene (Founder 5 7 8 18 19 20 24 25 31 33).

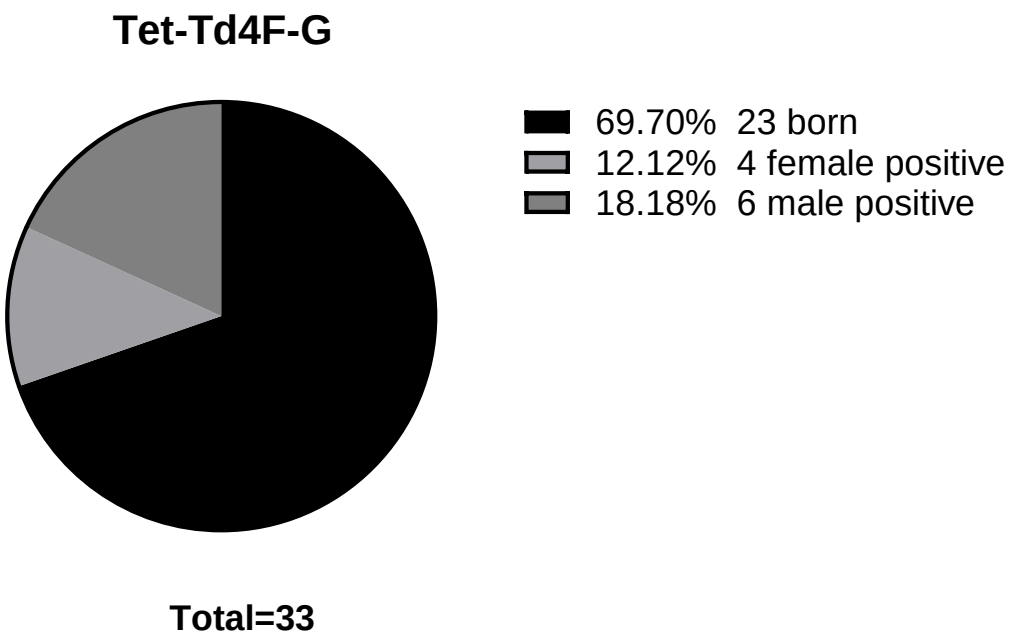

Figure 40. Founders of TetO-Td4F-GFP.
Thirty-three F0 mice were genotyping with ear samples taken at three weeks, ten of which carried the target gene.

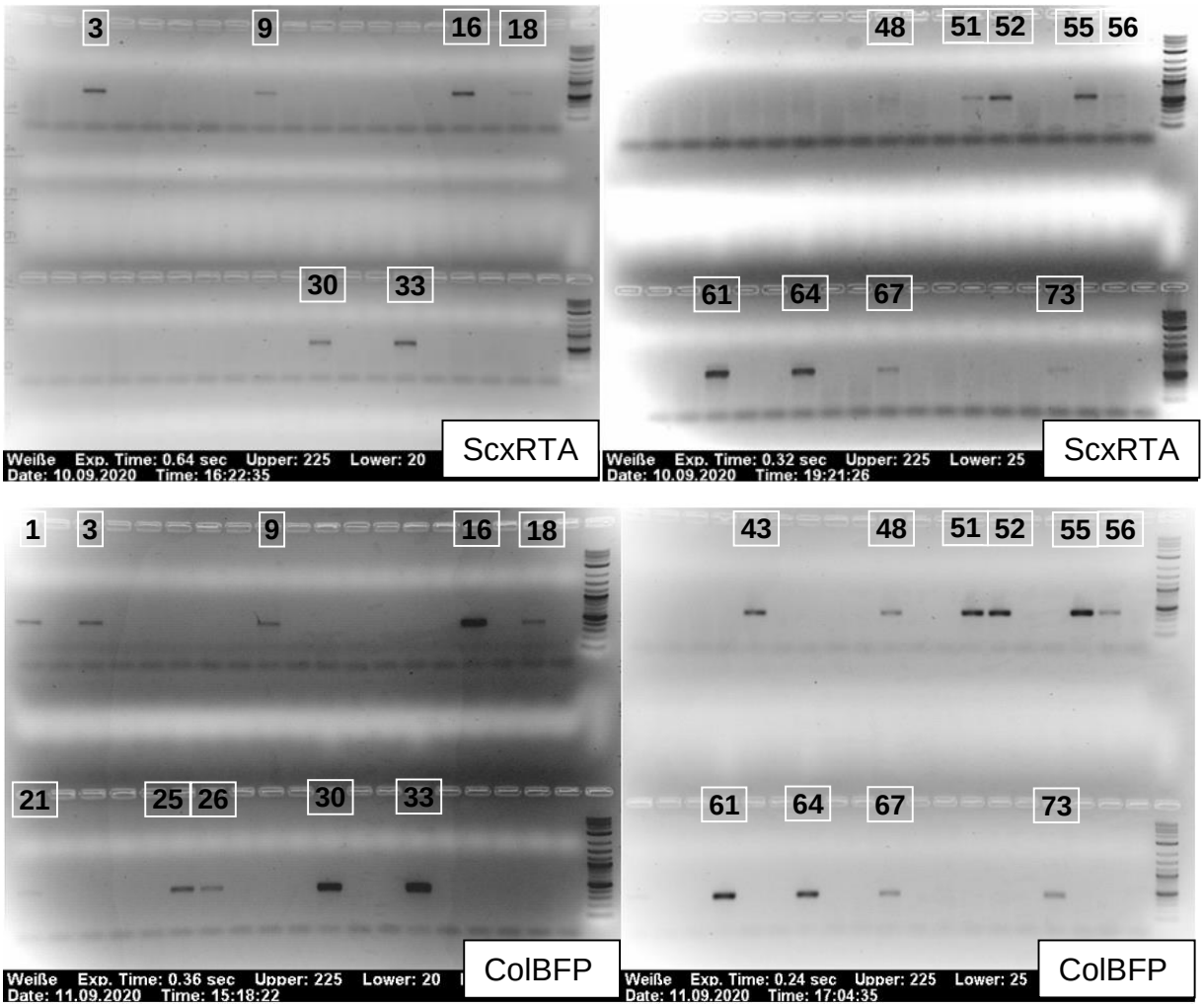

Figure 41. Founders of ScxRTA-ColB.
Seventy-six F0 mice were genotyping with ear samples taken at three weeks, 15 of which carried the ScxRTA as well as the ColB gene (Founder 3 9 16 18 30 33 48 51 52 55 56 61 64 67 73), and five carried only a single ColB gene (Founder 1 20 25 26 43).

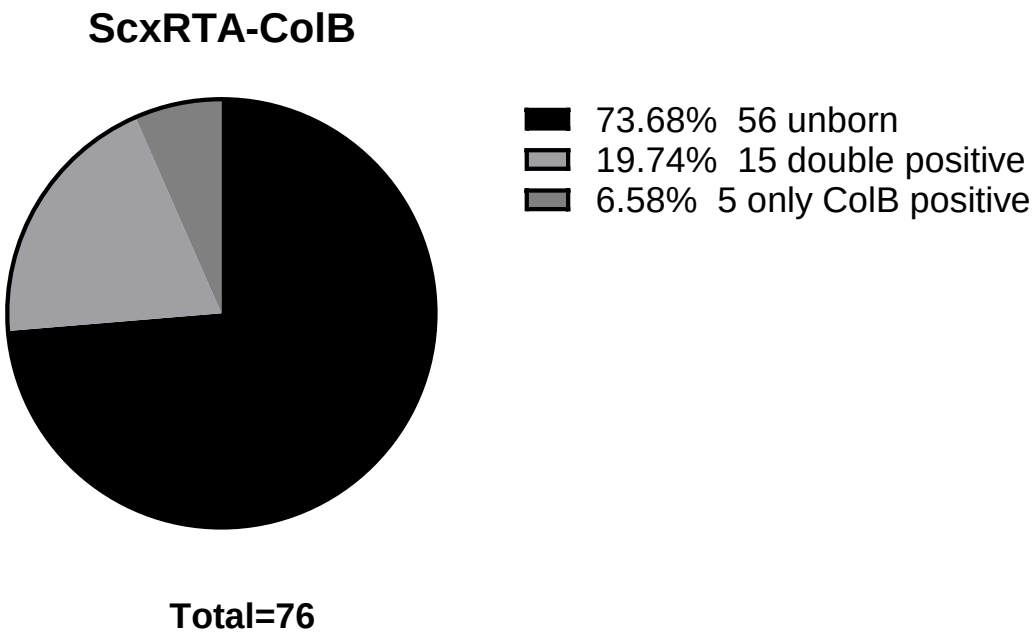

Figure 42. Genotyping results of ScxRTA-ColB.
Seventy-six F0 mice were genotyping with ear samples taken at three weeks, 15 of which carried the ScxRTA and the ColB gene, and five carried only a single ColB gene.

After screening the founders carrying the target genes, three identification strategies were developed to examine the expression of the genes separately:

a. Expression of the mScarlet and BFP reporter genes in the TdAR mouse line.

b. Expression of EmGFP in the Td4F mouse line (representing the expression of Achilles transcription factors).

c. Whether rtTAV16-mScarlet is expressed explicitly in the TdAR mouse line (tendon-specific).

3.2.2 Transgene expression analysis

3.2.2.1 Tendon reporter activator (TdAR) Transgene Expression Validation

Identification of mScarlet and BFP expression were carried on in fifteen double genes (Scx-ColB)-positive foundersF1 (F0 offspring) from mouse lines of TdAR. This part of the work was done by taking the Achilles tendon from 4-6 weeks genotyping positive offspring, stripping the outer tendon sheath of the Achilles tendon, and observing the expression of the two fluorescent reporter proteins under a fluorescent microscope. Five founder lines with working fluorescent reporter genes were identified. The Achilles tendon was fixed, and frozen sections were made to observe fluorescent reporter gene expression as a double-check (Figure 43-47).

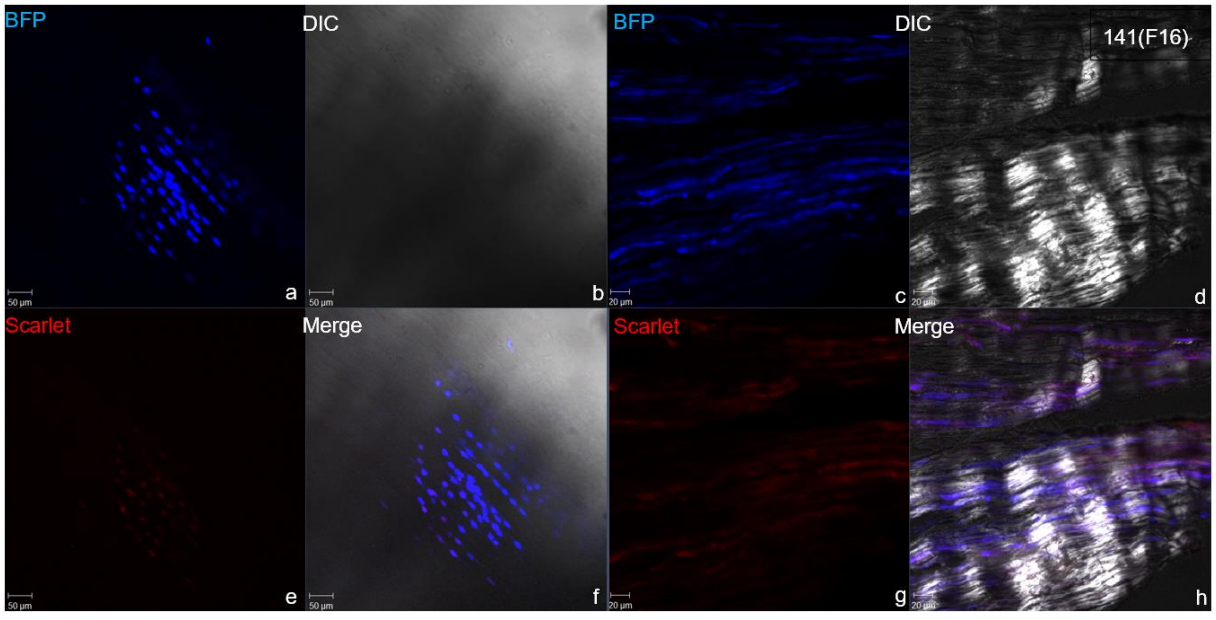

Figure 43. ScxRTA-ColB Lineage Founder 16 Validation.
Achilles tendon tissue was extracted from ScxRTA-ColB transgenic mouse strain founder 16 offspring #141. On the left are images of fresh Achilles tendon tissue after removing the tendon sheath (a,b,e,f), and on the right are images of frozen sections of fixed Achilles tendon tissue (c,d,g,h). Blue fluorescence is col1a1 expression, reported by BFP (a,c), and red fluorescence is Scx-rtTAV16 expression, reported by Scarlet (e,g). b,d are images of differential interference contrast. (medium fluorescent brightness)

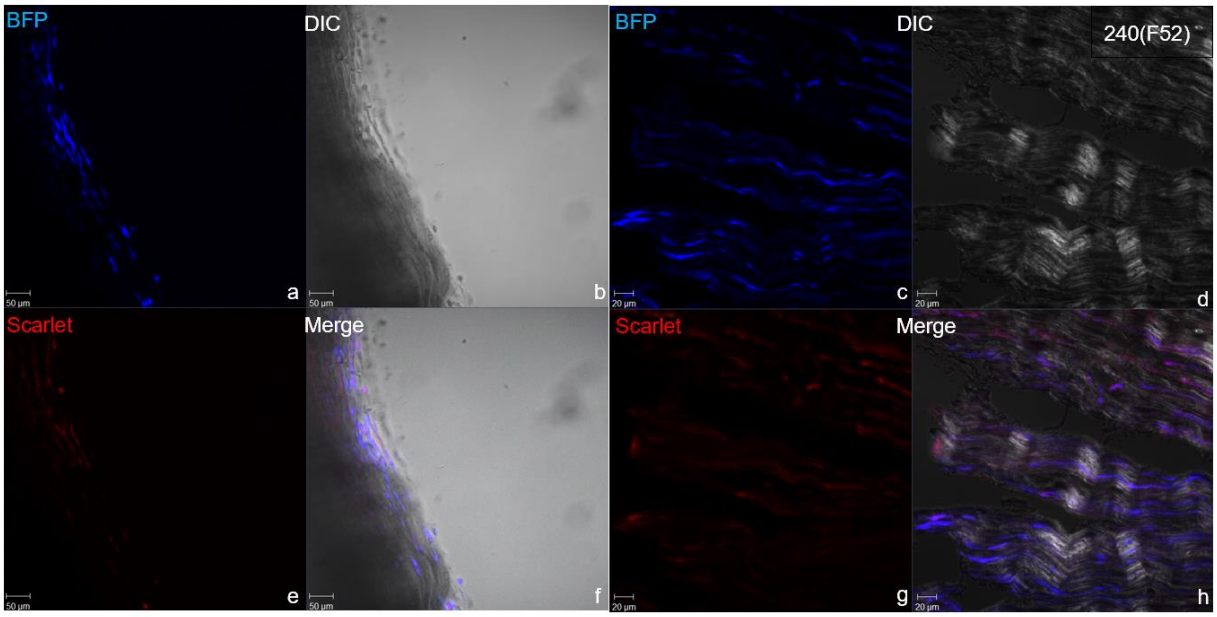

Figure 44. ScxRTA-ColB Lineage Founder 52 Validation.
Achilles tendon tissue was extracted from ScxRTA-ColB transgenic mouse strain founder 52 offspring #240. On the left are images of fresh Achilles tendon tissue after removing the tendon sheath (a,b,e,f), and on the right are images of frozen sections of fixed Achilles tendon tissue (c,d,g,h). Blue fluorescence is col1a1 expression, reported by BFP (a,c), and red fluorescence is Scx-rtTAV16 expression, reported by Scarlet (e,g). b,d are images of differential interference contrast. (low fluorescent brightness)

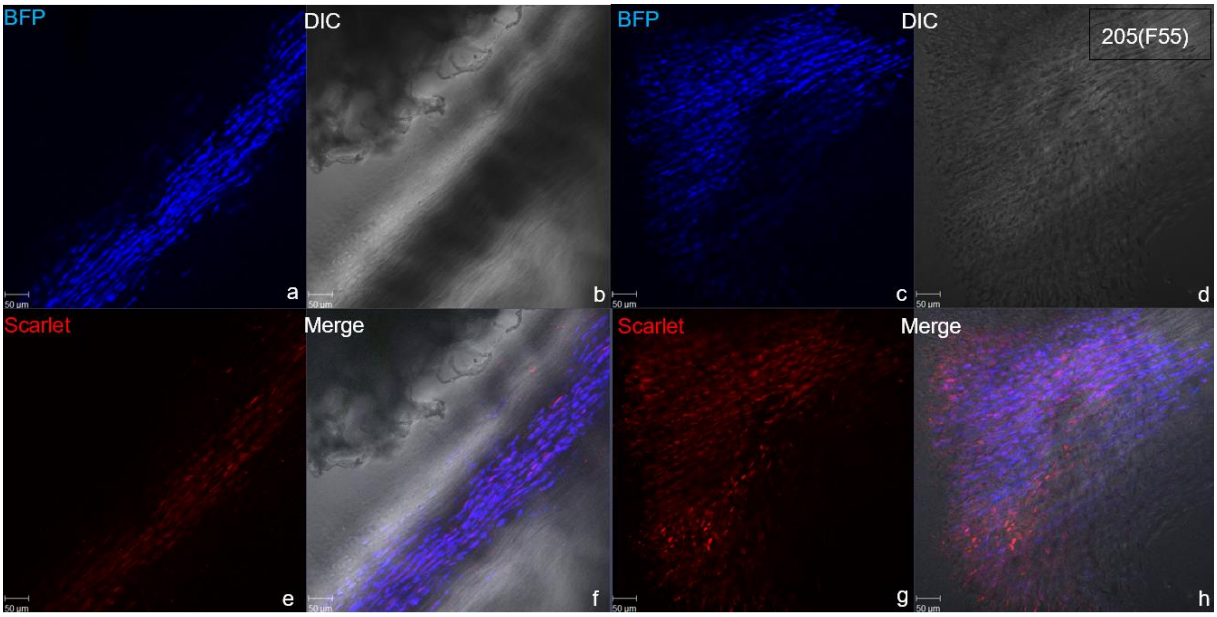

Figure 45. ScxRTA-ColB Lineage Founder 55 Validation.
Achilles tendon tissue was extracted from ScxRTA-ColB transgenic mouse strain founder 55 offspring #205. On the left are images of fresh Achilles tendon tissue after removing the tendon sheath (a,b,e,f),

and on the right are images of frozen sections of fixed Achilles tendon tissue (c,d,g,h). Blue fluorescence is col1a1 expression, reported by BFP (a,c), and red fluorescence is Scx-rtTAV16 expression, reported by Scarlet (e,g). b,d are images of differential interference contrast. (strong fluorescent brightness)

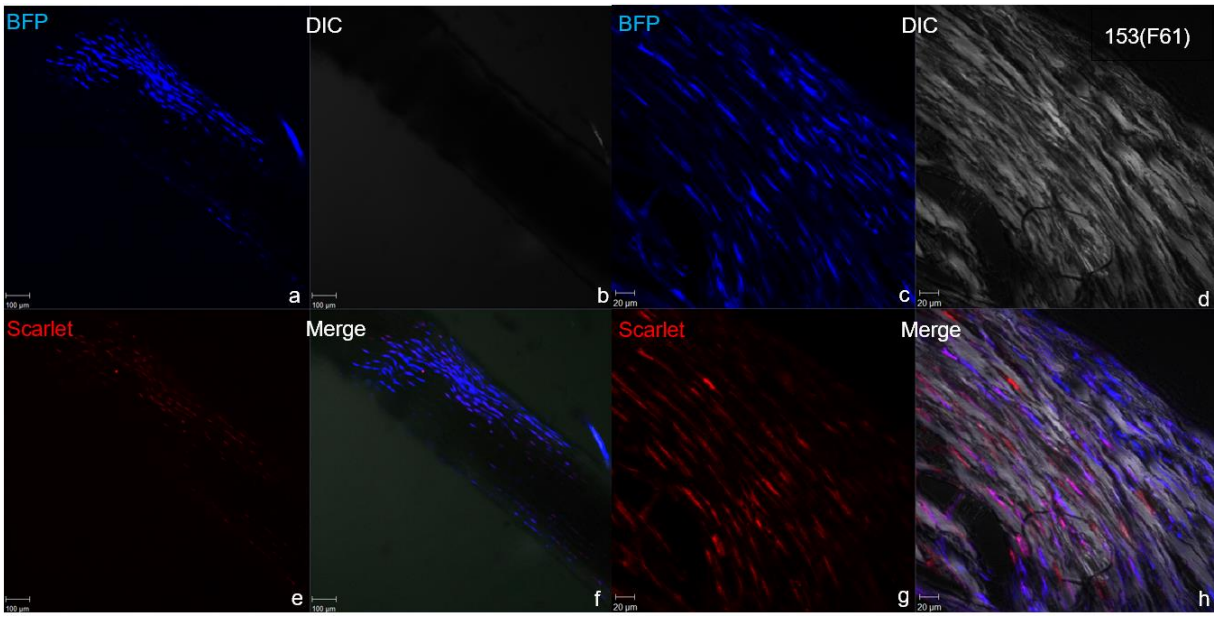

Figure 46. ScxRTA-ColB Lineage Founder 61 Validation.
Achilles tendon tissue was extracted from ScxRTA-ColB transgenic mouse strain founder 61 offspring #153. On the left are images of fresh Achilles tendon tissue after removing the tendon sheath (a,b,e,f), and on the right are images of frozen sections of fixed Achilles tendon tissue (c,d,g,h). Blue fluorescence is col1a1 expression, reported by BFP (a,c), and red fluorescence is Scx-rtTAV16 expression, reported by Scarlet (e,g). b,d are images of differential interference contrast. (strong fluorescent brightness) (Figure46 a,b,e,f are published in my Publication 1(Chen et al. 2021a))

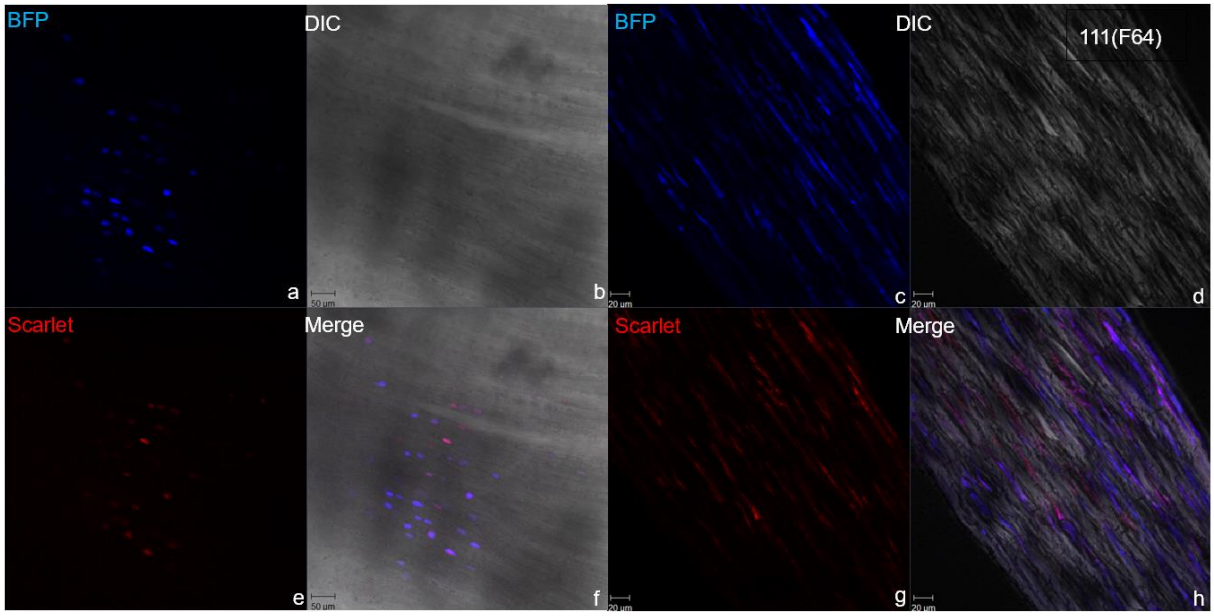

Figure 47. ScxRTA-ColB Lineage Founder 64 Validation.
Achilles tendon tissue was extracted from ScxRTA-ColB transgenic mouse strain founder 64 offspring #111. On the left are images of fresh Achilles tendon tissue after removing the tendon sheath (a,b,e,f), and on the right are images of frozen sections of fixed Achilles tendon tissue (c,d,g,h). Blue fluorescence is col1a1 expression, reported by BFP (a,c), and red fluorescence is Scx-rtTAV16 expression, reported by Scarlet (e,g). b,d are images of differential interference contrast. (low

fluorescent brightness)

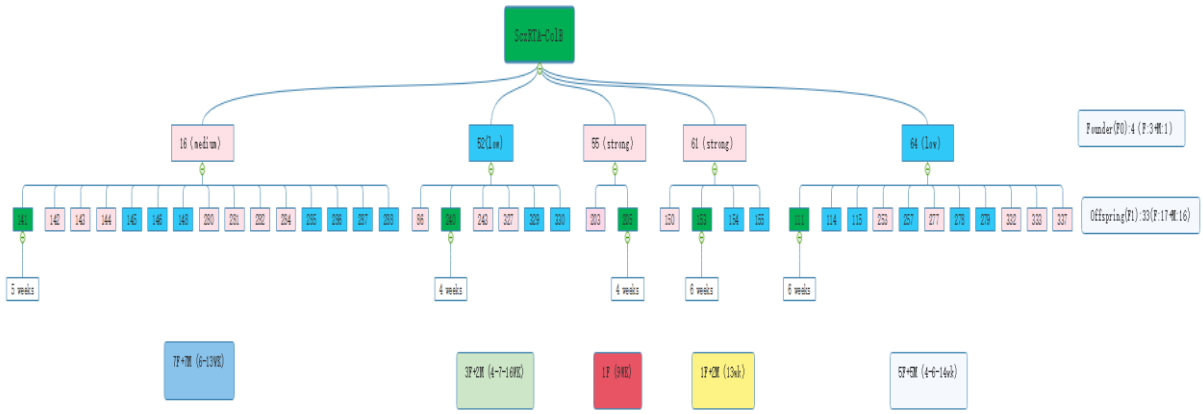

Figure 48. ScxRTA-ColB Lineage Founders' Gene Expression Validation.
The above is a summary of the fluorescence expression of the ScxRTA-ColB transgenic mouse line, with the founder number followed by the fluorescence expression level in parentheses; the number in the box is the mouse number, the blue box indicates male mice, the pink box indicates female mice, the green box indicates fonder offspring identified as fluorescence positive, and the colourless box below shows the age of the mice at the time of identification. The bottom sky blue, light green, red, yellow, and light blue boxes show the number and sex distribution of surviving positive offspring for founder 16, 52, 55, 61, and 64.

By identifying Achilles tendon fluorescence in F1 (Founder offspring) mice around four weeks after birth, five fluorescence expressing mice were determined from 15 genotyping double-positive founders. The more robust mice were founder16, 53, 61 (Figure 48).

3.2.2.2 Tendon Four Factors (Td4F) Transgene Expression Validation

To test the function of rtTA and tetO-OSKM in the OSKM mouse line for gene expression detection of the Td4F mouse line. MEF cells derived from E12.5 embryos of the Gt(ROSA)26Sor$^{tm1(rtTA*M2)Jae}$Col1a1 $^{tm3(tetO-Pou5f1,-Sox2,-Klf4,-Myc)Jae}$/J mouse line were cultured in MEF medium containing doxycycline (2 μgml $^{-1}$) to induce OSKM expression. Based on the following immunohistochemical staining of Oct4A, it is clear that this mouse line has the normal function of rtTA and tetO-OSKM (Figure 49). No events such as methylation silencing occurred.

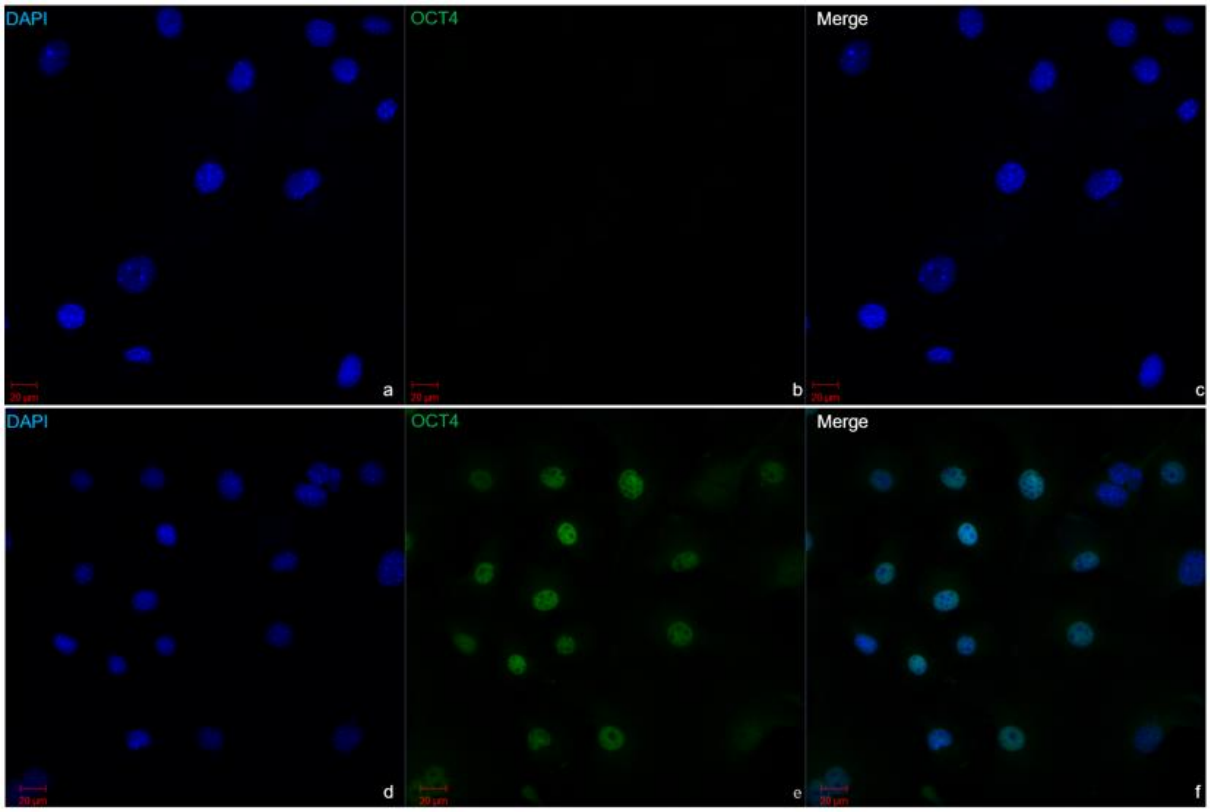

Figure 49. OSKM Expression Validation.
Embryonic fibroblasts from Gt(ROSA)26Sor$^{tm1(rtTA*M2)Jae}$Col1a1 $^{tm3(tetO-Pou5f1,-Sox2,-Klf4,-Myc)Jae}$/J transgenic mouse pins. Fig. a, b, c shows immunohistochemical images of embryonic fibroblasts without doxycycline induction, and fig. d, e, f shows immunohistochemical images of embryonic fibroblasts after two days of doxycycline induction. Embryo-specific OctA(Wang and Dai 2010) expression was evident after doxycycline induction.

Offspring (F1) with the genotype RosaM2rtTA-Td4F-OSKM were obtained by crossing Tg (Insulator-TetO-Tendon4F-GFP-Insulator)/SK(Td4F) with the mouse strain Gt (ROSA)26Sor$^{tm1(rtTA*M2)Jae}$Col1a1 $^{tm3(tetO-Pou5f1,-Sox2,-Klf4,-Myc)Jae}$/J from the Jackson Laboratory.

Fibroblasts from mouse offspring genotyped as RosaM2rtTA-Td4F-OSKM were extracted and cultured in a medium containing doxycycline, and M2rtTA of the Rosa26 locus was induced to be expressed. The rtTA activates the tetO promoter in the Insulator-TetO-Tendon4F-GFP-Insulator insertion fragment, and the transgenic mouse tendon transcription factor and EGFP were initiated. Thus, fibroblasts from RosaM2rtTA-Td4F-OSKM mice can be tested to express the Insulator-TetO-Tendon4F-GFP-Insulator insertion gene based on the expression of green fluorescence (Figure 50-54). The results are as follows:

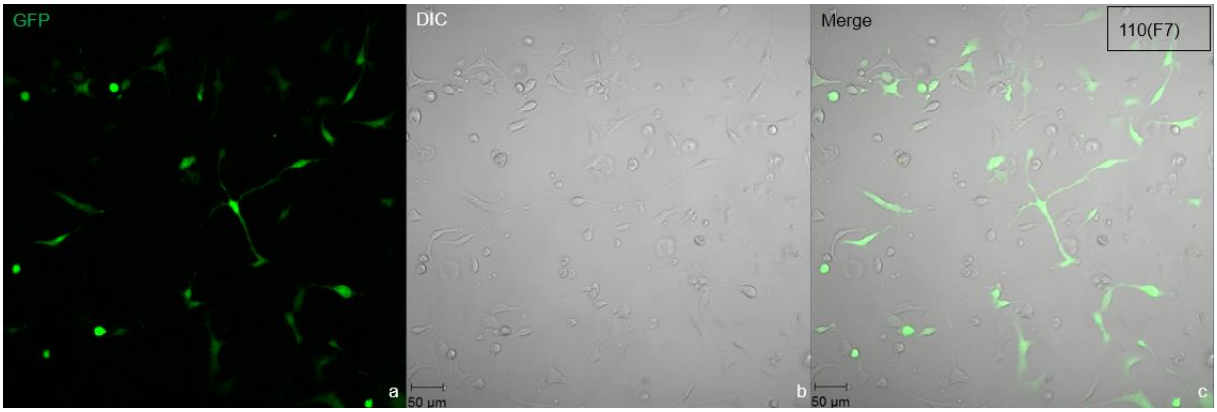

Figure 50. Td4F Lineage Founder 7 Validation.
Fibroblasts were obtained from the lungs of progeny #110 (RosaM2rtTA-Td4F-OSKM) from Td4F transgenic mouse strain founder #7 with Gt(ROSA)26Sor$^{tm1(rtTA*M2)Jae}$Col1a1 $^{tm3(tetO-Pou5f1,-Sox2,-Klf4,-Myc)Jae}$/J mouse line. The green fluorescence image on the left (a), in the middle, is an image of differential interference contrast. (Strong fluorescence brightness)

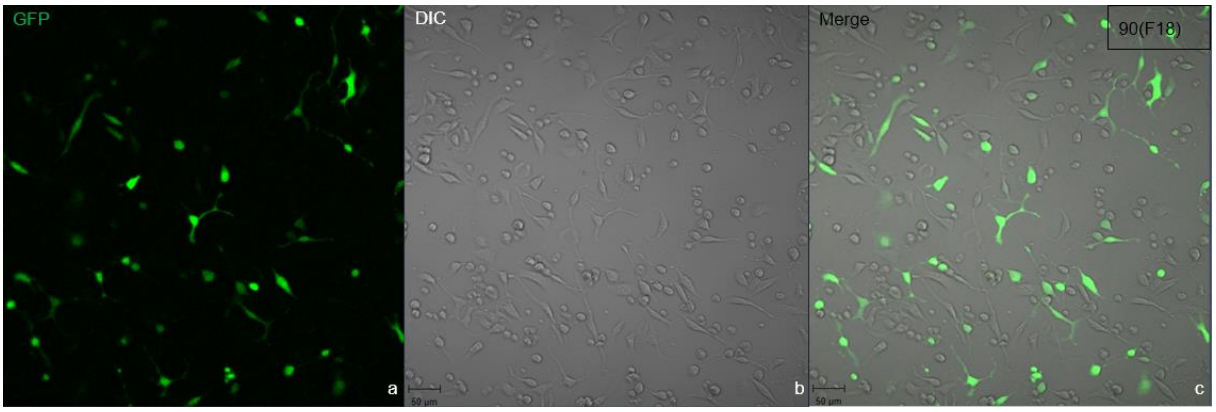

Figure 51. Td4F Lineage Founder 18 Validation.
Fibroblasts were obtained from the lungs of progeny #90 (RosaM2rtTA-Td4F-OSKM) from Td4F transgenic mouse strain founder #18 with Gt(ROSA)26Sor$^{tm1(rtTA*M2)Jae}$Col1a1 $^{tm3(tetO-Pou5f1,-Sox2,-Klf4,-Myc)Jae}$/J mouse line. The green fluorescence image on the left (a), in the middle, is an image of differential interference contrast. (Medium fluorescence brightness)

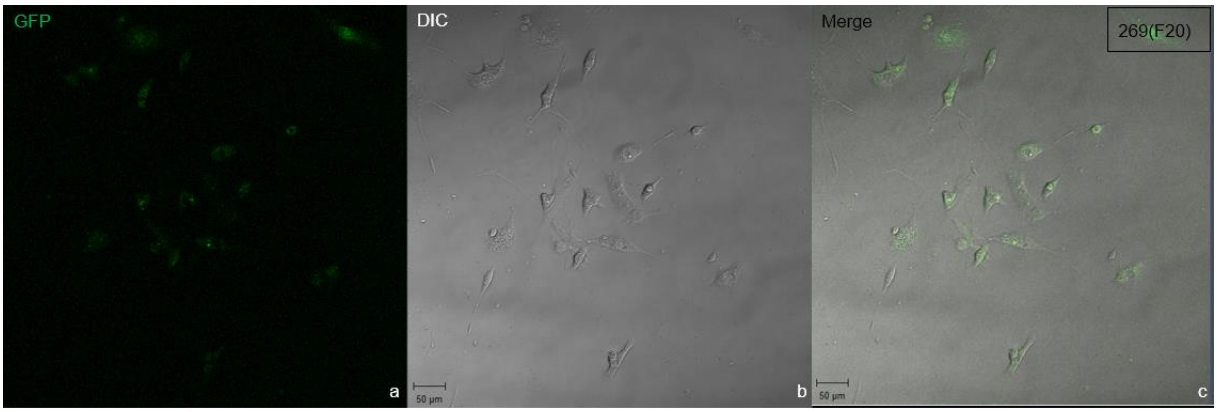

Figure 52. Td4F Lineage Founder 20 Validation.
Fibroblasts were obtained from the lungs of progeny #269 (RosaM2rtTA-Td4F-OSKM) from Td4F transgenic mouse strain founder #20 with Gt(ROSA)26Sor$^{tm1(rtTA*M2)Jae}$Col1a1 $^{tm3(tetO-Pou5f1,-Sox2,-Klf4,-Myc)Jae}$/J mouse line. The green fluorescence image on the left (a), in the middle, is an image of differential interference contrast. (Weak fluorescence brightness)

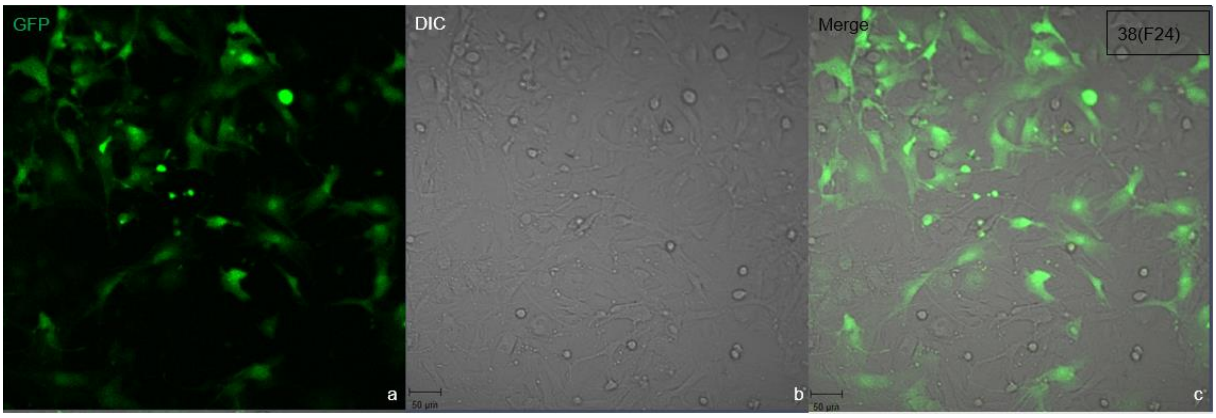

Figure 53. Td4F Lineage Founder 24 Validation.
Fibroblasts were obtained from the lungs of progeny #38 (RosaM2rtTA-Td4F-OSKM) from Td4F transgenic mouse strain founder #24 with Gt(ROSA)26Sor$^{tm1(rtTA*M2)Jae}$Col1a1 $^{tm3(tetO-Pou5f1,-Sox2,-Klf4,-Myc)Jae}$/J mouse line. The green fluorescence image on the left (a), in the middle, is an image of differential interference contrast. (Strong fluorescence brightness)

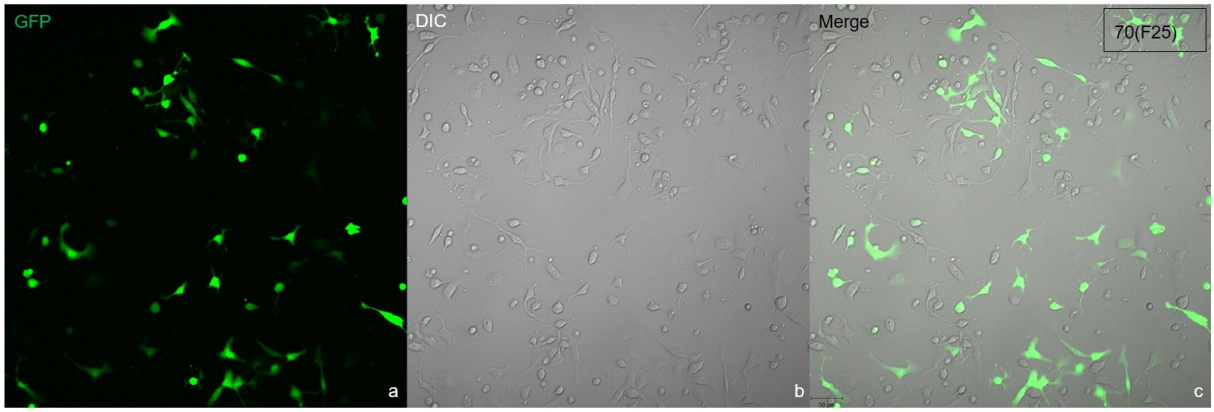

Figure 54. Td4F Lineage Founder 25 Validation.
Fibroblasts were obtained from the lungs of progeny #70 (RosaM2rtTA-Td4F-OSKM) from Td4F transgenic mouse strain founder #25 with Gt(ROSA)26Sor$^{tm1(rtTA*M2)Jae}$Col1a1 $^{tm3(tetO-Pou5f1,-Sox2,-Klf4,-Myc)Jae}$/J mouse line. The green fluorescence image on the left (a), in the middle, is an image of differential interference contrast. (Strong fluorescence brightness)

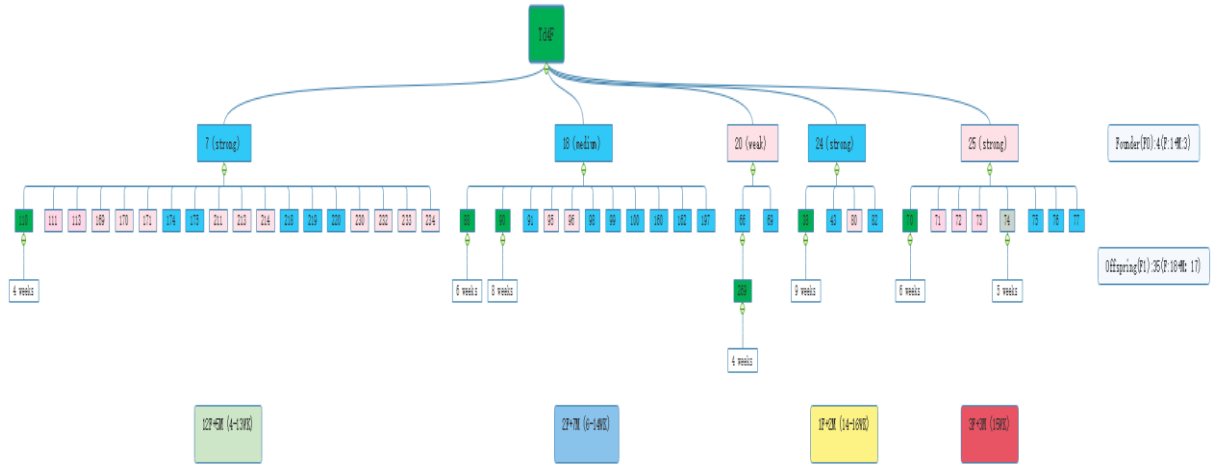

Figure 55. Td4F Lineage Founders' Gene Expression Validation.
Above is a summary of the fluorescent expression of the Td4F transgenic mouse strains, with the founder numbers in the second row of boxes followed by the fluorescent expression levels in

parentheses. The numbers in the boxes are mouse numbers; the blue boxes indicate male mice, the pink boxes indicate female mice, the green boxes indicate progeny identified as fluorescence positive, and the colorless boxes below show the age of the mice at the time of identification. The light green, sky blue, yellow, and red boxes at the bottom show the number and sex distribution of surviving positive offspring for founders 7, 18, 24, and 25, respectively.

By identifying green fluorescence of fibroblasts from F1 (founder offspring) mice four to six weeks after birth, five fluorescence positive, expressing mice were determined from 10 positive genotyping founders. The stronger founders are 7, 18, 24, and 25 (Figure 55).

3.3 Tendon specific transgene expression identification

Genotyping-positive zygotic embryos were identified as expressing red and blue fluorescence after the first screening step collected and observed. For red fluorescence expression distribution (blue fluorescence was a marker for mature Achilles tendon cells, no significant expression was observed) to determine whether the inserted gene faithfully followed its promoter (Scx promoter) expression specificity (Figure 56).

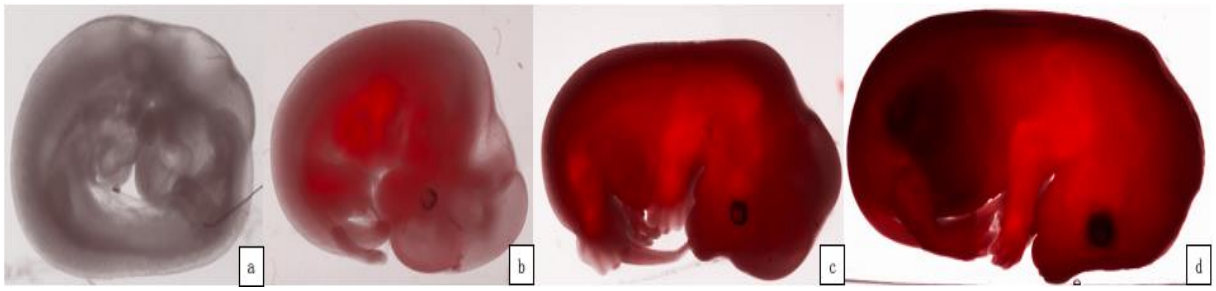

Figure 56. Scx-rtTAV16-Scarlet Expression Profile of E9.5, E 11.5, E13.5, and E15.5.
Figure a shows E9.5 embryos imaged whole mount under a binocular microscope with no significant expression of fluorescent Scx-scarlet signal. Figure b shows the fluorescent expression of the ScxRTA-ColB transgenic mouse strain embryos at E11.5. Figure b,d shows the strong expression of Scarlet at the forelimb and hindlimb tendon sites, the spinal to caudal intervertebral ligaments, and the rib and maxillofacial ligaments that can be clearly seen in mice at days E13.5 and E15.5, respectively. (Figure29 a b c are published in my Publication 1(Chen et al. 2021a))

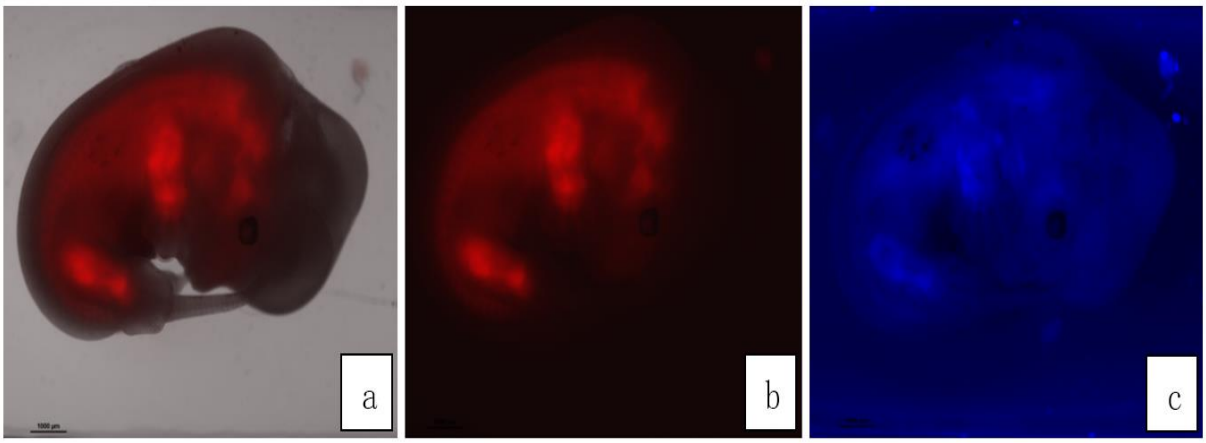

Figure 57. Scx-rtTAV16-Scarlet and Col1a1-BFP Expression Profile of E13.5.
Figure a shows whole-mount imaging of E13.5 embryos under a binocular microscope, and ScxRTA-ColB transgenic mouse strain embryos with fluorescent Scarlet expression at E13.5. Fig. b, c show the strong expression of Scarlet and BFP at day E13.5 can be clearly seen in mouse forelimb and hindlimb tendon sites, spine to caudal intervertebral ligaments, and rib and maxillofacial ligaments, respectively. (Figure30 b c are published in my Publication 1(Chen et al. 2021a))

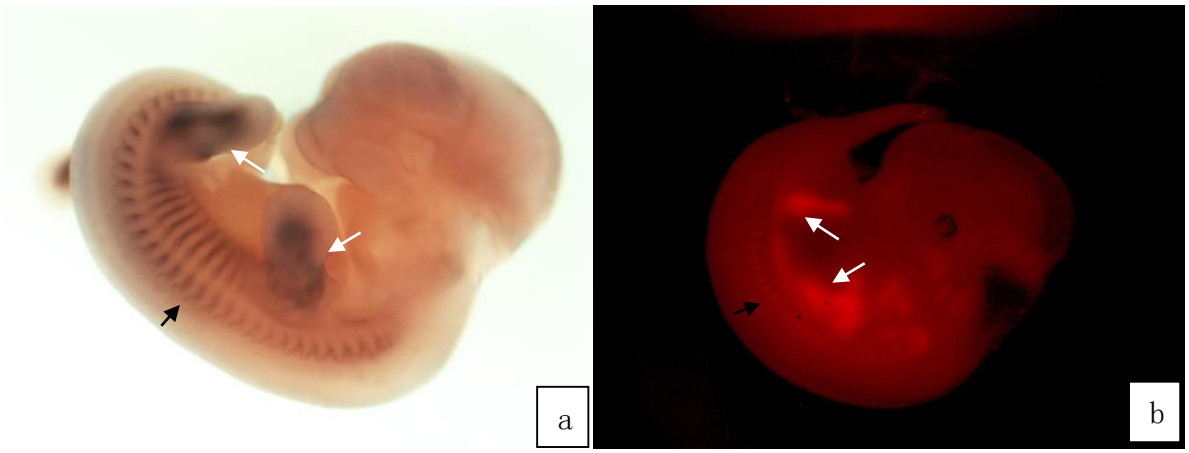

Figure 58. Scx-rtTAV16-Scarlet Expression Profile of E11.5.
Figure a shows whole-mount ISH for Scx on E11.5 wild-type(Pryce et al. 2009). Figure b shows the fluorescent expression of ScxRTA-ColB transgenic mouse strain embryos on 11.5. Scarlet's strong expression in the forelimb buds and hindlimb buds of mouse embryos can be seen at day E11.5, segmentally along the spine to the tail. Black arrows indicate the expression of Scarlet in somite. White arrows indicate the expression of Scarlet in the middle of the limb buds. (Figure A adapt from https://www.embrys.jp/embrys/2DView/pictures, on 6th. May 2021)

Scx has been reported to be highly expressed in tendon tissue since 2001(Schweitzer et al. 2001), and its expression distribution has been studied in a series of studies. In 2009, the same group reported that whole-mount in situ hybridization of E11.5 wild-type mouse embryos showed high expression of Scx in fore and hind limb buds and somites(Pryce et al. 2009). This figure is consistent with the distribution of scarlet expression in our ScxRTA-ColB transgenic mice.

Comparing with the latest in situ hybridization results of E11.5 wild-type mouse embryos (Figure 25, a), I found that scarlet reported scx expression faithfully (Figure 57,58)

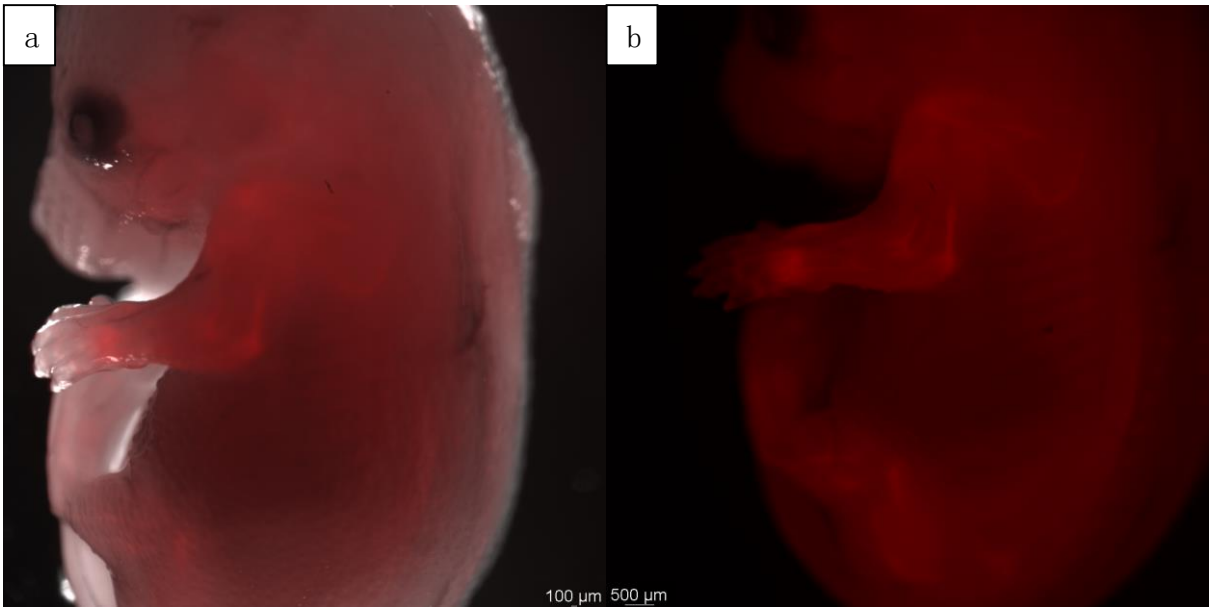

Figure 59. Scx-rtTAV16-Scarlet Expression Profile of E15.5.
Figure a shows fluorescent Scx-scarlet signals merged with brightfield for whole-mount imaging under a binocular microscope. Figure b shows the fluorescent expression of ScxRTA-ColB transgenic mouse strain embryos at 15.5. Scarlet's strong expression at the fore and hind limb tendon sites, spinal to caudal intervertebral ligament expression, and rib and maxillofacial ligament expression in mice can be clearly seen at day E15.5. (Figure 32 b is published in my Publication 1(Chen et al. 2021a))

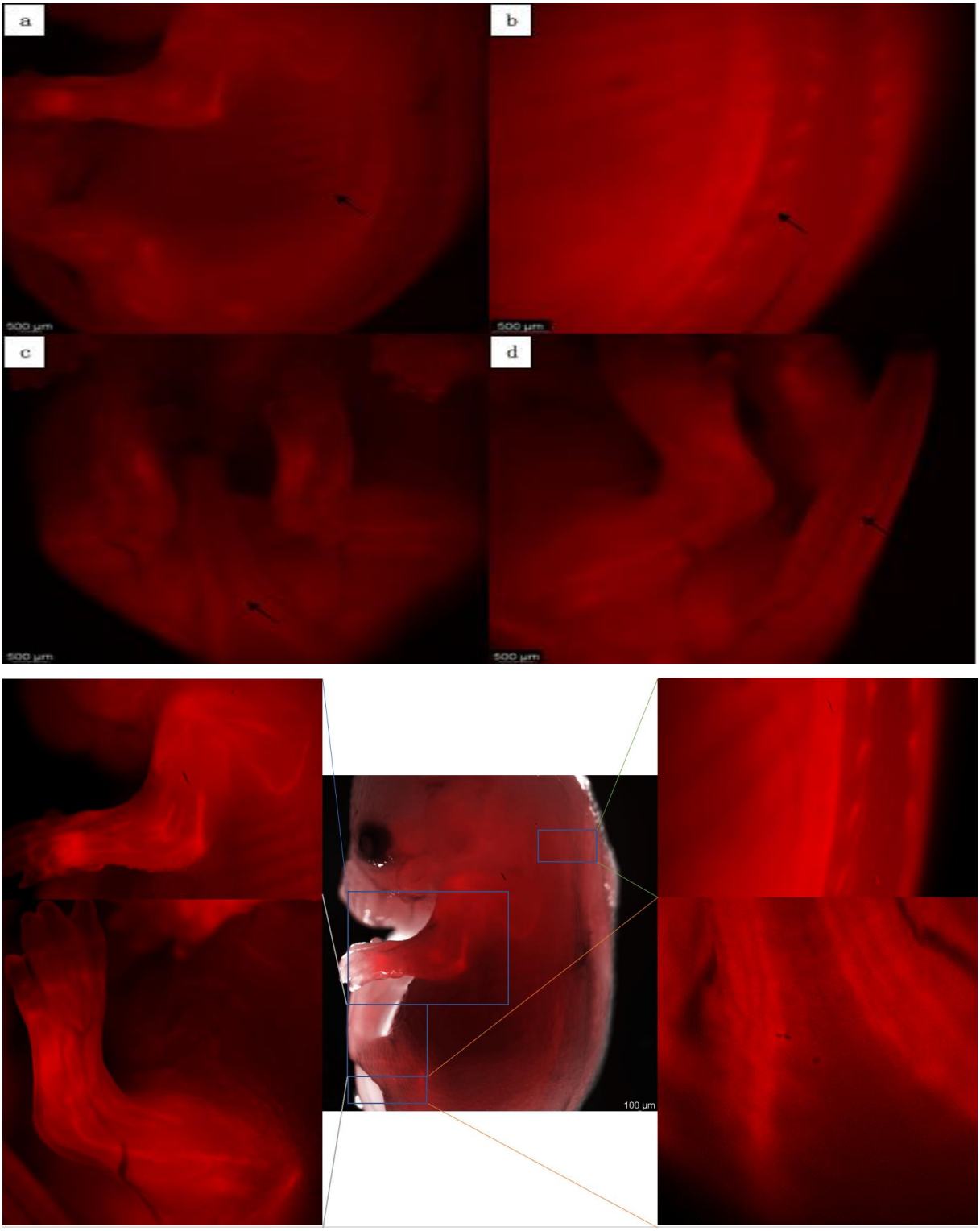

Figure 60. Scx-rtTAV16-Scarlet Expression Profile of the Vertebra, Rib, and Tail.
Figure a shows fluorescent Scx-scarlet signalling expressed at the rib site of E15.5. Figure b shows fluorescent Scx-scarlet signalling expressed fluorescently at the spinal area of the ScxRTA-ColB transgenic mouse strain embryos at 15.5. Figure c, d shows fluorescent Scx-scarlet signal expression at the tail site of E15.5.(The black arrows in Figures a, b, c, d point to the intercostal ligament, the supraspinal ligament, respectively. As well as sagittal and coronal images of the intercaudal ligament.) (Figure33 is published in my Publication 1(Chen et al. 2021a))

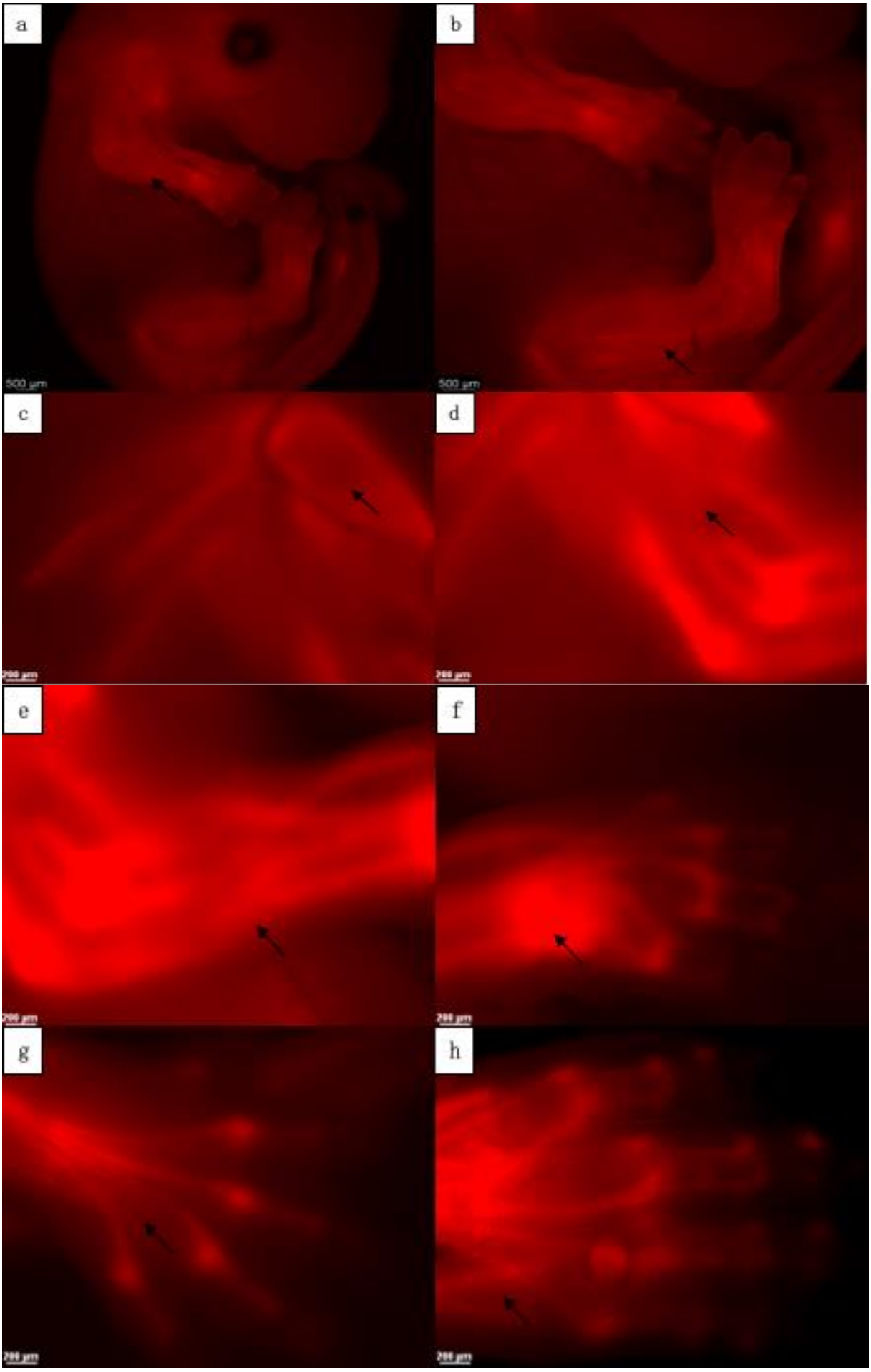
a
500 µm
b
500 µm
c
d
e
f
g
h

Figure 61. Scx-rtTAV16-Scarlet Expression Profile of the Limbs.
The black arrow in Fig. a points to the fluorescent Scx-scarlet signal expressed at the forelimb site of E15.5. Figure b shows fluorescent Scx-scarlet signal expression at the hind limb site of an embryo of the ScxRTA-ColB transgenic mouse strain at 15.5. The arrow in Fig. c points to the fluorescent Scx-scarlet signal expressed at the shoulder of E15.5. The arrow in Fig. d indicates the fluorescent Scx-scarlet signal express in the upper arm region of the forelimb of E15.5. The arrow in Fig. e indicates the fluorescent Scx-scarlet signal expressed in the lower arm of the forelimb at E15.5. The arrow in figure f points to the fluorescent Scx-scarlet signal expressed at the forelimb wrist of E15.5. The arrow in figure g points to the fluorescent Scx-scarlet signal expressed at the hinder paw of E15.5. The arrow in figure h points to the fluorescent Scx-scarlet signal expressed on the dorsal surface of the hinder paw of the skinned E18.5 embryo. (Figure34 g h are published in my Publication 1(Chen et al. 2021a))

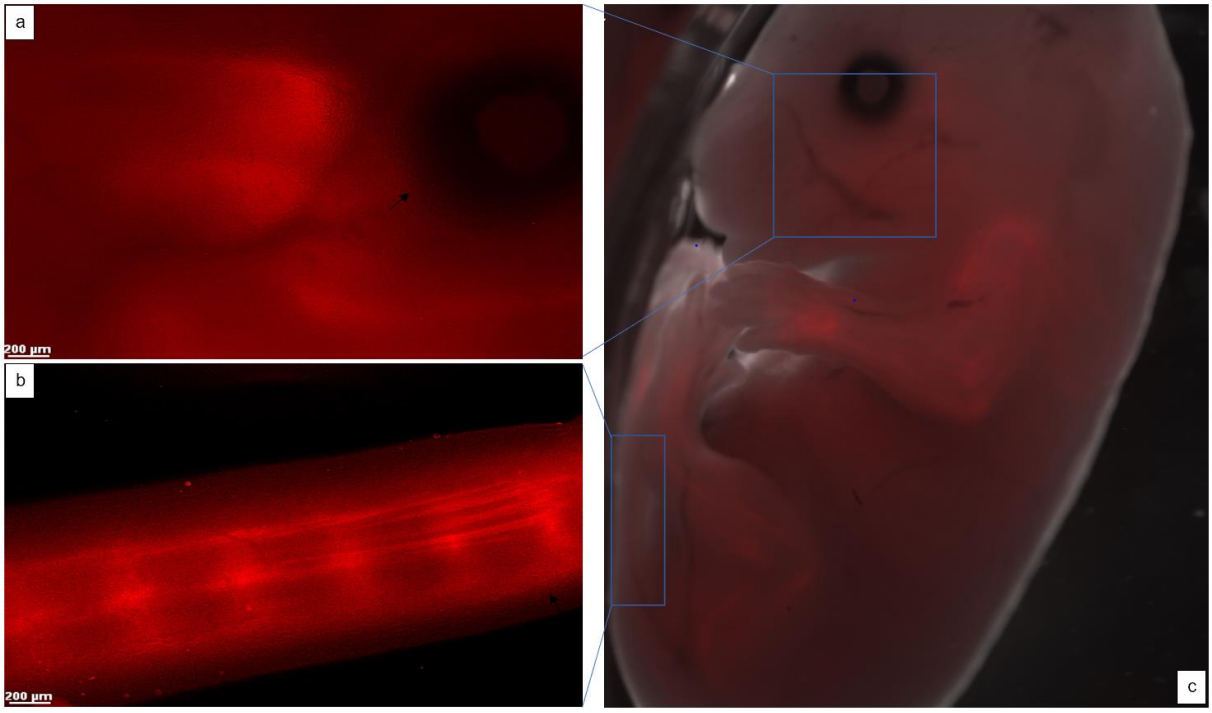

Figure 62. Scx-rtTAV16-Scarlet Expression Profile of the Neonatal Maxillofacial and Caudal Region.
Figure a show the fluorescent Scx-scarlet signal expression on the maxillofacial region of a neonatal mouse of the ScxRTA-ColB transgenic mouse strain. Figure b shows the fluorescent Scx-scarlet signal expression in the de-skinned tail of a newborn mouse of the ScxRTA-ColB transgenic mouse line. (In figure a, the arrow points to the mouse eye, and in figure b, the arrow indicates to the mouse tail tendon)

After verifying that Scx-Scarlet-rtTAV16 expression is tendon specific, theTg (Scx-Scarlet-2A-rtTAV16); Tg (Col1a1-BFP)/SK(TdAR) mouse line (verified Scarlet-rtTAV16 tendon-specific expression) was crossed with Tg (Insulator-TetO-Tendon4F-GFP-Insulator)/SK(Td4F) mouse line (validated for normal TetO-Tendon4F-GFP expression) to obtain progeny TdAR-Td4F with all three construct insertions (Figure 59-62).

TdAR-Td4F mice can be tested for rtTAV16 function by expression of EGFP after exposure to doxycycline. The rationale is that rtTA activates the tetO promoter in the

Insulator-TetO-Tendon4F-GFP-Insulator insertion fragment. EGFP will be expressed in transgenic mice capable of expressing both the tendon transcription factor and EGFP. Thus, when E12.5 days TdAR-Td4F mouse embryonic fibroblasts are extracted and cultured in a doxycycline-containing medium, the expression function of rtTAV16 can be detected based on the expression of green fluorescence (Figure 63). (The expression function of the Insulator-TetO-Tendon4F-GFP-Insulator insert has been tested previously.)

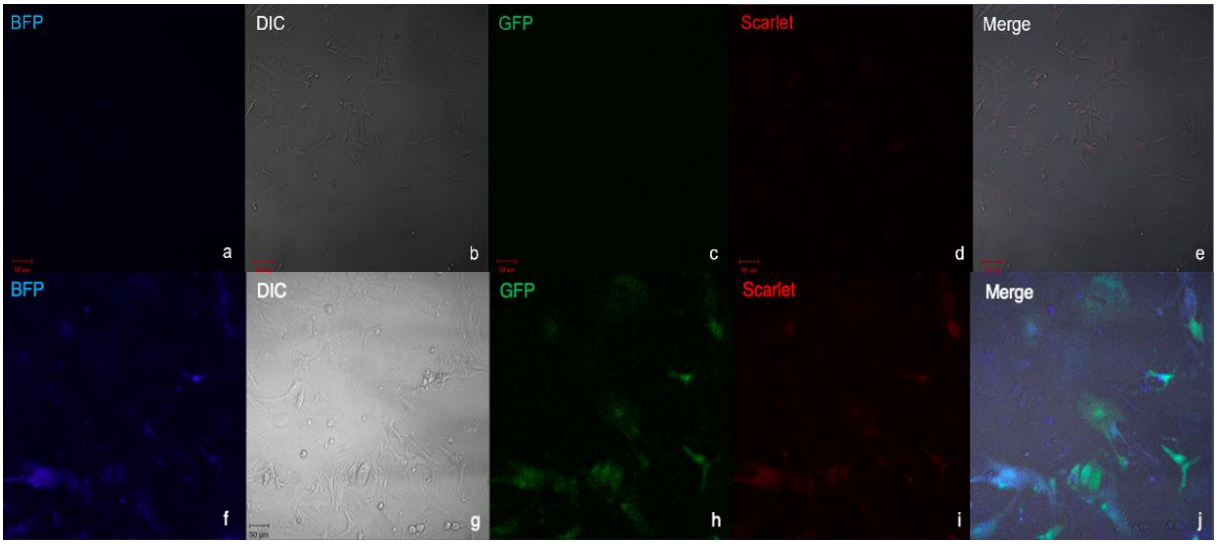

Figure 63. Fluorescence Expression in Embryonic Fibroblasts of TdAR-Td4F mice.
Two days after doxycycline induction, fibroblasts differentiated from TdAR mouse embryos expressed scarlet, whereas fibroblasts from TdAR-Td4F mouse embryos expressed scarlet EGFP and BFP. All three fluorescent proteins are expressed in the same population of cells. (Fig. a, b, c, d, e are fibroblasts from TdAR mouse; fig. f, g, h, i, j are are fibroblasts from TdAR-Td4F mouse)

I have thus completed our initial characterization of whether the new mouse lines correctly express. The results show that the new mouse line Scx-Scarlet-rtTAV16 is robustly expressed and tendon-specific.

CHAPTER 4: DISCUSSION

With increased age, the emergence of tendon ageing and injury is on the rise, causing great suffering to patients due to its intractability and impact on movement. It has been documented that Scx, a bHLH transcription factor expressed early in tendon cells (Schweitzer et al. 2001), plays an essential role in tendon development and repair after injury (Sakabe et al. 2018). Here, I constructed Scx promoter-driven mScarlet fluorescent reporter transgenic mice for early tendon repair and development analysis. Combined with the BFP reporter for the type I collagen (tendon cell function marker), I have developed animal models that can be used to study tendon regeneration.

Recent studies have found that the transcription factors (OSKM) for cellular reprogramming reverse cellular ageing and promote scar-free repair of injured organs (Doeser et al. 2018a; Ocampo et al. 2016). However, OSKM expression is at risk of reducing teratoma and tendon transcription factors (which are essential for tendon repair and tendon cell maturation) (Wang et al. 2021). Therefore, I have optimized the tendon reporter activator mice by introducing the tet-on system (Doxycycline-inducible Gene Expression) (Das et al. 2016). This optimization allows the expression of OSKM to be restricted to Scx-expressing tendon tissues, reducing the risk posed by OSKM. In combination with the construction of tendon transcription factors overexpression mice, I have created an animal model that can overexpress both the reprogrammed transcription factors and the tendon transcription factors.

The results of the identification strategy indicate that the new mouse line is functionally standard in terms of exogenous genes, creating an experimental model for further study of the mechanisms of tendon development, injury, and regeneration.

Therefore, I am going to discuss these viewpoints in this chapter as follows:

- Known Functions of Insulator
- Known Functions of Tendon-specific Cis-acting Elements
- Known Functions of 2A Self-cleaving Peptides
- Regulation of Transcription Factors Expression by Doxycycline
- Upstream Signal to Regulate Scx Genes in Tendon

- Four Tendon Transcription Factors as regulators of tendon development
- Conclusions and Outlook

4.1 Known Functions of Insulator

First of all, the insulator is a cis-regulatory element with an enhancer-blocker or barrier role. It acts as an enhancer-blocker (preventing distal enhancers from working on the promoters of neighbouring genes) or through loop formation and ribosomal modifications barrier action (containing euchromatin silencing by diffusion of adjacent heterochromatin) (Gaszner and Felsenfeld 2006; Valenzuela and Kamakaka 2006).

The insulator sequence I used is derived from the chicken β-globin locus near the 5' boundary of the chicken β-globin structural domain. Its prominent role is to keep the reporter gene from being activated by the nearby β-globin locus control region (5'HS2). The insulator itself does not significantly alter the expression levels of the genes within it. Instead, it allows the reporter protein to respond to the expression levels of the genes faithfully it reports by preventing aberrant activation. In the Drosophila transgenic model, the insulator precludes random insertion sites' effect on the inserted target gene (Chung et al. 1993).

The toxicity of high levels and continuous expression of reverse tetracycline blockers fused to the VP16 activation domain (rtTA) transactivator must be excluded to achieve precise regulation of the tet-on system (Anastassiadis et al. 2002). Otherwise, the problem of inability to switch off or over-expression of the inducible system would occur. As the integration into the genome is random, this may lead to issues where enhancers near the insertion site affect reporter genes and transcription factors and produce both basal expressions without induction. I, therefore, inserted insulator sequences derived from the chicken β-bead protein locus on either side of the Col1a1-BFP reporter gene and Td4F-GFP. The insulator prevents the automatic activation of the col1a1 promoter and the tetO promoter without impairing the promoter's inducible activation function (Zeng et al. 2008). In the Td4F mouse line that used the insulator, half of the ten founders carrying the insertion fragment clearly expressed the GFP, in line with the efficiency I expected.

4.2 Known Functions of Tendon-specific Cis-acting Elements

Type I collagen is the most crucial component that guarantees the strength of tendons. However, it is too widely distributed as a tendon-specific marker. Yet, it is the most critical indicator of mechanical tendon function and is difficult to replace by other molecular markers for tendon maturation and functional recovery after tendon injury.

However, the use of different fragments of the type I collagen promoter to drive fluorescent reporter genes allows for tissue-specific fluorescence expression (Rossert et al. 1995). The cis-acting element of the COL1a1 promoter, located between -3.2 and -2.3 kb, can drive specific expression of the reporter gene in tendons, fascia, and other tissues (Léjard et al. 2007). I, therefore, used two types I collagen promoter fragments named tendon-specific cis-acting elements (TSE1, TSE2) as promoters for the BFP reporter gene and separated them from the genomic DNA by insulators. I did not find significant widespread expression of BFP in TdAR embryos; instead, I found specific expression of BFP in tendon tissue. These results suggest that our Col1a1-BFP transgenic mice can specifically report tendon type I collagen expression and are suitable for use as a functional marker for tendon and as a marker for mature tendon.

4.3 Known Functions of 2A Self-cleaving Peptides

2A self-cleaved peptides are a class of short peptides (18-22 amino acids) that can be translated into two distinct proteins by inducing ribosomal jumping from consecutive sequences (Liu et al. 2017). The 2A peptide family consists of four members, P2A, E2A, F2A, and T2A, derived from porcine teschovirus-1, equine rhinitis A virus, foot-and-mouth disease virus, and thosea asigna virus, respectively. The 2A peptide family all share the core sequence of DxExNPGP (Liu et al. 2017). I selected T2A and P2A, and E2A with high self-cleavage efficiency and combined them with IRES elements to cleave the contiguous transcripts into five different peptides, whereas F2A was not selected because it is only about 50% efficient (Velychko et al. 2019). The 2A site has a 60% probability of causing glycosome

shedding. The peptide chain maybe only about 30% typically expressed because 10% of P2A and T2A are not cleaved (Liu et al. 2017).

In conclusion, the fluorescent reporter gene EGFP in the vector I constructed is theoretically expressed at a lower level than the first four transcription factors. So, I can predict that the transcription factors upstream of it are expressable by looking at the green fluorescence.

4.4 Regulation of Transcription Factors Expression by Doxycycline

Before performing damage repair experiments, transgenic animals should be protected from the effects of target genes integrated into the animal's genome. The reason is the potential for lethality and the potential for developmental effects that would prevent a valid control group. It is therefore essential to create transgenic models in which gene expression can be induced. Of all the inducible gene expression systems, the most commonly used is the corresponding doxycycline-inducible Tet system (Das et al. 2016). Tet systems can be divided into Tet-Off systems and Tet-On systems. The former prevents the tetracycline transactivator (tTA) protein, which is composed of the tetracycline deterrent protein (TetR) from E. coli fused to the VP16 activation domain from herpesvirus, from binding to the tetracycline response element (TRE) after exposure to doxycycline. This binding reduces the expression of genes downstream of the TetO sequence (Gossen and Bujard 1992). In turn, the latter can bind the TetO sequence and thus activate its downstream genes when exposed to tetracycline and its derivatives to make the rtTA protein (Gossen and Bujard 1992).

The Tet-On system is more responsive than the Tet-Off system. I, therefore, chose the former when creating the transgenic mice. Also, to further increase the sensitivity of the Tet-On system in response to doxycycline, I used the Tet-On 3G (rtTAV16), which consists of three minimal VP16 activation domains. (Zhou et al. 2006) This system significantly improved sensitivity to doxycycline while significantly reducing basal expression of its downstream genes.

My results show that gene induction in transgenic mice is effective in response to doxycycline induction and basal expression is not sufficient to elicit fluorescent protein expression (Figure 64)

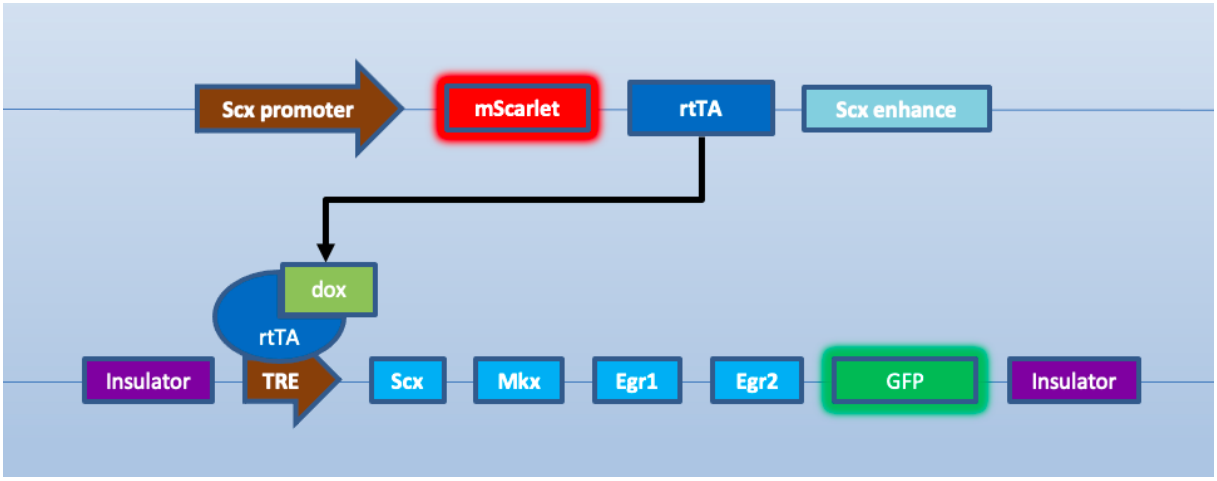

Figure 64. Inducible reporter system.
This construct consists of a tetracycline-inducible promoter and four tendon transcription factors (Scx, Mkx, Egr1, and Egr2) and GFP (Green fluorescent protein). When this line is crossed with a tendon promoter-driven reversed tetracycline trans-activator (rtTA) mouse line, the double-positive transgenic mice will express tendon transcription factors and reporter GFP when doxycycline is provided in drinking water.

4.5 Upstream Signal to Regulate Scx Genes in Tendon

Scx is a basic helix-loop-helix transcription factor, and it is one of the most important transcription factors known for tendon development, differentiation, and repair (Delgado Caceres et al. 2018; Nichols et al. 2018; Sakabe et al. 2018; Schneider et al. 2018). It is widely involved in tendon development and maintenance of mature tendon homeostasis by regulating the expression of Tnmd, Col1a1 in tendon cells (Shukunami et al. 2018). This raises a new question about what upstream signals regulate Scx.

Studies have shown that mechanical forces, TGFβ signalling, and Egr1 are the essential upstream regulatory signals for Scx expression (Havis and Duprez 2020). Mechanical forces are the most important in maintaining Scx expression among the physicochemical factors, which may regulate Scx expression and function through upregulation of TGFβ signaling and then phosphorylation by ERK1/2 (non-canonical signaling) or Smad3 (canonical signaling) (Subramanian et al. 2018). Among the growth factors, TGFβ signaling is the one that directly regulates Scx during

development, and the mechanism of regulation is the same as above (Kaji et al. 2020; Subramanian et al. 2018). Egr1 is involved in regulating Scx expression among the transforming growth factors through the regulation of TGFβ signalling (Gaut et al. 2016).

It has been shown that forced expression of Egr1 facilitates the recovery of tendon function and the expression of Scx in tendon repair cells (Gaut et al. 2016). Furthermore, tendon repair is mainly related to the successful differentiation of Scx-negative cells (but not Scx-lineage cells) that migrate into the injury site into Scx-positive repair cells (Best et al. 2021; Bobzin et al. 2021). Therefore, I have employed the inducible overexpression of Egr1 in mice, together with the aid of damage-induced Scx expression, to observe the combined effect of multiple transcription factors co-expressed. The transgenic mice also meet the need to study the regulatory mechanisms mentioned above, creating an effective experimental model for validating these mechanisms.

4.6 Four Tendon Transcription Factors Regulate Tendon Development

In mouse embryos, tendon development begins at approximately E9.5. At this point, Scx expression starts in response to TGFβ signaling (Brent et al. 2003); Mkx is then expressed at E12.5 and decreases after reaching a peak at E16.5. However, it continues to be expressed in tendon sheath cells (Liu et al. 2010). This finding suggests that the two cooperate during development and post-injury repair, regulating tendon maturation and post-injury repair through change the expression levels of the collagen I and tendon regulatory proteins. Egr1/2 are expressed in the Scx expression region of E12.5 and E14.5, respectively, and contribute to the regulation of collagen (types I, V, VI, XII, XIV, and XV) and proteoglycans (decorin, fibromodulin, lumican) by the former two (Lejard et al. 2011a).

Using a mouse model of forced overexpression of the four factors, I will test their effect on the direct conversion of fibroblasts into tendon cells; then in vivo on tendon repair by regulating the downstream products of the transcription factors. In summary, I can explore the above mechanisms and hypotheses through this mouse model. In particular, the introduction of the Scx-mScarlet, which detects cell identity

status, and the Col1a1-BFP reporter system, which detects the functional status of the cells, has made it possible to determine the various stages of repair and thus to analyze the downstream products of the transcription factors in the Achilles tendon (Figure 65).

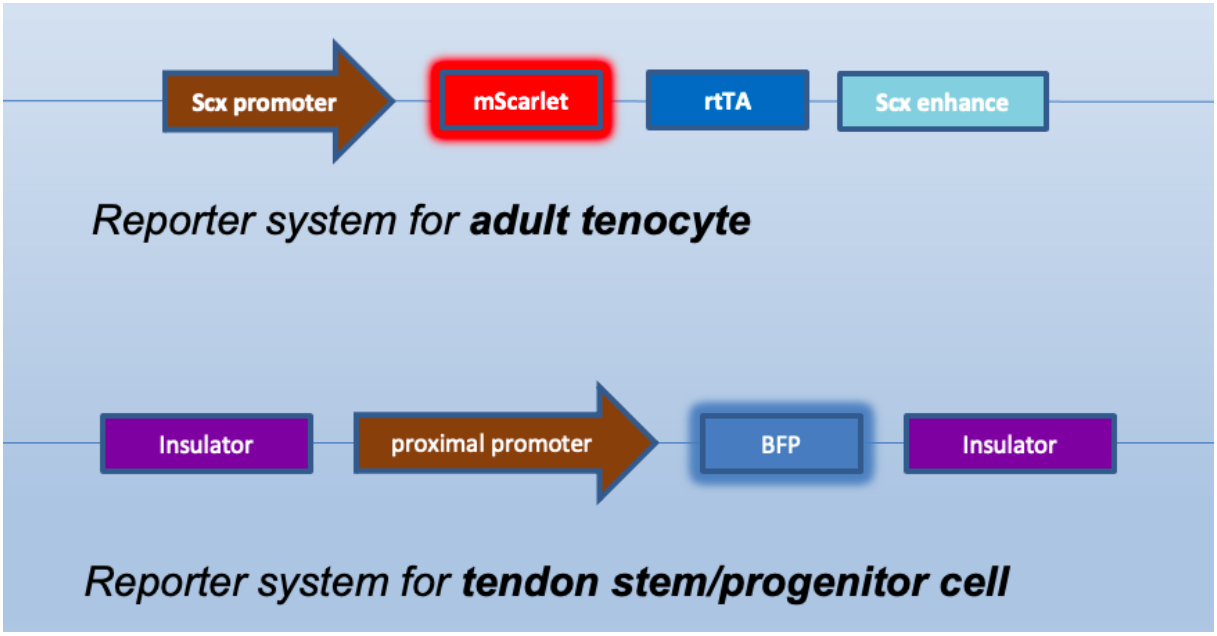

Figure 65. Reporter system.
The reversed tetracyclin trans-activator (rtTA) and mScarlet red fluorescent protein are expressed under the control of the Scx promoter. The Scx promoter is expressed in the tendon stem/progenitor cells. When the Tendon stem/progenitor cells appear during the differentiation or regeneration process, the mScarlet red fluorescent reporter will be switched on. The reversed tetracyclin trans-activator will be expressed. The mScarlet reports Scx activity, and the rtTA activates tetracyclin inducible promoter. The blue fluorescent protein reporter is expressed under the control of 3.6 kb Col1a1 proximal promoter. This region contains bone and tendon tissue-specific elements. When the tenocytes are mature from the differentiation of the tendon stem/progenitor cells, the blue fluorescent protein will be turned on.

4.7 Conclusions

The two core issues for tendon repair are facilitating the restoration and reducing scar formation. The former may be addressed by overexpression of tendon transcription factors that favour the promotion of set tendon repair, while the latter requires reprogramming factors. My work has created assay-ready and controllable mouse lines for the above hypotheses and has characterized the function of each component through an identification strategy. However, detection of the expression profile of the mouse line insertion gene and the insertion site will need to be the focus of further work.

4.8 Outlook

The tendon repair process will be studied in TdAR-containing mice after injury to the Achilles tendon by hemi-dissection under different combinations of transcription factors induced by doxycycline. Changes in situ tendon cells will be observed during the inflammatory phase dominated by immune cell infiltration; the source and number of Scx-positive cells will be analyzed during the proliferative phase dominated by migration of tendon progenitor stem cells; the head and number of Col1a1-positive cells will be documented during the integration phase dominated by type III collagen secretion, and the restoration of scar and fibre alignment will be observed during the maturation phase dominated by tendon fibre maturation and scar resorption (Figure 66).

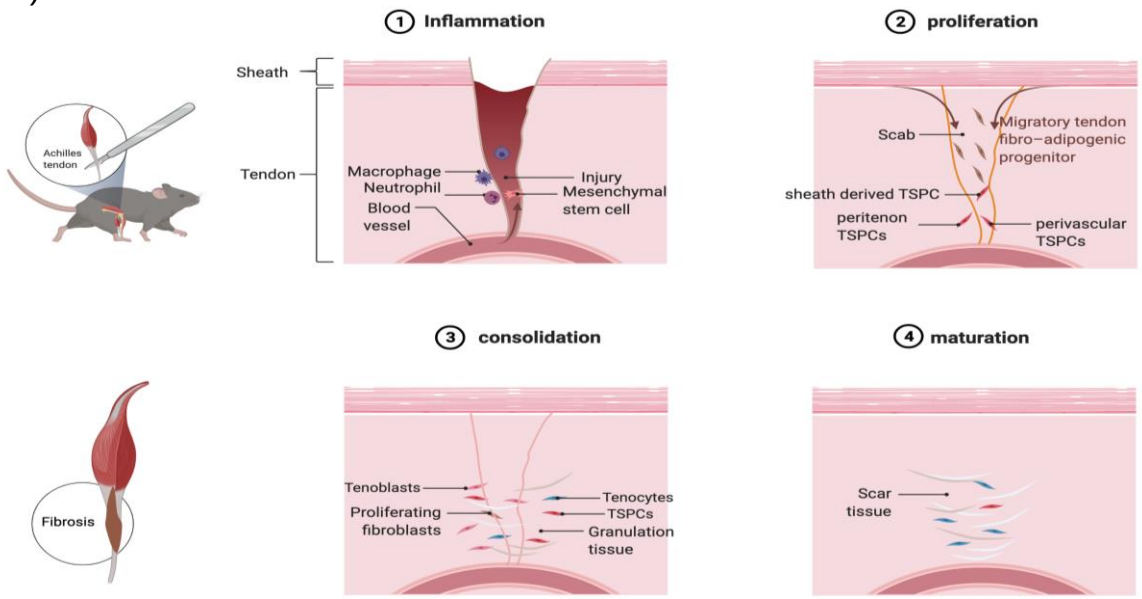

Figure 66. Anti-scarring and pro-repair research program in the TdAR mouse line.
Changes in the number and proportion of Scx-positive cells(red); Col1a1-positive cells(blue), and co-expressing cells(yellow) will be observed during the inflammation, proliferation, consolidation, and maturation phases after hemi-dissection of the Achilles tendon in the TdAR mouse line and will be assessed for scarification after repair.

Future studies will tell us about the role of different transcription factor combinations (Td4F/OSKM) on tendon repair and their mechanisms, laying the theoretical foundation for developing new clinical strategies to promote tendon repair and anti-scarring. The cells (Scx+/Col1a1+) that play a reparative function in the four tendon repair phases (inflammatory, proliferative, integrative, and mature) will be monitored based on a tendon reporter activator mouse line (TdAR). Transcriptomics will also be

performed to screen for therapeutic targets and to investigate repair mechanisms (Figure 67).

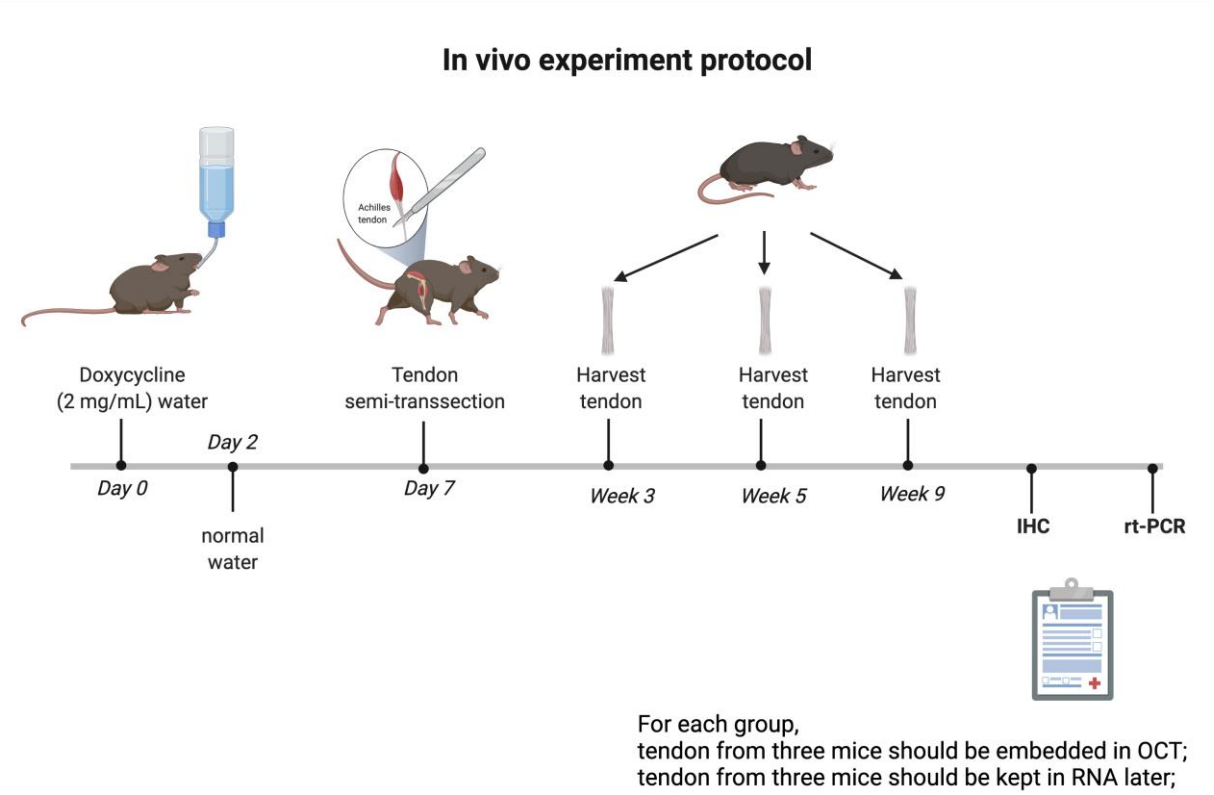

Figure 67. In vivo experiment protocol.
Mice 4-6, 10-12, and 24-26 weeks old will be experimented in vivo after drinking doxycycline-containing drinking water. The Achilles tendon injury will be modeled by a simple and reproducible model of partial transection of the Achilles tendon. Achilles tendon tissues will be taken at 2, 4, and 8 weeks post-operatively to analyze the effect of different transcription factors on tendon injury repair.

SUMMARY

Following injury, partial reprogramming of adult tendon cells by establishing a temporary and locally limited pluripotent state is a novel molecular therapeutic strategy to limit scar formation following injury and to promote regeneration of the affected tissues. Scaring and defective healing often occur after adult tissue injury, and their occurrence limits the regeneration and repair of affected tissues.

The basic principle of in vivo cellular reprogramming is to promote the proliferative capacity of reprogrammed cells through transformation/redifferentiation, resulting in the generation of precursor cells in the injured tissue that differentiate into the typical target cells of that tissue. Yamanaka factors generally achieve the de-differentiation process. Transcription factors that play an essential regulatory role in tissue development can facilitate the redifferentiation and trans-differentiation processes; or by transcription factors that can directly transform other cell types (stem cells or fibroblasts) into functional mature cells typical of the tissue.

After activation with doxycycline, endogenous Scleraxis-expressing (reported by Scarlet) tendon progenitor cells in the mouse line express Yamanaka factor (OSKM transcription factors: Oct3/4, Sox2, KLF4, and c-Myc). It resulted in a locally limited pluripotent state of the cells, increased proliferative capacity, and improved cellular status. The result is enhancing repair and reducing scarring. At the same time, the four most crucial transcription factors in embryonic tendon development, Td4F (Scleraxis, Mohawk Homeobox, Early growth response factor 1 and 2), are expressed in parallel with green fluorescent protein (EmGFP) anchoring cell fate, promoting efficiency of redifferentiation into target cells, and improving functional tissue repair. Expression of the mature tendon cell marker type I collagen (reported by blue fluorescent protein) is abundantly expressed and replaces type III collagen (a vital protein type for scar healing).

Accordingly, I intend to activate the Td4F-OSKM transcription factor in this mutant after experimental tendon injury. The aim is to induce OSKM factors in tendon lesions to reduce scar tissue formation and increase new tenocytes (tendon cells). I will also examine the extent to which OSKM-mediated partial reprogramming improves

the cellular state by the reporter protein Scarlet and the role of eight transcription factors in reducing in situ fibrosis by blue fluorescent protein, thus creating a basis that may allow tissue healing. For this reason, histological follow-up controls are performed to assess the pathology of the damaged tendon tissue. The study of the regulatory mechanisms of fibrosis, scarless repair, and embryonic tendon development validates the role of the transcription factors involved above for tendon repair.

It is essential to understand to what extent OSKM-mediated in situ partial reprogramming can reduce fibrosis and whether temporary and locally limited pluripotent state tendon cells can differentiate into mature tendon cells after overexpressing key transcription factors. I have established inducible transgenic mouse lines that specifically overexpress two classes of (dedifferentiated and re-differentiated) transcription factors and fluorescent reporters that eluminate Achilles tendon cell status by fluorescent proteins. The above work establishes the animal model and experimental platform for the second phase of the experiment.

ZUSAMMENFASSUNG

Die partielle Reprogrammierung von adulten Sehnenzellen nach einer Verletzung durch Etablierung eines temporären und lokal begrenzten pluripotenten Zustands ist eine neuartige zelluläre molekulare therapeutische Strategie, um die Narbenbildung nach einer Verletzung zu begrenzen und die Regeneration des betroffenen Gewebes zu fördern. Narbenbildung und fehlerhafte Heilung treten häufig nach Verletzungen von adultem Gewebe auf und ihr Auftreten begrenzt die Regeneration und Reparatur des betroffenen Gewebes.

Das Grundprinzip der zellulären Reprogrammierung in vivo besteht darin, die Proliferationsfähigkeit reprogrammierter Zellen durch Transformation/De-Differenzierung (Dedifferenzierung) zu fördern, was zur Bildung von Vorläuferzellen im verletzten Gewebe führt (Dedifferenzierung, Transdifferenzierung), die sich in die typischen Zielzellen dieses Gewebes differenzieren (Transdifferenzierung, Re-Differenzierung). Der Prozess der Dedifferenzierung wird in der Regel durch Yamanaka-Faktoren erreicht, und die Prozesse der Re- und Transdifferenzierung können durch Transkriptionsfaktoren erleichtert werden, die eine wichtige regulatorische Rolle bei der Entwicklung des Gewebes spielen, oder durch Transkriptionsfaktoren, die andere Zelltypen (Stammzellen oder Fibroblasten) direkt in funktionelle, reife, gewebetypische Zellen umwandeln können.

Nach Aktivierung mit Doxycyclin exprimieren endogene Scleraxis-exprimierende (von Scarlet berichtete) Achillessehnen-Vorläuferzellen in der Mauslinie Yamanaka-Faktoren (OSKM-Transkriptionsfaktoren), was zu einem lokal begrenzten pluripotenten Zustand der Zellen, einer gesteigerten Proliferationsfähigkeit und einem verbesserten zellulären Status (scheinbare Verjüngung) führt, wodurch die Reparatur verbessert und die Narbenbildung reduziert wird. Gleichzeitig werden die vier wichtigsten Transkriptionsfaktoren in der embryonalen Sehnenentwicklung, Td4F (Scleraxis, Mohawk Homeobox, Early growth response factor 1 und 2), parallel zu grün fluoreszierende Protein (EmGFP) exprimiert, wodurch das Zellschicksal eingeleitet, die Effizienz der Redifferenzierung in Zielzellen gefördert und die funktionelle Gewebereparatur verbessert wird. Die Nachbeobachtung wurde soweit fortgesetzt, bis reife Achillessehnenzellen den Marker Typ-I-Kollagen (angezeigt

durch blau fluoreszierende Protein) stark exprimierten und Typ-III-Kollagen, den wichtigsten Proteintyp bei der Narbenheilung, ersetzten.

Dementsprechend beabsichtigen wir, den Transkriptionsfaktor Td4F-OSKM in dieser Mutante nach experimenteller Sehnenverletzung zu aktivieren. Ziel ist es, OSKM-Faktoren in Sehnenläsionen zu induzieren, um die Bildung von Narbengewebe zu reduzieren, und Td4F-Faktoren, um die Differenzierung von Zellen in der Periwunde zu neuen Tenozyten (Sehnenzellen) zu fördern. Wir werden auch untersuchen, inwieweit die OSKM-vermittelte partielle Reprogrammierung den zellulären Zustand durch das Reporterprotein Scarlet verbessert und welche Rolle acht Transkriptionsfaktoren bei der Verringerung der In-situ-Fibrose durch blau fluoreszierende Protein spielen und damit eine Grundlage schaffen, die eine Gewebeheilung ermöglichen kann. Zu diesem Zweck werden histologische Nachkontrollen durchgeführt, um die Pathologie des geschädigten Sehnengewebes zu beurteilen. Diese werden durch die Untersuchung des Expressionsprofils der Fibrose und der embryonalen Sehnenentwicklung erreicht.

Um den oben genannten experimentellen Plan zu erreichen, ist es wichtig zu verstehen, inwieweit eine OSKM-vermittelte in situ Teilreprogrammierung die Fibrose reduzieren kann und ob temporäre und lokal begrenzte Achillessehnenzellen im pluripotenten Zustand durch Überexpression von Schlüsseltranskriptionsfaktoren in reife Achillessehnenzellen differenzieren können. Wir haben transgene Mauslinien mit induzierbarer Expression etabliert, die spezifisch zwei Klassen von (dedifferenzierten und re-differenzierten) Transkriptionsfaktoren überexprimieren, sowie transgene Mauslinien mit fluoreszierenden Reportergenen, die den Status von Achillessehnenzellen durch fluoreszierende Proteine anzeigen. Damit wurden die Tiermodelle und die experimentelle Plattform für die Phase-II-Experimente etabliert.

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-PUBLICATIONS-

LIST OF PUBLICATIONS BY THE CANDIDATE

1. **Chen R**, Zhou X, Skutella T. **A Tendon-Specific Double Reporter Transgenic Mouse Enables Tracking Cell Lineage and Functions Alteration In Vitro and In Vivo**. Int J Mol Sci. 2021, 22, 11189. doi: 10.3390/ijms222011189

2. **Chen R**, Thomas Skutella, **Synergistic anti-ageing through senescent cells specific reprogramming**. Cells. 2022, 11, 830. doi: 10.3390/cells11050830

3. **Chen R**, Sabine Conrad, Thomas Skutella, **Animal chimera for autologous organ transplantation, human animal chimeras for autologous organ transplantation**. 2022 (accepted)

This project was carried out independently by Rui chen under the supervision of Prof. Dr. Thomas Skutella and Dr. Xunlei Zhou. Microinjection and animal husbandry were done at IBF (The number of animals project G-305/19 permit is 624. Microinjection carried on in 15 mice. Newly generated 100 mice). Vector construction and sequencing were done by Rui Chen under the supervision of Dr. Xunlei Zhou. Genotypic identification and phenotypic determination of the animals was done independently by Rui Chen. which is the main result of this thesis.

Publication 1 is based on the results from the dissertation chapters 3.2.2.1 and 3.3. The discussion of the construction of TdAR transgenic mouse (chapter 4) was also mapped in this publication in terms of content. My own contribution to the publication extends to Conceptualization, R.C. and T.S.; methodology, R.C. and X.Z.; software, R.C.; validation, R.C.; formal analysis, R.C.; investigation, R.C.; resources, T.S., X.Z.; writing—original draft preparation, R.C.; writing—review and editing, R.C.; visualization, R.C.; supervision, T.S., X.Z.; project administration, T.S., X.Z.; funding acquisition, T.S.

Publication 2 and 3 are two review articles, the contents of which are briefly included in the discussion of this thesis in chapter 1. My own contribution to the publication comprised the literature research and almost all of the written text and schemes.

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