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Doctoral Thesis

ROLE OF NERVE-DERIVED CELLS OF NEURAL CREST ORIGIN IN SKIN REPAIR

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Summary

The skin is the largest organ of the human body and acts as the primordial barrier of the organism against the outside environment. In various occasions throughout life, acute injuries challenge the integrity of this frontline defence and trigger an immediate emergency response to establish a sealed environment and prevent blood loss and infection, but also slower, long-lasting repair mechanisms. The latter involve various cell types to restore, at least partly, the initial biological properties of the injured site. Endothelial cells, for example, participate in neovascularization that supports the increased proliferation of fibroblasts and keratinocytes. Skin is highly innervated with many sensory and autonomic nerve endings. And under normal circumstances hyperinnervation follows at the location of the injury. Among other disorders, peripheral neuropathies are known to severely impair wound healing capabilities of the skin, revealing the importance of skin innervation for proper repair. Here we report that peripheral glia are crucially involved in this process.

Using a mouse excisional wound healing model, in which the fate of genetically labelled peripheral glia can be followed during skin regeneration, we show that injury activates peripheral glia, thereby promoting de-differentiation of the cells, their re-entrance into the cell cycle, and dissemination from nerve bundles into the wound bed. Analysis of genetically traced cells demonstrated that injury-activated glia secrete a plethora of factors previously associated with wound healing and promote myofibroblast differentiation by paracrine modulation of TGF-β signalling. Accordingly, genetic depletion of these cells impairs epithelial proliferation and wound closure through contraction, while their expansion by genetic means promotes myofibroblast formation. Thus, injury-activated glia and/or their secretome might have therapeutic potential in human wound healing disorders.

Skin is also a source of different somatic stem cells. Here we characterize human skin dermis-derived cells, demonstrate that they possess mesenchymal stem cell features and differentiation potential. We have not observed formation of glial cells from endogenous dermis cells *in vivo*, however, their availability and differentiation potential, including ability to differentiate to Schwann cells *in vitro*, makes them good candidates for potential therapies and further research of glial cell involvement in wound healing.

Kopsavilkums

Ādas brūču dzīšana ir sarežģīts process, kura mērķis ir atjaunot ādas sākotnējo struktūru un funkcijas. Daudzi veselības traucējumi, tostarp perifērā neiropātija, var ietekmēt traumētas ādas atveseļošanas norisi. Šajā darbā mēs demonstrējam, ka perifēro nervu glijas šūnām ir svarīga nozīme ādas dzīšanas procesā.

Izmantojot peļu brūču dzīšanas modeli, kurā glijas šūnas ir ģenētiski iezīmētas, lai tās varētu izsekot ādas reģenerācijas laikā, mēs novērojām, ka ādas bojājums aktivē perifēro gliju, izraisot Švāna šūnu atgriešanos nediferencētā stāvoklī (dediferenciāciju) un migrāciju no nerva šķiedrām uz ievainotajiem audiem. Analizējot aktivētās glijas šūnas, mēs konstatējām, ka tās sekretē virkni faktoru, kam jau iepriekš novērota saistība ar brūču dzīšanu, un, aktivējot TGFβ signālceļu, tie veicina miofibroblastu veidošanos. Mēs arī novērojām, ka glijas šūnu skaita samazināšanās *Sox10* inaktivētā gēna pelēs kavēja jaunizveidotā ādas epitēlijslāņa augšanu un brūces aizvēršanos, vājinot brūces kontrakciju. Pretēji tam, glijas šūnu skaita pieaugums inaktivētā *Pten* gēna pelēs stimulēja miofibroblastu veidošanos brūcē. Tādējādi aktivētām glijas šūnām vai to izdalītiem faktoriem piemīt terapeitiskais potenciāls, ko varētu izmantot cilvēku ādas brūču dzīšanas uzlabošanai.

Āda ir dažāda veida cilmes šūnu avots. Šajā darbā mēs raksturojām no cilvēka dermas izdalītās šūnas un parādām, ka tām piemīt mezenhimālajām cilmes šūnām raksturīgās iezīmes un diferenciācijas potenciāls. Ņemot vērā ādas cilmes šūnu pieejamību un to spēju veidot Švāna šūnas *in vitro*, no dermas iegūtās mezenhimālās cilmes šūnas būtu labs kandidāts šūnu terapijai vai turpmākiem pētījumiem par glijas šūnu ietekmi uz brūču dzīšanu.

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Abbreviations

α-SMA – smooth muscle actin alpha

ANS – autonomic nervous system

CGRP – calcitonin gene related protein

cKO – conditional knockout

CNS – central nervous system

DRG – dorsal root ganglion

ECM – extracellular matrix

EMT – epithelial to mesenchymal transition

ENS – enteric nervous system

GFAP – glial fibrillary acidic protein

HF – hair follicle

NB – nerve bundle

NCSC – neural crest stem cells

NGF – nerve growth factor

NMJ – neuromuscular junction

NMSC – non-myelinating Schwann cells

LNGFR (p75, CD271) – low-affinity nerve growth factor receptor

PNS – peripheral nervous system

PSC – perisynaptic Schwann cells

SCP – Schwann cell precursor

SKP – Skin derived precursor cells

SNS – somatic nervous system

TGF-β – transforming growth factor β

Goals and objectives of the thesis

Aim of the thesis was to determine the potential role of neural crest-derived nerve associated cells in the adult skin repair process and to isolate, characterize and study differentiation of adult human dermis-derived cells.

Objectives

1. To follow the fate of neural crest-derived glial cells in normal and injured skin using genetic tracer in *Plp-Cre* and *Dhh-Cre* mice
2. To characterise neural crest derived cells in non-injured skin and in the wound *in situ* by performing immunolabeling
3. To design gain and loss-of-function situations where the pool of neural crest-derived cells in the wound bed could be genetically depleted or expanded and analyse the effect it has on wound healing process
4. To determine the differentiation potential of human dermis-derived cell towards mesenchymal and neuroectodermal lineages *in vitro*

1. Literature overview

1.1 Stem cells

Stem cells can be found in most of the mammalian tissues where they ensure that turnover of cells and homeostasis is maintained throughout life. They are traditionally described as cells that can self-renew for longer periods of time and in certain conditions are able to give rise to different cell types (Weissman 2000).

Differentiation ability of stem cells is at the basis of notions such as plasticity and potency. At the different developmental stages cells with different potency can be found throughout the organism (Fig. 1.1). Totipotent cells (latin.: *tōtus* – everything) are found at the very early developmental stages, when embryo consists of up to 4 cells. These cells are able to give rise to more than 200 distinct cell types to form all tissues in the body. Around the 8-cell embryo stage totipotency is lost and first differentiation occurs giving rise to extra-embryonic trophectoderm and pluripotent inner cell mass. Pluripotent cells (latin: *plurimus* – very many) are defined as being able to give rise to all cell and tissue types except early embryonic structures and thus cannot give rise to new animal. They can be found among the cells in the inner cell mass (Mitalipov & Wolf 2009). Multipotent stem cells possess more limited differentiation potential and can give rise to different cell types within one germ layer as well as identical daughter stem cell. These are, for example, tissue-specific cells like hematopoietic stem cells (HSCs) (Seita & Weissman 2010).

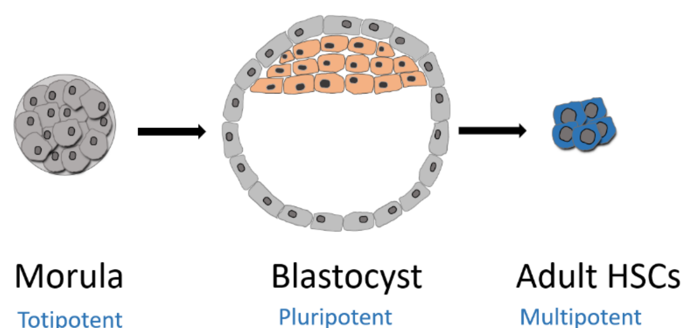

Figure 1.1 Stem cell potential. Depending on the age **s**tem cells with variable differentiation potential exist in the body.

1.1.1 Embryonic and induced pluripotent stem cells

The existence of multipotent stem cells was demonstrated in pioneering work by Till and McCulloch (Becker et al. 1963) and later by Friedenstein (Friedenstein 1970). Mouse pluripotent embryonic stem cells (ESCs) from the inner cell mass have been first isolated and cultured in 1981 (Evans & Kaufman 1981; Martin 1981), while human ESCs cultures where first established in 1998 (Thomson et al. 1998) and later shown to maintain pluripotency for a long time period also when clonally-derived (Amit et al. 2000).

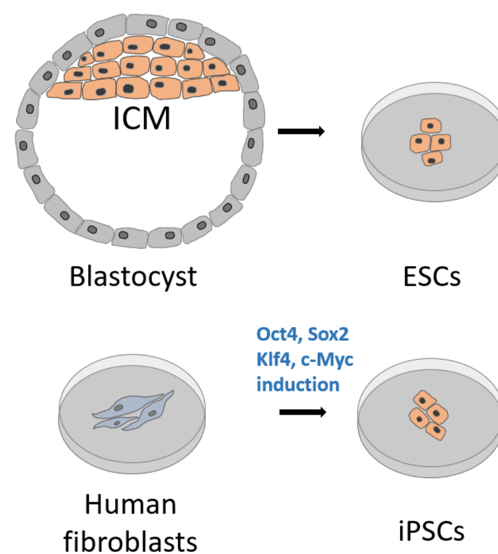

Figure 1.2 ESCs and iPSCs. Cells with features similar to ESCs can be induced from somatic cells.

ESCs provided great insights into the process of cell differentiation and embryonic development and, given their ability to give rise to all tissue types, have also been considered as promising source for regenerative medicine. The advance of ESCs into the clinics, however, has been hindered by safety issues related to spontaneous de-differentiation and teratoma formation (Murry & Keller 2008), in fact, the emergence of ESC biology itself is related to the studies on teratocarcinomas and embryonal carcinoma cells (Martello & Smith 2014). Immune rejection, differentiated cell purification and ethical problems in some countries are among other issues holding back the introduction of ESCs into clinics (Murry & Keller 2008; Puri & Nagy 2012).

Nobel Prize-winning work by Yamanaka and colleagues has revolutionized the stem cell field by demonstrating that overexpression of transcription factors Oct4, Sox2 Klf4 and c-Myc can reprogram adult mouse and human fibroblasts into pluripotent stem cells with differentiation

potential similar to ESCs (Fig. 1.2) (Takahashi & Yamanaka 2006; Takahashi et al. 2007). These cells termed induced pluripotent stem cells (iPSC) provided alternative to ESCs solving ethical and immune rejection problems and opening new possibilities for personalized medicine, drug screening and disease modelling (Takahashi & Yamanaka 2013; Zeltner & Studer 2015). Since iPSCs can be derived from patient fibroblasts, this offers opportunities to study genetic disorders including rare diseases for which there are currently no animal models. Modelling of monogenic diseases, such as spinal muscular atrophy using patient-derived iPSCs was achieved first (Ebert et al. 2009). Recent reports demonstrate that even complex and multifactorial psychiatric disorders, such as schizophrenia can be modelled (Brennand et al. 2011).

Despite previously mentioned issues, both ESCs and iPSCs preparations are used in clinical trials. So far, the most success has been achieved in treatment of eye disorders. Several ongoing trials using ESCs, patient-derived and allogenic iPSCs-based products for treatment of age-related macular degeneration and Stargardt disease are under way. Two follow-up studies reported improved vision acuity in most of the patients treated for macular degeneration (Schwartz et al. 2014; Song et al. 2015). Patients are being recruited for other trials using pluripotent stem cell products dealing with conditions like heart failure, type 1 diabetes and spinal cord injury, while many more trials for other diseases are in development (Kimbrel & Lanza 2015).

1.1.2 Tissue-resident stem cells and regeneration

A whole different approach is studying tissue-resident stem cells such as adult stem cells. These cells exist in the body as constantly active cells, for example in the epidermis and bone marrow or in a dormant state and can only be activated periodically or upon injury (Hsu & Fuchs 2012). It is now accepted that stem cells in the body exist in microenvironments called niches, which was first postulated for HSCs (Schofield 1978). Niches are dynamic systems that can instruct stem cells by combining signals from neighbouring cells, ECM substrate, inflammatory cells, innervation and other sources. A major problem is associated with precise identification of stem cell populations *in vivo* and is further complicated by the fact that stem cells in the niche don't represent homogeneous population, however, during the past years routine use of *in vivo* tracing techniques has advanced the field (Schroeder 2008; Kretzschmar & Watt 2012).

Understanding how stem cells behave within the niche and which signals send and respond to might open up possibilities to manipulate these cells directly or through niche modification *in vivo*. In ideal situation this could help to reverse pathology or promote endogenous regeneration potential (Morrison & Spradling 2008; Lane et al. 2014).

Regeneration is needed to replace the lost tissues and thus can be a result of proliferation of existing differentiated cells, as in liver regeneration, activation of intrinsic tissue stem cells as in the case of blood formation or transient de-differentiation of cells as in the peripheral nerve injury (Jopling et al. 2011). Many of the things we know about regeneration come from the studies of animals that have significant endogenous regeneration capacity like planarians and amphibians. Some planarians are unique in their ability to regenerate the whole organism from a small original part (Reddien & Sánchez Alvarado 2004), while some amphibians like xenopus frogs and salamanders are able to regenerate whole limbs and other organs including brain, heart and the eye (Godwin & Rosenthal 2014). Regeneration of the limbs in amphibians can be divided in several stages – wound healing, activation of tissue progenitors and formation of new limb, a process highly reminiscent of original development. Second stage – activation of progenitors, depending on the site of regeneration, can go one or several different ways including cell de-differentiation, transdifferentiation and reprogramming (Jopling et al. 2011). It can be influenced by immune system and other cells or signals coming, for example, from the nerve (Kumar & Brockes 2012). Progenitor cells that would form different limb tissues during regeneration are located in a special zone called blastema. Cells within the blastema seem to be morphologically homogeneous, which previously led to assumption that these cells could be pluripotent. Experiments where different cell types were tracked in the regenerating salamander limbs proved, however, that blastemal cells represent a set of restricted tissue progenitors that “remember” their tissue of origin and in the case of cartilage progenitors even maintain positional memory (Kragl et al. 2009).

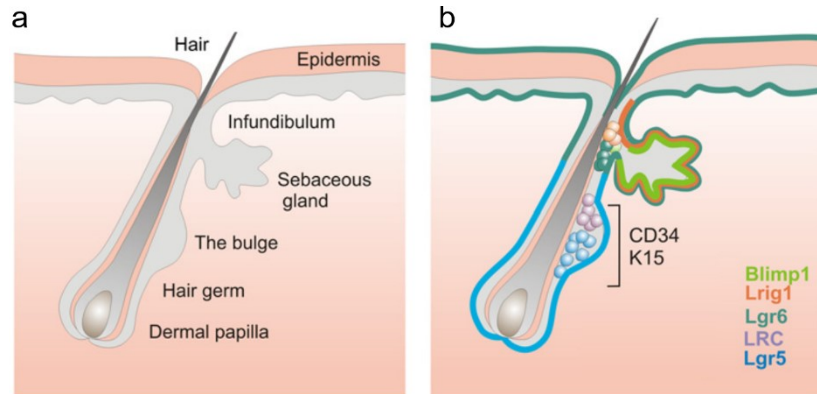

Figure 1.3 Tissue resident cells in the skin that ensure constant epidermal and sebaceous tissue turnover. (**a**) Adult hair follicle structure. (**b**) Different stem cells of the hair follicle and their distinct markers. Adapted from Barker et al. 2010.

Mammals including humans also possess limited ability to regenerate and some regeneration processes such as replacement of the lost bone, skin keratinocytes, and gut epithelia as well as regeneration in the brain to certain extent are constantly ongoing (Fig 1.3) (Barker et al. 2010; Poss 2010). Wounds in humans at the early foetal stages heal by completely restoring the original tissue, this ability is lost with the onset of immune system and wounding leads to scar-forming healing (Larson et al. 2010). Interestingly, also in xenopus frogs this regeneration capacity is inversely-correlated with the maturity of immune system (Godwin & Brockes 2006). Apart from that, there are several prominent examples of injury-triggered tissue regeneration in adult mammals, for example, yearly deer antler regeneration (Price et al. 2005), de-novo hair follicle formation after large skin wounds in mice and rabbit (Ito et al. 2007), complete regeneration of skin in certain rodent species after skin shedding (A. W. Seifert et al. 2012), ear punch regeneration, first described in rabbits and later in other lagomorph and rodent models like MRL/MpJ mice (Clark et al. 1998; Metcalfe et al. 2006) and the generation of mouse digit tip after amputation (Borgens 1982). Spontaneous regeneration of human digit tips has also been documented in several cases both in children and adults (Illingworth 1974; Lee et al. 1995; Rinkevich et al. 2015).

Digit tip regeneration model has lately received particular attention with several groups identifying important components for the regeneration (Han et al. 2008; Fernando et al. 2011; Rinkevich et al. 2011). During this process a structure similar to lower vertebrate blastema is formed. Takeo and colleagues have identified nail stem cells to be important drivers of this process. After the amputation of the finger at the level of the terminal phalange but distal from

the nail base, nail stem cells get activated in a Wnt-dependent manner and new nail is being formed with differentiation gradient starting from the base of the nail. Newly-forming nail acts in a similar manner as blastemal epidermis in lower vertebrates and signals to the blastema, attracts nerves by secreting nerve-guiding cues and nerves in turn provide FGF-2 for proliferating mesenchymal blastemal cells (Takeo et al. 2013) Intriguingly, this study pointed at the importance of innervation in mammalian regeneration, something that has been long observed for amphibians, where the extent of innervation can be directly correlated with regeneration ability (Endo et al. 2004; Kumar & Brockes 2012). This finding was further confirmed by denervation experiments in the same model, where loss of innervation led to disturbed patterning of the bone and nail (Rinkevich et al. 2014).

Moreover, a role for neural crest-derived cellular component of the nerve – Schwann cell has recently been shown in regeneration process. In salamander wounds Schwann cells have been demonstrated to separate from nerves, migrate into blastema and secrete newt anterior gradient protein (nAG) that mediates blastemal epithelium formation (Kumar et al. 2007). A recent study showed that Schwann cells upon digit amputation in mice are found within the blastema and secrete factors – PDGF-AA and Oncostatin M that promote mesenchymal linage proliferation and that digit regeneration in the denervated limb can be rescued to a certain extent by rat Schwann cell precursor transplantation or by the addition of the mentioned factors (Johnston et al. 2016).

1.2 Neural crest and its derivatives

1.2.1 Origins of the neural crest. Neural crest stem cells

Neural crest is a transient embryonic population of precursor cells unique to vertebrates that was first identified by Swiss anatomist Wilhelm His in 1868. Even though it is derived from the ectoderm, for its uniqueness and importance it is often called the fourth germ layer. Appearance of neural crest has dramatically influenced evolutionary development of chordate and is at the basis of genesis of such structures as vertebrate craniofacial skeleton and PNS (Green et al. 2015). It forms at the end of gastrulation and during neurulation and is located on the dorsal neural primordium on the border with non-neural ectoderm. Neural crest specification is induced by combination of Wnt, BMP and FGF signalling and neural crest

marker expression can be detected across (Jacob 2015). Responding to the activating signals neural crest undergoes the process of epithelial to mesenchymal transition (EMT) during which neural crest cells get activated, are individualized and leave the neural tube to settle in virtually all organs of the body (Sauka-Spengler & Bronner-Fraser 2008). Neural crest is not a uniform structure and, based on the position in the rostro-caudal axis, it is subdivided into cranial, cardiac, vagal and trunk neural crest. Depending on the compartment in which neural crest cells are located they migrate to different parts of the body giving rise to different progeny including cartilage and bones of the head, different types of glia, neurons, and fibroblasts of autonomous, sensory and enteric parts of the PNS, melanocytes, smooth muscle cells and glandular cells (Fig. 1.4) (reviewed in Dupin & Sommer 2012).

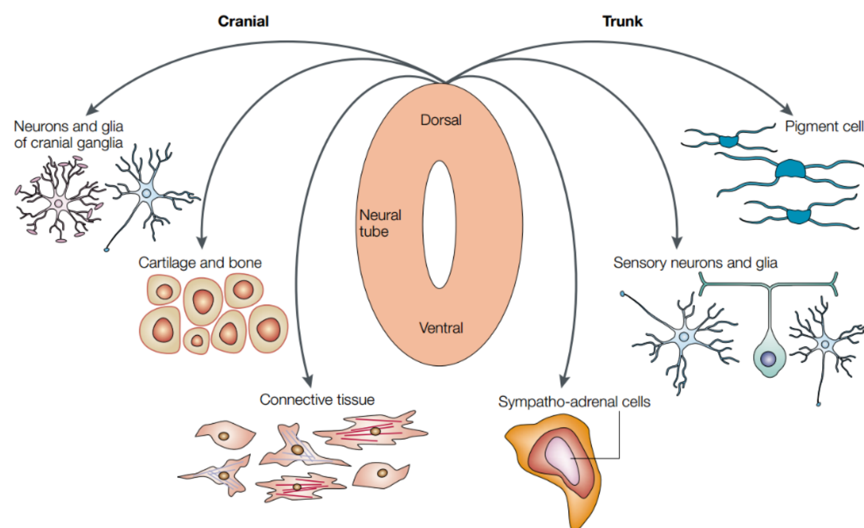

Figure 1.4 Neural crest cells and their progeny. Neural crest cells are multipotent, but differences exist in the progeny they give rise to depending on axial localization in the structure. Adapted from Knecht & Bronner-Fraser 2002.

It was long debated whether neural crest cells in these compartments represent heterogeneous intrinsically-restricted populations predisposed to give rise to certain progeny or they behave according to environmental cues and are in essence multipotent cells. Stem cell nature of migrating neural crest cells has been shown *in vivo* first in chimeras (Couly & Le Douarin 1985) and later in tracing experiments of individual cells in avian models (Bronner-Fraser & Fraser 1989). Neural crest cells isolated based on surface marker low-affinity nerve growth factor receptor (LNGFR or p75) were shown to be multipotent *in vitro* and termed neural crest stem cells (NCSC) (Stemple & Anderson 1992). Later elegant tracing experiments proved the

multipotency of migrating trunk and cranial neural crest cells in mice (Baggiolini et al. 2015; Kaucka et al. 2016).

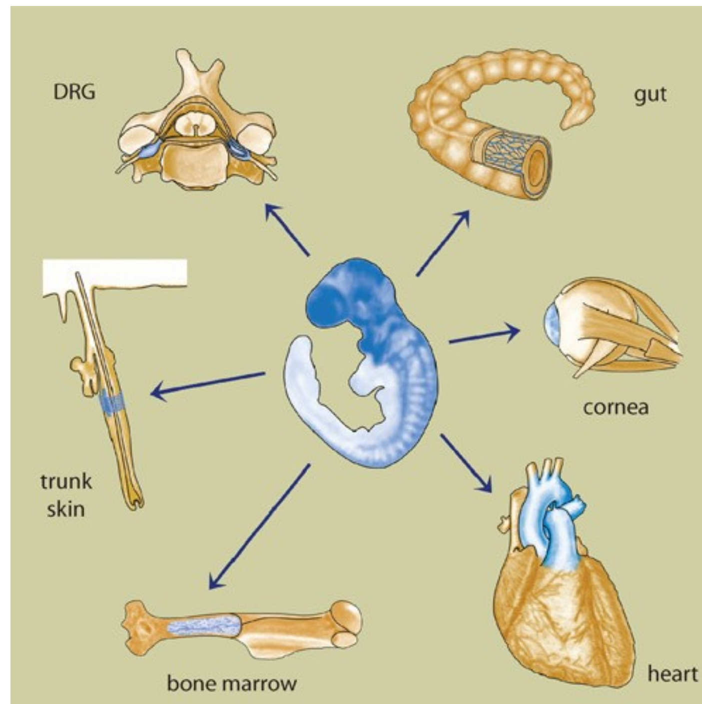

Figure 1.5. NCSC-like cells in the adult. Cells with self-renewal capacity similar to NCSC can be isolated from various adult organs, including skin, gut, DRG, cornea, bone marrow and heart. Adapted from Shakhova & Sommer 2010.

Another long-standing question was whether NCSC are only transiently present in the embryo and irreversibly lose plasticity with time or they also persist after migration and in the adult and could potentially contribute to tissue regeneration. More than 15 years ago post-migratory NCSC were first isolated from foetal peripheral nerve, which was prior considered to contain only Schwann cell precursors. These cells were sorted based on $p75^{+}/P0^{-}$ phenotype, they proliferated *in vivo* and, when transplanted, could give rise to both neurons and glia. This demonstrated the persistence of neural stem cells in the PNS to late embryonic stages (Morrison et al. 1999). Later it was demonstrated that NCSC-like cells could be isolated from the adult gut and *in vitro* as well as *in vivo* after injection into chick embryos displayed differentiation potential similar to the same cells isolated from the foetal gut (Kruger et al. 2002). Since then cells with NCSC properties displaying intrinsic differences in their differentiation potential have been isolated from other adult tissues including bone marrow, heart, dorsal root ganglion (DRG), cornea, tooth and carotid body (Fig. 1.5) (Shakhova & Sommer 2010). Since multipotent neural crest cells are a transient embryonic population, a definition question exists and there is no consensus whether to refer to these adult-derived cells as to NCSC or progenitor

cells. In many cases it remains unclear whether displayed differentiation potential of isolated NCSC/progenitor cells is an *in vitro* culture phenomenon of unmasked plasticity, which asks to be properly demonstrated *in vitro* and further analysed *in vivo*. (Achilleos & Trainor 2012).

1.2.2 NCSC-like cells in the peripheral nervous system

The neural tube forms the central nervous system (CNS), while neural crest cells that are located at the dorsal-most part of the neural tube, migrate to give rise to most of the peripheral nervous system (PNS). At cranial level of the neural crest PNS originates from both neural crest cells and ectodermal placodes, further down all the PNS is formed by neural crest. At vagal level neural crest cells form parasympathetic innervation of the heart and all the enteric nervous system, while trunk neural crest cells located caudal to seventh somite originate major part of the PNS including sensory and autonomic neurons and glia (Butler & Bronner 2015).

PNS, a common feature of all jawed vertebrates (Green et al. 2015) is an extensive structure consisting of ganglia and nerves outside of the CNS reaching to all distant organs. PNS functions to convey information form peripheral tissues to the CNS and ensure proper control over limbs and organs. PNS is usually divided into somatic nervous system (SNS), autonomic nervous system (ANS) and ENS. Somatic nervous system is mainly associated with voluntary control over body movement. It consists of nerves carrying information from sensory receptors to the CNS (afferent nerves) and motor neurons carrying information from CNS to effector organs like muscles (efferent nerves). ANS is associated with non-voluntary control over internal organ functions such as heart rate and breathing. ANS is subdivided into quick response sympathetic branch responsible for 'fight-or-flight' system and slower response parasympathetic responsible for 'rest and digest' system. ENS is another branch of PNS responsible for the function of gastrointestinal system. ANS is highly sophisticated and contains interneurons, harbours intrinsic microcircuits and is able of autonomous function independent of the CNS (Gershon & Ratcliffe 2004). The fact that PNS similar to vasculature reaches to all body parts and recent *in vivo* tracing observations allowed to propose non-canonical function of PNS nerves as a source of plastic progenitor cells potentially important in regeneration and repair (Kaucká & Adameyko 2014).

As mentioned previously post-migratory p75^{+}/P0^{-} NCSC-like cells were first isolated from foetal peripheral nerve (Morrison et al. 1999). Another study published the same year showed that non-neural P0^{+}/PMP-22^{+} cells from embryonic rat DRG were multipotent and could form neurons glia and smooth muscle cells (Hagedorn et al. 1999). In 2002 NCSC-like cells with *in vivo* neurogenic and glial potential were first isolated from the adult gut (Kruger et al. 2002) and in the same Neuron issue same group demonstrated that cells isolated from different parts of the foetal PNS exhibit cell-intrinsic differences when transplanted to *in vivo* structures – gut NCSC from the ENS preferentially differentiated to neurons, while sciatic nerve NCSC give rise exclusively to glia (Bixby et al. 2002). Confirming *in vitro* data on existence of cells displaying NCSC properties in the ENS, neural crest derivatives in the gut have been shown to exhibit neurogenesis also *in vivo* when stimulated with 5-HT_4 antagonists (Liu et al. 2009).

It is not known whether NCSC exist in adult sciatic nerves. Unlike from foetal nerves (Morrison et al. 1999), spheres could not be grown from dissociated adult sciatic nerves (Toma et al. 2001; Wong et al. 2006), but could be established from a distal part of a crushed nerve (Takagi et al. 2011), suggesting that sphere-forming cells might reside in the nerve endings, but not sciatic nerves or could be enriched for upon injury. In recent years the remarkable plasticity of neural crest-derived Schwann cell precursors (SCPs) became evident. It was first shown in tracing experiments with *Dhh-Cre* animals that SCPs apart from different Schwann cell types give rise to other cell type found within the nerve – endoneurial fibroblast (Joseph 2004). During development they give rise to melanoblasts, which arrive with nerves to distant locations in the skin and develop into melanocytes. This switch of SCPs to ventrally migrating melanoblasts is thought to occur before embryonic day (E) 12.5 and thus using tracers active in Schwann cells past this point, such as *Dhh-Cre* does not lead to traced melanocytes. Surprisingly, injury to adult sciatic nerve yields formation of ectopic pigmented melanocytes around the crushed nerve, suggesting unstable and plastic identity of Schwann cells in the adult nerves (Adameyko et al. 2009; Ernfors 2010).

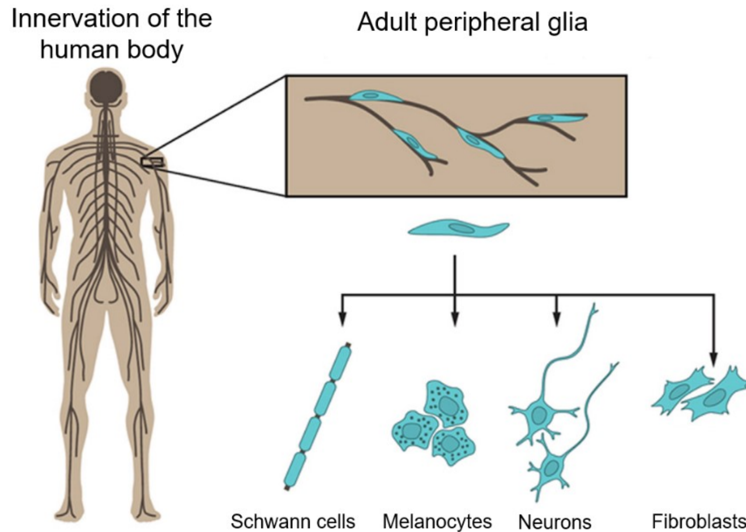

Figure 1.6. Plasticity of peripheral glia. Both embryonic SCPs and adult glial cells in certain conditions exhibit remarkable plasticity and form different cell types. Adapted from Kaucká & Adameyko 2014.

Apart from mature Schwann cells, endoneurial fibroblasts (Joseph 2004) and melanocytes (Adameyko et al. 2009; Adameyko et al. 2012) arising from embryonic SCPs, cells expressing markers characteristic to SCP have been shown to give rise to parasympathetic neurons (Dyachuk et al. 2014; Espinosa-Medina et al. 2014) and, alongside enteric neural crest cells, perform neurogenesis in the adult gut (Uesaka et al. 2015). Glial cells from cranial nerves are also a source of mesenchymal progenitor cells in the tooth (Kaukua et al. 2014). Recently several groups demonstrated that environmental insult can lead to peripheral glia plasticity *in vivo* – melanocytes (Adameyko et al. 2009), neurons (Pardal et al. 2007; Laranjeira et al. 2011) and mesenchymal cells (Kaukua et al. 2014) have all been demonstrated to arise from cells expressing SCP markers (Fig 1.6). This injury induced plasticity can be beneficial in regeneration (Mirsky et al. 2008), but, on the other hand, can relieve tumour-suppressive properties of the nerve in cooperation with neurofibromin loss and promote neurofibroma formation (Ribeiro et al. 2013). Taking together, the accumulating evidence suggests that plastic SCP-like cells could be the likely candidates for adult NCSC role.

1.2.3 Schwann cell origins and biology

Schwann cells originate from the neural crest and represent the majority (~90%) of nucleated cells in the peripheral nerves. Embryonic gliogenesis happens in two waves. First, distal peripheral nerve SCPs, satellite cells and some ventral root SCPs differentiate directly from neural crest cells. A second wave of gliogenesis comes from boundary cap cells, a population of glial cells derived from neural crest cells. Boundary cap cells give rise to all the dorsal root SCPs, fraction of ventral root SCPs and satellite cells (Fig. 1.7) (Jacob 2015). Gradual Schwann cell differentiation during development and into adulthood has been well described (Jessen & Mirsky 2005; Woodhoo & Sommer 2008). Migrating neural crest cells in mice at around E12 establish connection with the axons and form SCP, which later, at approximately E15, develop into immature Schwann cells, that in turn at birth become either myelinating Schwann cell or a non-myelinating Schwann cell (NMSC) (Mirsky et al. 2008; Woodhoo & Sommer 2008).

Adult myelinating Schwann cells ensure large nerve fibre myelination, whereas NMSC surround small umyelinated axons. Around 75% of axons in the skin and most of the postganglionic axons are unmyelinated (Geuna et al. 2009). Axons provide important factors that control Schwann cell development, survival and myelination process, such as neuregulin 1 and Notch signaling ligands (Birchmeier & Nave 2008; Woodhoo et al. 2009). A small subset of NMSC cells called perisynaptic Schwann cells (PSC) is found at the neuromuscular junction (NMJ), where PSC participate in synapsis formation and remodelling (Ko & Robitaille 2015).

Mechanisms regulating neural crest cell fate decision towards glial lineage are gradually being understood. Persistent expression of transcription factor Sox10 is required for Schwann cell specification and maintenance (Britsch et al. 2001). Sox10 is important for upregulation of Neuregulin-1 receptor ErbB3, whereas neuregulin-1 signaling through ErbB3 suppresses neural crest cell differentiation towards other linages. Notch is another glial fate-promoting signalling that can override neuronal differentiation-promoting BMP-2 signals (Jessen & Mirsky 2005; Jacob 2015). Adameyko et al. showed that in neural crest ventral migratory pathway SCPs arise as a result of lack of neuronal specification by Hmx1 homeobox gene and that these cells are bipotent and can give rise to melanocytes (Adameyko et al. 2009). Maintenance of FoxD3 expression was correlated with glial fate and identified as regulating the switch between glial vs. pigment and neuronal fates (Thomas & Erickson 2009; Nitzan et al. 2013). Recently histone deacetylases HDAC1 and HDAC2 have been shown to be upstream of Sox10, interacting with it to promote *Pax3* expression, which in turn leads to higher Sox10

levels and FABP7 and P0 expression. Genetic ablation of HDACs results in loss of SCP and satellite cells (Jacob et al. 2014).

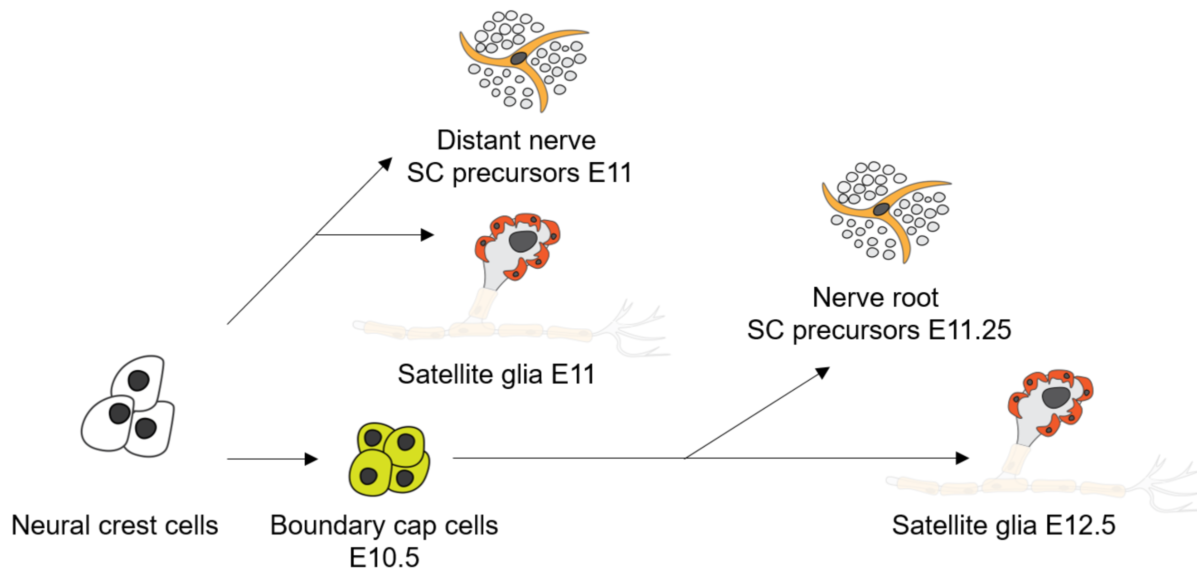

Figure 1.7. PNS gliogenesis. Distant nerve Schwann cells and part of satellite glia arise directly from neural crest. Nerve root Schwann cells and part of satellite glial cells arise from intermediate progenitors – boundary cap cells. Adapted from Jacob 2015.

SCPs are found in the early embryonic nerves and express several common markers with NCSC like Sox10, p75 and ErbB3. SCPs can be distinguished by expression of BFABP, Cadherin 19 and other markers, as well as their dependence on the axon-derived neuregulin 1 type III. SCPs disappearance from the developing nerves coincides with the appearance of immature Schwann cells and endoneurial fibroblasts, which are also SCP-derived. (Jessen et al. 2015).

Immature Schwan cells can be distinguished from SCPs by absence of markers such as BFABP and Cadherin 19 and expression of more mature Schwann cell markers, for example glial fibrillary acidic protein (GFAP), S100B and Oct6. Immature Schwann cells participate in radial axon sorting prior to their myelination. At their later developing stages and after nerve injury Schwann cells are less dependent on axons and can initially survive and proliferate without neuregulin 1 thanks to autocrine ligands, such as neurotrophin 3 (NT3), PDGF-B, insulin growth factor 2 (IGF2) and leukemia inhibitory factor (LIF). This axon independence becomes particularly important after nerve injury (Fig.1.8) (Jessen et al. 2015; Fricker et al. 2013).

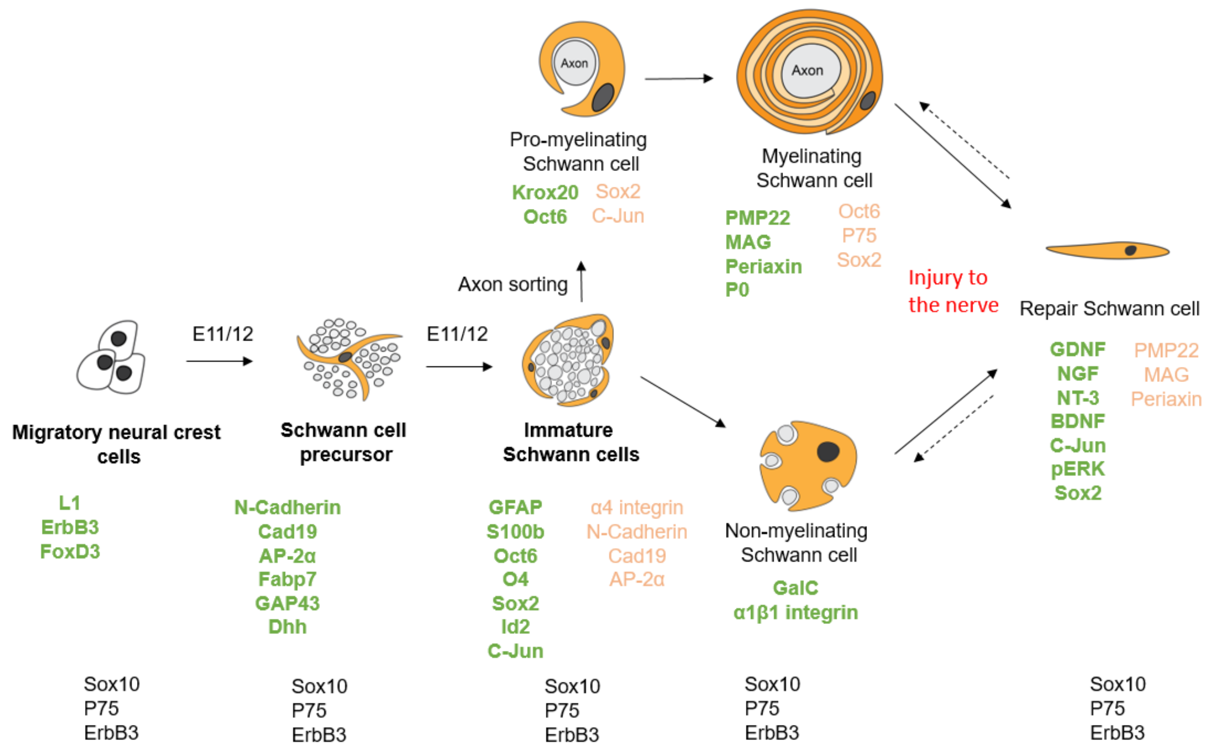

Figure 1.8. Development of Schwann cell lineage. Schwann cells arise from neural crest cells and eventually for mature myelinating of non-myelinating Schwann cells. Injury can trigger de-differentiation of mature Schwann cells and formation of repair cell. Several markers that help to identify different stage Schwann cells are shown. Upregulated markers are shown in green, some of those that are known to be downregulated compared to previous Schwann cell state are shown in orange. Sox10, p75 are ErbB3 are found on Schwann cells through lineage progression. Based on Mirsky, Jessen et al.

Of note, not all Schwann cells of the PNS follow the same rules and can be identified by the same markers. For example, a subset of Schwann cells found in the DRG and on the border between PNS and CNS called boundary cap cells express S100 earlier than Schwann cells of the spinal nerves. Boundary cap cells can be identified by their expression of Krox20 (*Egr2*), a transcription factor that in other Schwann cells is found before the onset and during myelination (Maro et al. 2004; Jessen & Mirsky 2005).

1.2.4 Schwann cells after nerve injury

Unlike the CNS, PNS possesses significant regeneration potential. Two models of nerve injury are often used. Peripheral nerve crush preserves basal lamina tubes of the nerve, and results in rapid regeneration and restoration of the function. Nerve cut, depending on the size of the gap leads to formation of the tissue bridge that connects both ends of the nerve and usually results in insufficient functional recovery (Jessen et al. 2015). In both injury scenarios a complex process called Wallerian degeneration that aims at restoring the structure and function of the nerve takes place. At the proximal stump axons first degenerate and retract and within hours after injury sprouting of new axons starts (Geuna et al. 2009). At the site distal to the injury, where axons die and at the tip of a proximal stump Schwann cells and go through a process commonly referred to as de-differentiation to eventually form bands of Bungner and help to guide newly formed axon sprouts. As a result of this changes Schwann cells first lose their myelinating ability and acquire a phenotype more resembling immature Schwann cell (Mirsky et al. 2008).

Recent reports involving sequencing of distal stump of the injured nerve rather suggest that the cell type formed during repair process is different from Schwann cells found during any of the embryonic stages and thus a process of trans-differentiation or adaptive reprogramming takes place which results in formation of a repair cell called Bungner cell (Arthur-Farraj et al. 2012; Fontana et al. 2012). Key regulators of this process are ERK-signaling pathway and transcriptional factor c-Jun (Napoli et al. 2012; Arthur-Farraj et al. 2012) Apart from axon guiding activities, Schwann cells at the injury site initiate myelin clearance by autophagy and recruit macrophages to help clear the debris (Gomez-Sanchez et al. 2015; Tofaris et al. 2002). It is also suggested that depending on the location and time after the injury several “activated” cell types may exist (Jessen et al. 2015) and that response of myelinating Schwann cells and NMSC to the injury can be different (Griffin & Thompson 2008). For example, NMSC start proliferating even if the neighbouring myelinated nerve is damaged (Murinson et al. 2005).

1.3 Skin innervation, structure and functions

One organ that harbours numerous PNS extensions is the skin. Human skin is often regarded as the largest body organ. It is a complicated structure that has many functions and possesses

remarkable renewal capacity (Kanitakis 2002). Skin protects us from the insults such as radiation, injury and infection, enables thermoregulation and serves as a water barrier. It is also a crucial member of a somatosensory system that allows us to perceive external stimuli. Classical cutaneous senses include the sensing of touch, temperature, pain, itchiness (McGlone & Reilly 2010) as well as so-called affective touch (Lloyd et al. 2015). All these diverse functions are possible due to highly organized and specialized structure of the skin comprising different cell and tissue types (Fig. 1.9). Even though skin is not a uniform organ with many regional differences, usually it is structurally subdivided in three parts – epidermis, dermis and hypodermis (Kanitakis 2002).

1.3.1 Innervation of the skin

Skin is one of the most densely innervated organs. Major nerve bundles in the skin are located on the border between epidermis and dermis, dermis and subcutis and next to the muscle layer found in the skin (panniculus carnosus). Nerve endings stemming from these bundles (often unmyelinated and lacking Schwann cells) are found throughout the skin terminating freely in the epidermis and dermis as well as around structures such as hair follicles and blood vessels. Nerves in the skin are part of the PNS and are classically divided in afferent responsible for sensations like touch itch and pain and efferent governing smooth muscle contraction and gland function (Hendrix et al. 2008).

Apart from these functions axons found in the nerve bundles in the skin are also a source of non-synaptically released neuropeptides such as substance P (SP), calcitonine gene-related peptide (CGRP) etc. (Peters et al. 2006). These neuropeptides in turn can regulate several processes in the skin including hair cycle, wound healing and inflammation (Peters et al. 2001; Cruise et al. 2004; Chéret et al. 2013; da Silva et al. 2010). Levels of neuropeptides, neurotrophins and other regulatory nerve derived factors dramatically increase within the skin after nerve stimulation that can be caused by physical, chemical or other stimuli like trauma, allergens or inflammation. Neural signalling components, for example, neuropeptide receptors are also found on non-nerve-related cells, further proving neurocutaneous interactions and involvement of the PNS in skin homeostasis. (Roosterman et al. 2006)

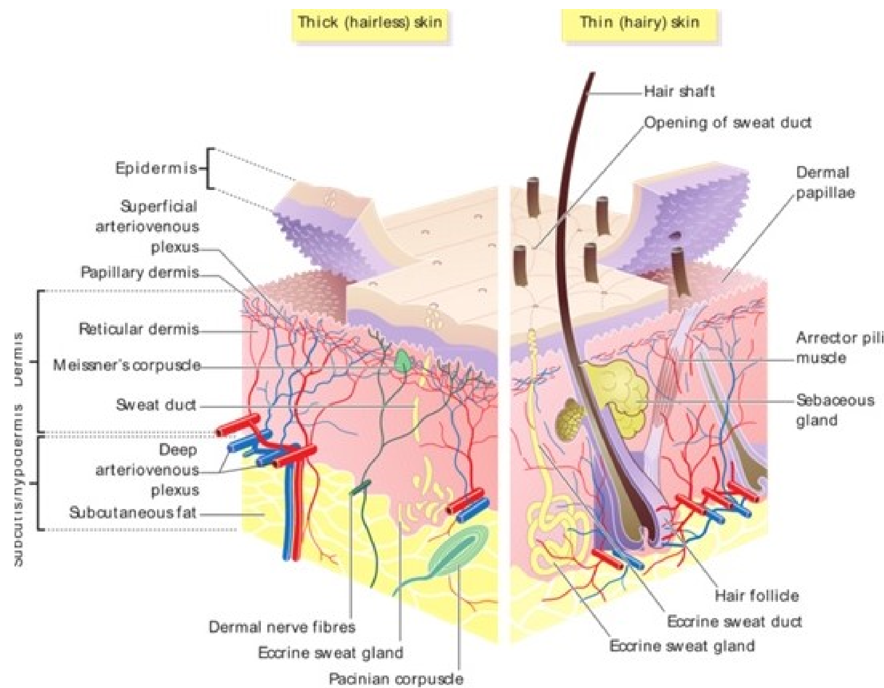

Figure 1.9. Skin comprises several layers consisting of many appendage structures and numerous cell types. Note the presence of nerves in all layers of the skin (Picture from Wikimedia commons).

1.3.2 Epidermis

Epidermis is the outermost layer of the skin and is in essence a stratified epithelium that goes through constant cycle of self-renewal. Around 95% of all cells in the epidermis are keratinocytes which have an ectodermal origin. Other major cell types found in the epidermis of human skin are Langerhans cells, Merkel cells and melanocytes. Langerhans cells are dendritic antigen-presenting cells of hematopoietic origin which become activated and mature upon contact with antigen. Melanocytes descend from the neural crest cells and in human skin are found among keratinocytes in the basal layer of the epidermis. Melanocytes are pigment cells that produce melanin, provide protection from UV radiation and are also responsible for the colour of the skin. Origin of Merkel cells has been long debated, however recent tracing studies suggest that in mammals they are of epidermal origin (Morrison et al. 2009; Van Keymeulen et al. 2009). Merkel cells are found in especially large numbers in the mechanosensitive parts of the skin such as fingertips, they form complexes with Aβ sensory neurons and ensure touch transduction (Woo et al. 2015). Apart from the fore mentioned cell types, epidermis might harbour other immune cells and it is also a place where lots of smaller

neurons terminate ensuring extensive innervation of this uppermost layer of the skin (Kanitakis 2002; Hendrix et al. 2008).

Epidermis also harbours skin appendages such as the upper part of the sebaceous glands and hair follicles that both extend down to the dermis. Hair follicles normally don't develop after birth, however Ito and colleagues has shown that in mice models, depending on the size of the wound the generation of new hair follicles was observed in larger wounds (Ito et al. 2007).

1.3.3 Dermis

The dermis is another major layer of the skin that is composed of many distinct cell types including fibroblasts, endothelial cells, immune cells, nerve-associated cells, muscle cells and adipocytes. These components interact to support the epidermis that forms the barrier with the outside environment and are in turn affected by the signals coming from the epidermis. Fibroblasts are most populous cells in the dermis producing the extracellular matrix (ECM) that is the basis for the elasticity of the skin and play important role in adult wound healing by contributing to rapid wound closure and formation of the scar (Sorrell & Caplan 2004). Dermis is usually anatomically subdivided into upper or papillary dermis and lower reticular dermis which contains considerable numbers of adipocytes (Kanitakis 2002).

Developmental origins of the dermal tissue vary depending on the part of the body, for example, fibroblasts found in the facial skin don't have mesodermal origins, but are rather derived from the neural crest (Dupin & Sommer 2012). Advances were made in understanding the nature of fibroblasts which made it clear that fibroblasts within the dermis are not a heterogeneous population (Sorrell & Caplan 2004). These distinct populations have surprisingly different roles in tissue homeostasis and repair process and tumour environment formation. Interestingly, even upon isolation fibroblasts display positional memory and are either more or less prone to support hair follicle formation (Driskell & Watt 2014; Hanahan & Weinberg 2011).

Tracing experiments in mice have shown that trunk dermal fibroblasts originate from a common multipotent precursor found as early as E12.5 that at E16.5 segregates into different lines with distinct and dynamic marker expression characteristic to fibroblasts of upper or lower

dermis. Upper reticular dermis can be distinguished based on the expression of CD26, Blimp1 and Integrin α8, while lower dermis expresses Dlk1, Podoplanin and Sca1. The same precursor cells also give rise to other dermal structures such as dermal papilla found at the base of the hair bulb and arrector pili muscle, a structure important in hair erection. Upper papillary dermis is important for new hair follicle generation through interaction with epidermis, and when β-catenin signalling is boosted in keratinocytes it forms new hair follicles upon wounding, while lower reticular dermis is expressing α-smooth muscle actin (α-SMA) and is responsible for ECM deposition and scarring (Driskell et al. 2013).

1.4 Stem cells in the adult skin

Notion of stem cells has been revolutionized since the reports of discovery and isolation from various adult tissues of cells able to self-renew in clonal assays and differentiate to form functional cells of other types than the original isolated cell. And skin was not an exception in this regard (Hsu et al. 2014). It is an organ that possesses remarkable capacity for regeneration. In part it can be explained by the fact that it harbours different types of endogenous stem cells found in several compartment of the skin including epithelial stem cells (Arwert et al. 2012), melanocyte stem cells (Nishimura 2011), skin precursor cells (Toma et al. 2001), mesenchymal stromal cells (MSCs) (Dimarino et al. 2013), recruited MSCs during injury repair (Sasaki et al. 2008) and others.

1.4.1 Epithelial stem cells

Epidermal layer of the skin is in need for constant renewal. Thus the notion of epithelial stem cell has been around for a long time. This understanding has shifted from the single epidermal stem cell hypothesis to a no-stem-cell view, and recently thanks to genetic tracing techniques it has been shown that, in fact, several compartmentalized and devoted stem cell pools with limited differentiation potential exist within the epidermis. Epithelial tissues of the skin are organized as layers connected by tight intercellular junctions attached to the basement membrane with less differentiated cells and stem cells located closer to the basement membrane (Blanpain & Fuchs 2006; Donati & Watt 2015).

Stem cells of the epidermis exist in different compartments and adnexal structures such as hair follicles, sebaceous glands and can be distinguished using specific markers (Fig. 1.3). There are several epithelial stem cell niches in the skin including hair follicle bulge, isthmus, sebaceous gland and interfollicular epidermis. Epithelial stem cells to a certain extent are governed by the signals coming from these niches. Despite being restricted in homeostatic conditions, epithelial stem cell populations upon injury display plasticity and contribute to other compartments than in homeostasis condition. For example, tracing *Lrig1*$^{+}$ cells after wounding unexpectedly reveals their presence across the newly formed epidermis and outside of pilosebacious space (reviewed in Donati & Watt 2015)

Stem cells have to be viewed in their environment and for tissue stem cells it is their niche with neighbouring cells, soluble factors and the surrounding matrix. In the case of insult to the skin epithelial stem cell niche is also altered, and additional factors like immune cells and cytokines are involved (Lane et al. 2014). Stem cells in the niche can be in direct contact with other cell types or can receive signals from neighbouring cells in form of secreted factors. Schwann cells, for example have been shown to be such niche regulators maintaining dormancy of the hematopoietic cells by TGF-β secretion (Yamazaki et al. 2011). ECM is prominent epidermal regulator acting as growth factor depository or through, for example, the stiffness of the substrate in a process called mechanotransduction (Gattazzo et al. 2014). Another example of such niche modifier in the hair follicle is innervation. Sonic hedgehog factor (SHH) derived from nerve endings around the bulge have been shown to maintain a population of cells capable of becoming epidermal stem cells and contribute to the wound healing (Brownell et al. 2011).

1.4.2 Melanocyte stem cells

Melanocytes are specialized cells that produce pigment that defines skin and hair colour, depletion of this population leads to age-related hair greying. Melanocytes arise from stem cells (MelSC) residing in hair follicle bulge, which are of neural crest origin (Nishimura 2011). NCSC after migration form melanoblast/glial progenitor cells, which are later committed to melanoblasts that migrate dorsolaterally to populate the skin. As previously mentioned, ventral migration route for melanocytes also exists – within nerves Schwann cell precusors can form melanoblasts that would eventually reach destination sites with peripheral nerves (Sommer 2011; Ernfors 2010). In culture melanoblasts are multipotent and can generate several cell types

including melanocytes, glia, neurons, and smooth muscle cells (Motohashi et al. 2009). MelSC have also been shown to leave their compartment, migrate to newly-forming epidermis to participate in wound healing and differentiate to functional melanocytes to protect the skin from radiation (Chou et al. 2013).

1.4.3 Mesenchymal stromal cells

The basic knowledge about mesenchymal stem cells or mesenchymal stromal cells (MSCs) (Caplan 2010) first came from the pioneering work by Friedenstein in the 1960s – 70s who viewed bone marrow as a source of adult stem cells used for maintenance and repair of mesenchymal structures. Friedenstein and colleagues isolated non-haematopoietic cells from the bone marrow and described this population as being able to adhere to the tissue culture plastics and to grow as clonal colonies calling it colony forming unit fibroblasts (CFU-F). Friedenstein also demonstrated osteogenic potential of these cells in transplantation experiments (Friedenstein et al. 1968; Friedenstein 1970).

Nowadays, according to the statement of International Society for Cellular Therapy MSC are defined as cell culture plastic adherent cells expressing surface markers CD73, CD90 and CD105, and lacking hematopoietic markers CD45, CD14/CD11b, CD34, CD79a/CD19 and HLA-DR able to form clonal colonies and differentiate towards osteocytes, chondrocytes and adipocytes (Fig. 1.10) (Dominici et al. 2006). Several groups have also reported a more broad *in vitro* differentiation potential of MSC towards Schwann cells (Caddick et al. 2006) and neurogenic phenotypes (Shih et al. 2005).

Cells with MSC properties have been so far identified and isolated from various tissues including adipose tissue, skin, dental pulp, umbilical cord blood and Wharton`s jelly, heart, nose mucosa etc. (Zuk 2002; Shih et al. 2005; Gronthos et al. 2002; Erices et al. 2000; Wang et al. 2004; Beltrami et al. 2003; Murrell et al. 2005; Riekstina et al. 2008). MSCs share some common marker with fibroblasts (Haniffa et al. 2009). There have also been reports claiming that MSC reside in virtually all organs (da Silva Meirelles et al. 2006). Based on the findings from Crisan and colleagues this indeed could be true, since MSC have been demonstrated to be of a perivascular origin, and hence could be present in all organs that contain blood vessels. (Crisan et al. 2008) This finding has even allowed to assume that all MSCs could be pericytes (Caplan 2008).

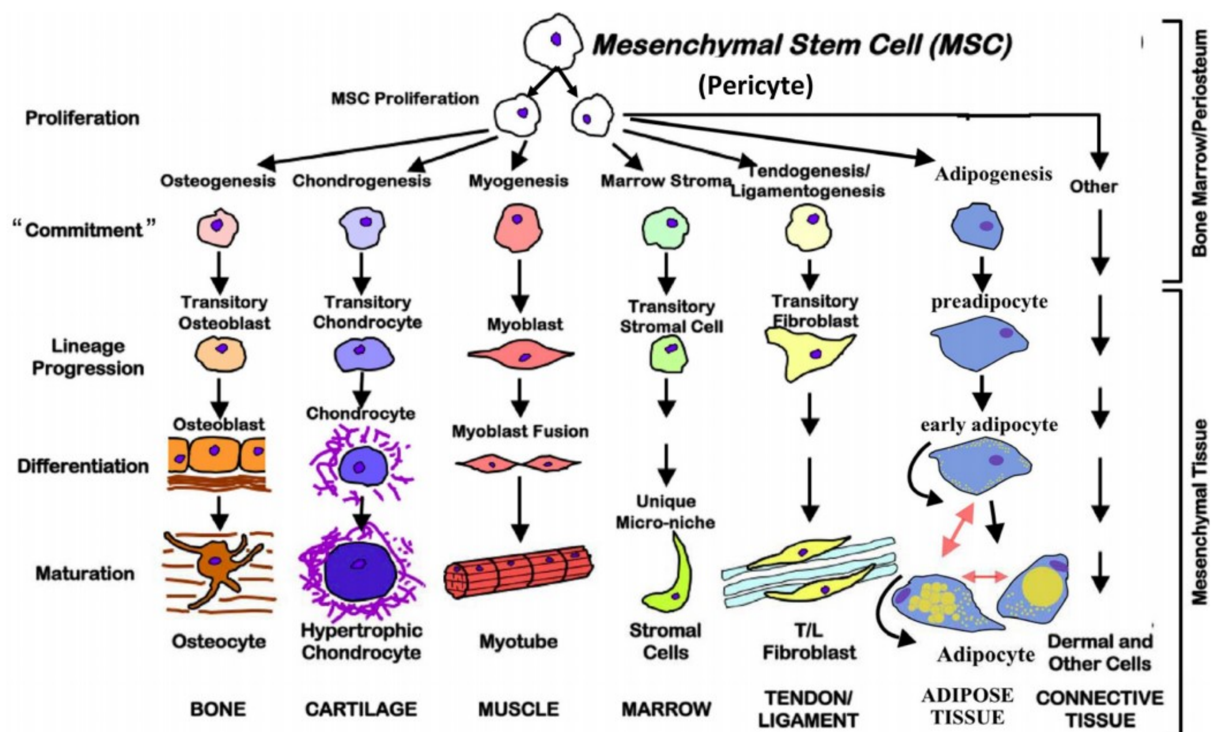

Figure 1.10. Formation of different mesenchymal tissues from MSCs. Bone MSCs are considered multipotent and give rise to various cell and tissue types both *in vitro* and *in vivo*. Image adapted from Dimarino et al. 2013.

Despite the demonstration of multi-linage potential of MSCs *in vitro*, and the promise for the regenerative medicine, the endogenous localization and role of MSCs in tissue homeostasis and regeneration, as well as their mode of action and contribution upon transplantation are more difficult to assess, especially since there is no specific marker to identify MSC. What is clear however is that heterogeneity of MSCs exists also *in situ* and the attempts to trace

different populations with a number of different gene promoters also reveals plasticity of these different populations. The acquired knowledge on the function of endogenous MSCs point toward their role in hematopoietic niche maintenance and development of skeletal system (Kfoury & Scadden 2015).

Isolated MSCs have found therapeutic application in several conditions and large number of clinical trials is currently ongoing. According to ClinicalTrials.gov at the present moment (May 2016) there are more than 150 completed trials that used MSCs (mesenchymal stem/stromal cells) and more than 400 studies that are currently open for various conditions. Indeed the application of MSCs has been ahead of the science and understanding of mechanisms how these cells might function (Prockop & Oh 2012). Initially it was proposed that transplanted MSCs could home to injury site, differentiate and reconstitute the damaged or even lost tissue. Later it was demonstrated in animal models, that these cells don`t make it to the injury site if infused or are quickly eliminated, but the positive effects are still observed (reviewed in Prockop 2016).

One current understanding is that endogenous MSCs are most likely not circulating cells monitoring the organs and are not recruited from bone marrow, since no circulating MSCs were found in several studies (Roufosse et al. 2004; Hoogduijn et al. 2014), but are rather residing in their niche e.g. pericyte niche, get locally activated and differentiate to MSCs upon insult to the organ. This in concert with the fast clearance of infused MSCs, instant blood-mediated inflammatory reaction and thrombotic risk, if infused in large numbers of late passage MSCs, (Moll et al. 2012) speaks against their systemic application and in favour of a local delivery (Caplan 2015).

MSCs are highly secretory cells and among their proposed mechanisms of action is immunomodulation through secretion of chemokines and cytokines, regulation of inflammatory cytokine secretion by other cells, T cell suppression as well as induction of either pro-inflammatory or ant-inflammatory state of MSC by activation of Toll-like receptors on the surface of MSCs (reviewed in Keating 2012). In this regard cell cultivation *in vitro* prior to transplantation could serve as intervention window to manipulate immunomodulatory properties of MSCs, for example, through Toll-like receptor ligand addition to the medium (De Witte et al. 2016). Other effects of transplanted MSCs are also attributed to their secretome and possible contribution to various processes such as angiogenesis, differentiation and endogenous stem cell activation (Keating 2012).

1.4.4 Skin-derived precursor cells

During the last 15 years several independent groups using different approaches (e.g. enrichment through sphere formation, explant culture assay or sorting based on markers and reporters) have isolated and characterized multipotent cells from adult mammalian skin with mesenchymal and neuroectodermal differentiation potential (Toma et al. 2001; Belicchi et al. 2004; Sieber-Blum et al. 2004; Amoh et al. 2005; Wong et al. 2006).

Skin derived precursor cells or SKPs first isolated by Freda Miller's group are a stem cell population found in the dermis and thought to reside in different niches and most prominently in the dermal papillae of the hair follicles (Toma et al. 2001; Fernandes et al. 2004). These cells have initially been described as being different from the skin MSCs. Unlike MSCs they formed spheres and displayed different potential differentiating towards mesenchymal and ectodermal cell types, most importantly, to functional Schwann cells thus resembling NCSC in terms of their plasticity (McKenzie et al. 2006; Wong et al. 2006). Upon injection into the migratory pathway of embryonic NCSC, SKPs were able to migrate and populate multiple destination sites of the neural crest cells. Importantly, cells with the same localization could be found and isolated from human skin and displayed similar differentiation potential *in vitro* (Toma et al. 2001; Joannides et al. 2004; Hunt et al. 2008).

SKPs from different parts of the body in mammals, despite being similar in their differentiation capacity and gene signature, are different with respect to their origin. SKPs found in facial skin have been shown by tracing experiments to be of neural crest origin (Fernandes et al. 2004; Wong et al. 2006), the SKPs found in the trunk skin, however, where not of neural crest origin and where later traced to the mesenchymal origins (Wong et al. 2006; Jinno et al. 2010). These dermal stem cells reside in dermal papilla, lower dermal sheath and in the hair bulge area and have been shown to participate in new hair follicle generation, tissue maintenance and injury repair (Biernaskie et al. 2009).

The group of Lukas Sommer has also reported the isolation of sphere-forming multipotent stem cells from adult trunk skin (Wong et al. 2006). Unlike previously reported (Toma et al. 2001) these cells were positive for neural crest markers p75 and Sox10 and traced with β-galactosidase suggesting neural crest origin. However, both *in vivo* genetic tracing using *Wnt1*-Cre and marker analysis showed that in the trunk skin these cells are not confined to known mesenchymal structures. This suggested presence of different sphere-forming cells in the adult

skin possibly arising from known cutaneous neural crest-derived components like peripheral nerves and melanocytes. Indeed, Etxaniz and colleagues have shown that in the human dermis cells with neurogenic potential are limited to $p75^{+/}CD56^{+}$ Schwann cells (Etxaniz et al. 2014). Later report employing *Dermo1*-Cre mesenchymal reporter mice showed that, nevertheless, spheres of clearly-mesenchymal origin formed from dermal papilla *in vitro* do give rise to neural crest derivatives like Schwann cells without the need for genetic reprogramming. Despite this fact, traced *Dermo1*-Cre SKPs failed to display this plasticity *in vivo* upon injury (Krause et al. 2014).

Interestingly, SKP-derived Schwann cells where shown to be fully functional and, when used in murine peripheral nerve injury model, even able to surpass the myelinating properties of peripheral nerve-derived Schwann cells (Kumar et al. 2016). These cells combined with bio-reactor provided expansion possibilities thus present a promising source of Schwann cells for peripheral nerve injury therapies.

Hoffman lab described Nestin expression in the hair bulge and outer root sheath and took advantage of GFP-expressing Nestin-Cre mice to trace and isolate $Nestin^{+}/CD34^{+}$ pluripotent cells from the hair follicle. These cells displayed mesenchymal and neurogenic potential *in vitro* and where also able to form neurons *in vivo* and new blood vessels if transplanted as a whole follicle (Li et al. 2003; Amoh et al. 2004; Amoh et al. 2005). Another group demonstrated presence of pluripotent cells in the same structure of a mouse whisker follicle and was able to trace them back to neural crest and label these bulge stem cells epidermal NCSC (EPI-NCSC) (Sieber-Blum et al. 2004). Human EPI-NCSC as directly originating from neural crest are also suggested to be a suitable source of Schwann cells for peripheral nerve injury repair (Sakaue & Sieber-Blum 2015).

1.5 Cutaneous wound healing model

Upon injury to the skin a cascade of highly regulated and evolutionary optimized events aiming at renewing the integrity of the tissue are set in motion. There are several key processes involving different cell types that are classically grouped in three overlapping stages – formation of a blood clot and inflammation, new tissue formation and remodelling (Gurtner et al. 2008; Shaw & Martin 2009). In the adult mammal skin wound healing process does not lead

to complete regeneration. Usual outcome of the healing process is formation of a scar. Scar is a collagen-rich relatively acellular tissue that is devoid of appendages usually found in normal skin such as hair follicles and sebaceous glands. The outcome of the wound healing depends on the way different healing stages are carried out but is also highly dependent on many other individual factors including, for example, location of the injury, age and health state of the individual, care and medication used. Abnormal wound healing can lead to formation of chronic wounds as in the case of diabetes (Falanga 2005) or, on the contrary, to the formation of hypertrophic and keloid scars (English & Shenefelt 1999).

1.5.1 Wound healing stages – fibrin clot formation and inflammation

Most rapid in wound healing is the platelet response. Within minutes after the injury thrombocytes are recruited to form a blood clot by transforming fibrinogen to fibrin. This prevents initial blood loss and bacteria from invading the tissue (Nurden et al. 2008). In the meantime, platelets also secrete number of factors including platelet derived growth factor (PDGF), transforming growth factor β (TGF-β) and stromal cell-derived factor 1 alpha (SDF1α) which are important for the fibroblast response initiation and angiogenesis (Golebiewska & Poole 2015). Fibrin clot also serves as a provisional matrix for the migration of other cell types including keratinocytes. Thrombocytes, however are not essential and their depletion does not significantly alter the general dynamics of wound repair (Szpaderska et al. 2003).

The onset of the inflammation is also immediate. It is aimed at fighting the invading bacteria and continues until keratinocytes have fully closed the wound, which in excisional mice wound models occurs at around day 5 – 7 after injury. Neutrophils are the first to arrive at the wound site, followed shortly by leukocytes and monocytes, which will later give rise to macrophages (Eming et al. 2007). Neutrophils are the key cells in initial pathogen response, however their positive function might be outbalanced by adverse toxic effects due to unspecificity of their action (Wilgus et al. 2013). Macrophages are phagocytic cells and are important for the clearance of fibrin clot, dead cells and other tissue debris, while also contributing to the high levels of cytokines and growth factors at the wound site (Koh & DiPietro 2011).

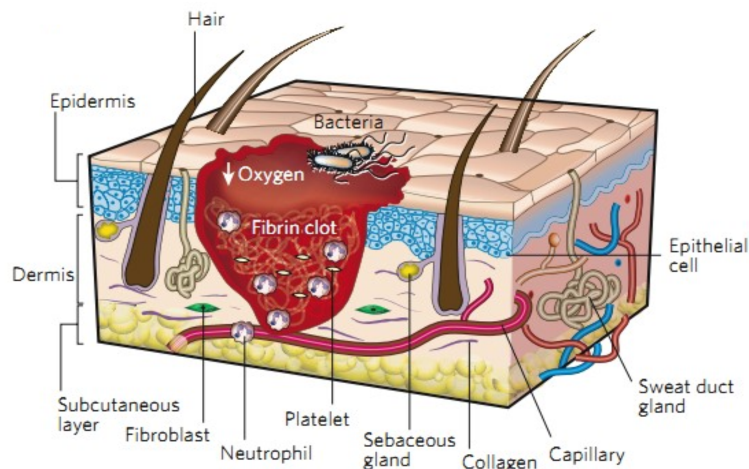

Figure 1.11. Coagulation and inflammation stage of wound repair. It is the first stage of wound healing with immediate onset. This step is crucial to fight the invading bacteria and provides the first matrix for migrating epithelial cells. More evidence is accumulating that points towards importance of immune cells for different tissue stem cell activation. Image adapted from Gurtner et al. 2008.

In the aseptic age the importance of strong immune response is seen to be secondary. Experiments where different subsets of leukocytes were depleted led to surprising results and depletion of neutrophils in sterile conditions in fact even promoted wound closure. There is evidence that reducing inflammation by means of depleting both macrophages and neutrophils leads to faster re-epithelialization and, more importantly, to scarless healing reminiscent of embryonic wound healing (Martin & Leibovich 2005).

1.5.2 Wound healing stages – proliferation

Proliferation stage of the wound healing is another dynamic process during which new tissue called granulation tissue is formed. The onset and timeframe of this stage can vary. During this stage epithelial barrier and tissue homeostasis are restored – keratinocytes lose their tight contacts to each other and to the basal membrane, form lamellopodia and migrate from the edges of the wound on the matrix provided by the granulation tissue (Kuipers et al. 2014). Granulation tissue fills the gap created by the injury and is comprised mainly of migrating fibroblasts and myofibroblasts, endothelial cells and immune cells.

Fibroblasts are highly-secretory cells that deposit large quantities of extracellular matrix, most importantly, collagen type I and fibronectin. Myofibroblasts are one of the key players in proliferation phase and can have multiple origins arising from fibroblasts or blood vessel

associated cells – pericytes. TGF-β1 is a crucial factor for their formation. Myofibroblasts are highly secretory cells that continue ECM deposition they also possess contractile properties and are able to promote wound closure by physically contracting the wound. Aberrant activation or function of these cells, in turn, can lead to adverse effects such as fibrosis due to excessive ECM deposition or to wound contractures (Hinz et al. 2012).

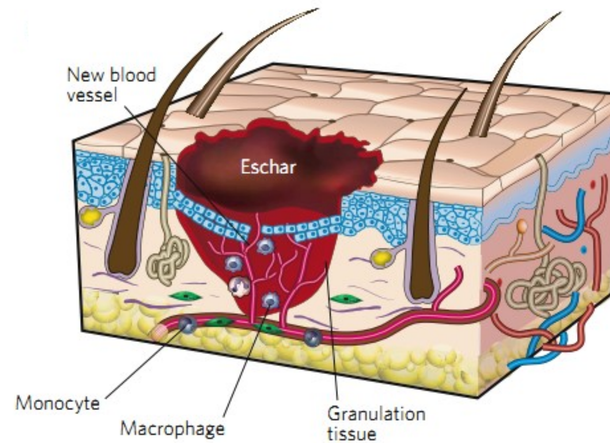

Figure 1.12. Proliferation stage of wound repair. Proliferation stage continues for several days as the initial fibrin clot is replaced by granulation tissue, consisting of fibroblasts, myofibroblasts, endothelial and immune cells. Keratinocytes continue to migrate to seal the wound and inflammation processes are ongoing as neovascularization peaks. Image adapted from Gurtner et al. 2008.

Proliferation stage also exemplifies how important the interactions between different cell types can be for the wound healing process. In the case of mesenchymal-epithelial interactions also remarkable in the way different cell types participating in it influence each other, sometimes in a double-paracrine manner, as in the case of keratinocyte-fibroblast interactions (Werner et al. 2007).

1.5.3 Wound healing stages - remodelling

The last and longest stage is the remodelling stage. Most of the cells that have migrated to or formed within the wound are going through apoptosis leaving behind tissue rich in collagen formed by fibroblasts and myofibroblasts. It is during this stage that scar in its final mature shape is formed and disorganised collagen III is replaced by cross-linked collagen type I, giving wound more tensile strength (Gurtner et al. 2008).

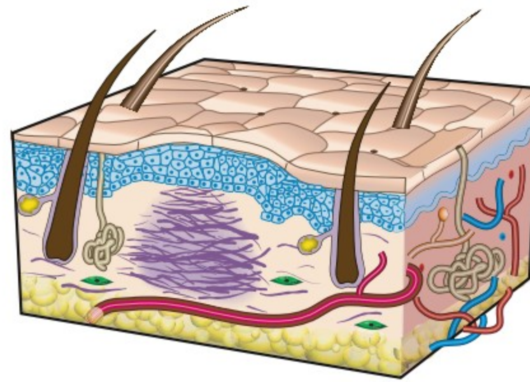

Figure 1.13. Remodelling stage of wound repair. Longest stage of wound healing that can take up to several months. During this time collagen deposited by fibrotic cells in the wound is being replaced by more elastic forms. Cells that have expanded during proliferation stage leave the wound through apoptosis and leave relatively acellular scar tissue devoid of skin appendages. Image adapted from Gurtner et al. 2008.

Despite the fact that in the adult tissues during wound repair regeneration occurs only to a limited extent, wound healing is often used as a model to better understand regeneration mechanisms and processes underlying both chronic wound formation and "overhealing", such as formation of a hypertrophic scar. There are several approaches to creating a wound depending on the aim of the study. Incisional wounds are superficial and preserve the original tissue, whereas excisional full-thickness wounds are characterized by loss of original tissue and thus provide more newly-formed tissue material to analyse.

One drawback of using certain animal models is a considerable difference in skin anatomy compared to human skin. Murine skin, for example is characterised by presence of an underlying muscle called panniculus carnosus, which allows for rapid tissue contraction and re-generation as such is less significant component of the healing process. Thus, to better mimic the human wound healing some groups employ tissue splinting techniques or porcine models (Ansell et al. 2012).

1.5.4 Wound healing and cancer

Many parallels exist between wound healing and other biologic events, including some embryonic processes and cancer. Indeed, cancer is sometimes seen as an overhealing wound and some benign wound lesions or non-healing ulcers exposed to prolong inflammation can

eventually lead to formation of malignant tumours (Schäfer & Werner 2008; Arwert et al. 2012). Excessive cell migration is one exemplary process that is normally not associated with healthy tissues but is observed during embryogenesis, in adulthood during wound healing and in cancer, for example, in metastasis formation (Shaw & Martin 2016).

1.5.5 Wound healing and innervation

Medical conditions that are accompanied by neuropathy are also often associated with reduced wound healing capacity and formation of poorly healing ulcers for example, in case of diabetes and in patients with paralysis (Basson & Burney 1982) It is still however being debated whether this is due to direct secretory contribution of the axons/nerve-derived cells or can be simply explained by the loss of sensation and consequent self-harming. Innervation in context of tissue repair has received relatively little attention until recent, even though the link between innervation and regeneration capacity has been known since 19th century

Another intriguing possibility apart from secretory function is the involvement of innervation in maintenance of various tissue stem cell niches. Schwann cells, for example have been demonstrated to maintain the dormant state of hematopoietic stem cells in the bone marrow (Yamazaki et al. 2011), while loss of innervation in the hair follicle leads to depletion of Gli1^{+} epidermal stem cells during skin regeneration (Brownell et al. 2011).

Another study suggests that Lrg6 marker expression in skin epithelial stem cells is dependent on cutaneous innervation and Schwann cell actions (Liao & Nguyen 2014). Depletion of Sox2-linage of neural crest origin that presumably represents de-differentiated Schwann cells has been recently shown to interfere with normal wound healing. Authors argue that this effect could be exerted through secretion of certain factors, that promote wound healing (Johnston et al. 2013).

2. Methods

2.1 Mouse wound healing model related experimental procedures

Mouse Strains

Dhh-Cre: Tg(Dhh-cre)1Mejr (Jaegle et al. 2003). A transgene consisting of a cre coding sequence inserted at the start codon of a *Dhh* genomic clone. (Strain of origin: FVB/N).

Plp-CreER^{T2}: Tg(Plp1-cre/ERT2)1Ueli (Leone et al. 2003). A transgene consisting of the mouse promoter of the *Plp* gene driving the expression of a TM inducible Cre recombinase. (Strain of origin: (C57BL/6 x DBA/2)F1).

Pten^{lox}: Pten^{tm1Hwu} (Lesche et al. 2002). A floxed allele of *Pten* allowing the suppression of its phosphatase activity upon Cre-mediated recombination. (Strain of origin: 129S4/SvJae).

Sox10^{lox}: Sox10$^{tm7.1(Sox10)Weg}$ (Finzsch et al. 2010). A floxed allele of *Sox10* allowing the conditional KO of the gene upon Cre-mediated recombination. (Strain of origin: 129P2/OlaHsd).

tdTomato: Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$ (Madisen et al. 2010). A *tdTomato* sequence preceded by a loxP-flanked STOP cassette was targeted to the Rosa locus. This allows Cre reporting and subsequent lineage tracing by following tdTomato expressing cells (Strain of origin: (129S6/SvEvTac x C57BL/6NCrl)F1).

Tyr-CreER^{T2}: Tg(Tyr-cre/ERT2)13Bos (Bosenberg et al. 2006). A transgene consisting of 5.5 kb fragment of the Tyr promoter and a 3.6 kb fragment of the Tyr enhancer driving expression of a TM inducible form of Cre recombinase.

Experimental mouse lines

For genetic tracing of neural crest derived population mice with the following genotypes were used: *Plp-CreER^{T2}*; *Rosa-LacZ, Plp-CreER^{T2}*; *tdTomato*, *Dhh-Cre*; *tdTomato* and *Tyr-CreER^{T2}*; *tdTomato*.

For ablation of neural crest derived cell population mice with the following genotype were used: *Plp-CreER*^{T2}; *Sox10*$^{lox/lox}$; *Rosa-LacZ.*

Expansion of neural crest derived cell population mice with the following genotype were used: *Plp-CreER*^{T2}; *Pten*$^{lox/lox}$; *tdTomato.*

Genotyping

For mice identification toe clipping method was used. DNA extraction was performed on tissue samples obtained from the toe clipping. Briefly, tissue samples were placed on a Thermomixer (Eppendorf) with 500 µl tissue lysis buffer per sample containing Proteinase K (Roche) and incubated overnight at 55ºC with constant shaking. Isopropanol was added after incubation and samples inverted several times and centrifuged for 15 min. Supernatant was removed and 500 µl of 70% EtOH added followed by 5-minute centrifugation. Supernatant was removed and pellet allowed to dry for 20 minutes at room temperature. Pellet containing DNA was dissolved in 300 µl distilled water and stored at 4ºC. DNA extracted by these procedures was analysed by PCR, using gene specific primers (see below).

Experimental animals

In our wound healing experiments, we used animals aged between 2 and 6 months of several combined transgenic lines, with their skin hair cycle in the late anagen to telogen phases. Due to the various strains of origin of our transgenes, the genetic background is empirically considered mixed. Cell population characterization by immunolabeling was performed on a cohort mixed gender. Littermates were evenly distributed across control and cKO experimental groups. All quantifications were performed on tissue samples obtained from males only.

Cre-mediated allele recombination

cKO of *Sox10* using the *Plp-CreER*^{T2} driver was performed using i.p. injection of a 10 mg/ml Tamoxifen (TM) solution in sunflower oil/EtOH 9:1. Animals were injected daily with 200 µl

TM for 5 consecutive days for a total of 10mg TM per animal. To obtain efficient depletion of the Sox10 expressing population in the skin, cKO of *Sox10* was performed 3 weeks prior to injury.

Pten cKO was performed using a 3-day treatment of daily i.p. injection of 10mg/ml TM solution. Operative procedure was performed 5 days after the initial injection.

Excisional wounding of murine skin

Following a protocol approved by the veterinary office of Kanton Zurich, general anaesthesia of the animals was induced by 5% Isoflurane in 100% O_2 and subsequently maintained using 3% Isoflurane. The back skin was shaved, thoroughly cleaned and disinfected prior the surgery using antibacterial soap solution and 75% EtOH solution. Preoperative analgesia was applied using a subcutaneous injection of Buprenorphine (0.1mg/kg). 4 circular full thickness excisional wounds of 5mm of diameter were generated on the lower back skin of each animal, 2 on each side, 1cm of the midline of the animal and roughly 2cm apart from each other. Post-operative analgesia was performed by a 5-day treatment of Buprenorphine through the drinking water. Wounds were allowed to heal without dressing.

Macroscopic wound size measurement

Wound area was determined for each wound by quantification of wound area based on pictures taken from day 0 to day 14 post surgery. Area of wounds was measured using ImageJ 1.49 software (National Institutes of Health USA). The area at different time points after wounding were then reported to the original area value at day 0 for each wound.

Histological analysis of murine skin samples

At day of collection, animals were euthanized using CO_2 and skin samples were excised post-mortem, fixed in a buffered 4% formaldehyde solution at 4°C, overnight for samples to be embedded in paraffin or for 4 hours for samples to be embedded in tissue freezing medium for

cryosection. Paraffin blocks were processed into sections of 5µm while cryoblocks were processed into 12µm sections. For immunofluorescence, protocols described elsewhere were applied. Briefly, sections were deparaffinized and subjected to an antigen retrieval step using citrate buffer (S2369, Dako). Primary antibodies (see below) were applied in blocking buffer (2% BSA in PBS and 0.05% Triton X-100) overnight at 4°C and visualized using secondary antibodies (see below) in blocking buffer for 1 h at room temperature. For pERK, c-Jun and pSMAD2 staining biotin streptavidin and TSA amplification kit was used (PerkinElmer). Hoechst 33342 (14533, Sigma-Aldrich) usually was used as nuclear counterstain at a 1 µg/ml working concentration, unless stated otherwise.

Haematoxylin and Eosin (H&E) and Herovici-stained slides were processed according to standard protocols. Immunofluorescence-, H&E- and Herovici-stained sections were imaged using either a DMI 6000B microscope (Leica) or an Axio Scan.Z1 slide scanner (Zeiss). Image analysis and quantifications were performed using ImageJ 1.49 (National Institutes of Health USA) and ZEN (Zeiss) imaging software. For pSMAD2 quantification 3 high magnification fields per wound were randomly selected within the area positive for α-SMA, percentage of nuclei positive for pSMAD2 was quantified and averaged.

Morphometric analysis

Morphometric analysis was performed on 5µm sections obtained from the middle of D7 wounds and stained with H&E. Measurements of wound closure, area of hyperproliferative epidermis, distance between wound border hair follicles (contraction assessment) and length of newly-formed wound epithelium (re-epithelialization) were carried out using ImageJ as described in Fig. 3.17.

Expression profiling of genetically traced cells

The skin of 3 non-wounded control *Dhh-Cre; tdTomato* animals and the 7 day old wounds of their 3 littermates were collected post mortem and pooled in two groups. Subsequently, the tissue samples were mechanically chopped using a disposable scalpel and further dissociated to a single cell suspension using 0.25 mg/ml Liberase DH Research Grade (05401054001,

Roche) in RPMI 1640 (42401, Life Technologies) for 45min at 37°C with gentle rocking followed by a treatment with 0.55mg/ml Dispase II (17105, Life Technologies) and 0.2 mg/ml DNase I (10104159001, Roche) for 15min at 37°C. The cell suspension was then passed through a 30 μm cell strainer and sorted for tdTomato positivity using FACSAria III (BD Biosciences).

Sorted cells were directly placed into RNA lysis buffer solution for subsequent RNA isolation. Total RNA obtained from 3 control and 3 wounded skin samples (pools from 4 wounds) were then amplified using Ovation Single Cell RNA-Seq System (NuGEN) Kit and subjected to sequencing on Illumina Hiseq platform at the Functional Genomics Center Zurich (http://www.fgcz.ch/). Differential gene expression analysis was performed using a minimum fold change of 1.5 and a False Discovery Rate inferior to 0.05. Gene ontology network analysis was performed with MetaCore (Thomson Reuters).

Statistical analyses

Statistical analysis was performed and graphs created using Prism 6 software (GraphPad Software Inc, San Diego, CA). Statistical significance between two groups was calculated using unpaired Student's *t*-test. For wound area measurements in Fig. 3.16 nonparametric Mann-Whitney test was used.

2.2 Human skin sample derived cell characterization and differentiation

Human skin primary culture

Human skin samples were obtained from plastic surgery post-operation material of anonymous donors in accordance with Latvian Central Ethics Committee authorized approval (Riekstina et al. 2008). All patients signed an informed consent form. Human skin tissue samples were transported to laboratory in ice-cold $Ca2^{+}/Mg2^{+}$ ion free PBS, containing 2% antibiotic mix (penicillin/streptomycin) and fungizone 2 μg/ml. The specimens were washed with cold PBS, cut into 4–6 mm^2 pieces and incubated in dispase 0.6 U/ml for 1–3 h at 37°C.

SKP cultures were obtained as described earlier (Riekstina et al. 2008). The epidermis was manually removed from tissue pieces after dispase incubation and dermis was cut into 1 mm^3 pieces following enzymatic digestion with 0.62 U/ml Liberase Blendzyme 1 (Roche) for 30–40 min at 37°C. Afterwards, tissue pieces were dissociated by pipetting vigorously using 5ml pipette, passed through a 70µm cell strainer into 50 ml tubes (BD Falcon, USA), and centrifuged at 1500 rpm for 5 min. The pellet was re-suspended in cell growth media DMEM-F12 (3:1) containing penicillin and streptomycin 100 u/ml and 100 µg/ml respectively, supplemented with 20% FBS. Cell suspensions were transferred into 25-cm^2 tissue culture flasks (T-25) or 24-well plates (Sarstedt) and grown until confluence. Several primary skin cell culture samples from female donors (age from 29 to 65 years, passage number one) were selected for in-depth characterization.

Morphological analysis

Cell morphology was analysed on sub-confluent cell monolayers at 100x magnification on a phase-contrast microscope (Leica). Photos were taken by Kodak camera. Cells were counted in haemocytometer (Marienfeld) and cell viability was assessed by Trypan blue staining.

FACS analysis

Adherent skin-derived precursor cells were trypsinized and stained with primary antibodies. Briefly, $1–2\times10^5$ cells per sample were incubated with the following primary antibodies: CD90-FITC, CD34 (all from Dako), CD73 (Abcam), CD105 (R&D Systems), CD45-FITC, CD14-APC, HLA-DR-APC (all from BD Biosciences) and isotype controls IgG1-FITC (Dako), IgG1-PE, IgG2A-APC (both from BD Biosciences), and IgG1 (R&D Systems). Secondary goat anti mouse IgG-PE and goat anti mouse IgG-APC (R&D Systems) were used where appropriate. For p75 analysis in dermal cells and ReNcells PE-conjugated CD271 antibody (Clone C40-1457) (BD Biosciences) was used. Flow cytometry data were acquired using FACSCalibur and analysed by CellQuest (BD Biosciences) software.

Cytochemistry and immunocytochemistry

Cytochemical staining techniques were used to detect mesenchymal differentiation. For Oil Red O staining cells were fixed with 60% isopropanol and stained for 10 minutes with staining solution. For Alizarin Red staining cells were fixed for 60 minutes with 70% ice-cold ethanol, followed by rinse with distilled water and 2-minute incubation with 1% staining solution. Subsequently cells were washed with 60% and 100% isopropanol. Alcian blue staining was used for detection chondrocyte differentiation in cell pellets. Slides with 8 µm thick cell pellet cryosections were incubated with staining solution for 15 minutes, rinsed with 100% ethanol and mounted.

Prior to immunocytochemical staining procedure, samples were rinsed with PBS. Afterwards, specimens were fixed in 4% paraformaldehyde (PFA) for 10 min at room temperature. Cells were rinsed with PBS and incubated with blocking/permeabilization buffer consisting of 5% bovine serum albumin (BSA), 0.3% TritonX-100 in PBS for 45 min in room temperature. Endogenous peroxidase activity was blocked with 3% H_2O_2 peroxide for three minutes. Cells were subsequently incubated with primary antibodies. IgG1 and IgG3 mouse antibodies were used for isotype control (R&D Systems) A negative control with no primary antibodies was also included. Samples were incubated at +4°C overnight. After incubation, cells were rinsed three times in PBS. Horse-radish peroxidase (HRP) conjugated secondary reagent and diaminobenzidine (DAB) substrate were applied to the cells according to the manufacturer's instructions (LSAB+ System-HRP, Agilent). Specimens were counterstained with Mayer's haematoxylin (Lillie's modification, DakoCytomation), mounted (GelMount, DakoCytomation) and analysed under microscope (Leica DMI4000 B). For image analysis, five randomly selected fields per tissue were photographed and recorded using Image-Pro® Express software.

For immunofluorescence analysis, cells were grown in a 4-well chamber slide (Nunc) until 50% confluence was reached. Specimens were rinsed with PBS and fixed with 4% PFA for 10 min at room temperature. After rinsing with PBS, cells were blocked and permeabilized with 5% BSA, 0.1% Triton X-100 in PBS for 45 min. Samples were subsequently incubated with primary antibodies overnight at +4ºC. Cells were rinsed three times in PBS and incubated with secondary antibodies at room temperature for 1h in the dark. Specimens were rinsed with PBS three times and counterstained with DAPI diluted in PBS. Then cells were rinsed, mounted with Fluoromount (Dako) and analysed under the microscope. For image analysis, five

randomly selected fields per tissue were photographed and recorded. Image overlay was performed using Image-Pro® Express software.

Differentiation of human dermis-derived cells

Mesenchymal differentiation was performed as previously described (Shih et al. 2005; Riekstina, Parfejevs, et al. 2009).

Adipogenesis. After trypsinization and cell counting cells were seeded on 24-well plates (Nunc) at $2.17x10^4$ cells/mm^2 and cultivated in 0.5 ml of 10% FBS MEM medium supplemented with penicillin and streptomycin (100 u/ml and 100 µg/ml) (all Invitrogen) until they reached 100% confluence. The medium was then changed to one that additionally contained 0.2 mM Indomethacin, 1µM dexamethasone, 0.5 mM 3-isobuthyl-1-methylxanthine and 10 µg/ml human insulin (all Sigma-Aldrich). Differentiation medium was changed every 3-4 days.

Osteogenesis. After trypsinization and cell counting cells were seeded on 24-well plates (Nunc) at $4.2x10^3$ cells/mm^2 and cultivated in 0.5 ml of 10% FBS αMEM medium supplemented with penicillin and streptomycin (100 u/ml and 100 µg/ml) (all Invitrogen) until they reached 70% confluence. The medium was then changed to one that additionally contained 10^{-7}M dexamethasone, 50 µM ascorbic acid-2-phosphate, 10 mM ß-glycerolphosphate (all Sigma-Aldrich). Differentiation medium was changed every 3-4 days.

Chondrogenesis. Dermal cells ($250x10^3$) were centrifuged in 15 ml tube for 5 minutes, supernatant removed and cell pellet cultured 0.5 ml chondrocyte differentiation medium containing DMEM/F12 (3:1) 0.5 FBS, 2mM L-Glutamine, 100, 100 µg/ml sodium pyruvate (all Invitrogen), 50 nM ascorbic acid-2-phosphate (Sigma-Aldrich), 10^{-7}M dexamethasone (Sigma-Aldrich), 10 ng/ml TGF-β1 (RnD Systems) and 10 µg/ml human insulin (RnD Systems).

Human skin-derived cell neuroectodermal differentiation. Cells were seeded into tissue culture flasks or multiple well plates (Sarstedt) and allowed to adhere in serum-supplemented medium for 24 hours. Then, cell culture medium was changed to serum-free neural progenitor expansion medium DMEM-F12 (3:1) containing FGF-2 (20 ng/mL), EGF (20 ng/mL), and B27 (2%). After seven days of differentiation, with differentiation media being changed once every three

days, cells were ready for fixation and subsequent immunophenotyping. For FT-IR analysis cells were cultivated in 75 cm^2 tissue culture flask (Sarstedt) and harvested each week during six-week period (w0, w1, w2, w3, w6).

FT-IR analysis

The amount of cells in the sample, 1×10^6 cells, was determined according to the absorption spectra intensity 0.35–1.25 to fulfil Lambert-Buger-Beer Law endowing that the intensity of band is proportional to the concentration. 3–10 μl of sample were dropped on a silicon plate and dried at room temperature. FT-IR analysis was carried out using microplate reader HTS-XT (Bruker, Germany) over the range 4000–600 cm^{-1} in absorption mode and data processed using OPUS 6.5. Spectra were evaluated by vector normalization, 2nd derivative, hierarchical cluster analysis (HCA) and quantitative analysis (Grube et al. 2002; Heraud et al. 2010).

Genotyping primers

Allele	Forward sequence	Reverse sequence
Cre	CTATCCAGCAACATTTGGGCCAGC	CCAGGTTACGGATATAGTTCATGAC
Pten^{lox}	CAAGCACTCTGCGAACTGAG	AAGTTTTTGAAGGCAAGATGC
Rosa-LacZ	GGTCGGCTTACGGCGGTGATTT	AGCGGCGTCAGCAGTTGTTTTT
Sox10^{lox}WT	CAGGTGGGCGTTGGGCTCTT	TCCCAGGCTAGCCCTAGTG
Sox10^{lox}pos		GTGAGCCTGGATAGCAGCAG
TdTomato null	AAGGGAGCTGCAGTGGAGTA	CCGAAAATCTGTGGGAAGTC
TdTomato pos	CTGTTCCTGTACGGCATGG	GGCATTAAAGCAGCGTATCC

Primary antibodies

Epitope	Reactivity	Producer	Localization	Catalog Nr.	Dilution
CD271/p75	human / mouse	Alomone Labs	membrane	ANT-007	IF, 1:200
CD31	human / mouse	Abcam	membrane	ab28364	IF, 1:200
CD45	Mouse	BD Pharmingen	membrane	550539	IF, 1:200
C-JUN	human / mouse	SC Biotechnology	nucleus	SC-1694	IF, 1:200
DCT	human / mouse	SC Biotechnology	membrane	SC-10451	IF, 1:250
GFAP	human / mouse	Dako	melanosome membrane	Z0034	IF, 1:300
KI67	Mouse	BioLegend	nucleus	652402	IF, 1:100
Laminin	human / mouse	Sigma	ECM	L9393	IF, 1:200
MBP	human / mouse	Abd Serotec	membrane	aa82-87	IF, 1:300
Neurofilament-M	human / mouse	EMD Millipore	cytoskeleton	AB1987	IF, 1:300
pAKT	human / mouse	Cell Signaling	nucleus	4058	IF, 1:200
pERK1/2	human / mouse	Cell Signaling	cytoplasm/ nucleus	9101	IF, 1:300
pSMAD2 (138D4)	human / mouse	Cell Signaling	nucleus	3108	IF, 1:300
RFP	NA	EMD Millipore	cytoplasm/ nucleus	AB3216	IF, 1:100
α-SMA	human / mouse	Sigma	cytoskeleton	A2547	IF, 1:100
Sox10	human / mouse	SC Biotechnology	nucleus	SC-17342	IF, 1:100
TdTomato	NA	LS Bio	cytoplasm/ nucleus	LS-C340696	IF, 1:200
SSEA-4	human / mouse	R&D Systems	membrane	MAB1435	FC ICC 1:100
GFAP	Human	R&D Systems	cytoskeleton	SC028	IF ICC 1:10
Tubulinβ III	human / mouse	R&D Systems	cytoskeleton	SC028	IF ICC 1:10
Nestin	Human	R&D Systems	cytoskeleton	SC028	IF ICC 1:10
IgG1 mouse	Human	R&D Systems	membrane	MAB002	ICC 1:100
IgG3 mouse	Human	R&D Systems	membrane	MAB007	ICC 1:100

Secondary antibodies and amplification reagents

Secondary antibody	Producer	Catalog Nr.
Cy3 Donkey anti-goat IgG (H+L)	Jackson	705-165-147
Alexa Fluor 488-conjugated AffiniPure Donkey anti-goat IgG (H+L)	Jackson	705-545-147
Cy3 Donkey anti-rabbit IgG (H+L)	Jackson	711-165-152
Alexa Fluor® 488-AffiniPure Donkey Anti-Rabbit IgG (H+L)	Jackson	711-545-152
Alexa Fluor 647-conjugated AffiniPure Donkey anti mouse IgG	Jackson	715-605-150
Alexa Fluor® 647-AffiniPure Donkey Anti-Rat IgG (H+L)	Jackson	712-605-153
Biotin-SP Donkey anti-rabbit IgG (H+L)	Jackson	711-065-152
FITC Goat anti mouse IgG (H+L)	Jackson	115-095-146
HRP Streptavidin	Jackson	016-030-084
LSAB+ detection system	Agilent	K5001
Tyramide Signal Amplification kit (TSA)	PerkinElmer	NEL744001KT

3. Results

3.1 Tracing nerve-derived cells in the skin upon wounding

Skin is a densely innervated organ (Hendrix et al. 2008) with major nerve bundles (NB) visible in both intact skin and in the skin healing from full-thickness excisional wounds. Higher cellularity compared to normal skin, presence of eschar and thick newly-formed epidermis lacking normal appendages, such as hair follicles and sebaceous glands are all characteristic features of adult wound at this stage (Fig. 3.1 a,b) (Martin 1997; Eming et al. 2014).

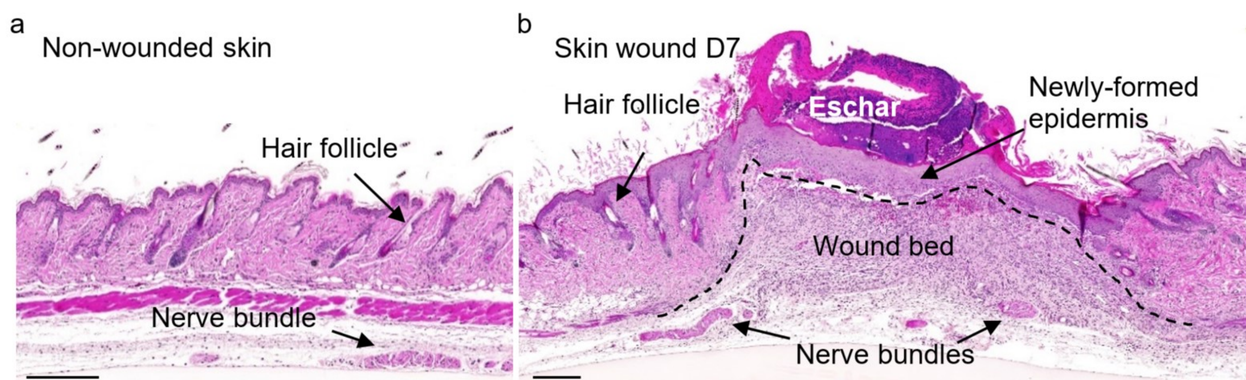

Figure 3.1. **Histological comparison of normal and wounded adult mouse skin.** Haematoxylin-Eosin stained sections of intact skin **(a)** and full-thickness excision wound at D7 after injury **(b)**. Scale bars, 50µm.

To determine the potential involvement of skin innervation in wound healing, we first employed genetic lineage tracing to follow the fate of nerve-derived cells upon skin wounding. Consistent with previous reports (Leone et al. 2003; Adameyko et al. 2009; Hari et al. 2012), Tamoxifen (TM)-mediated activation of CreER^{T2} in the intact skin of *Plp-CreER^{T2}; tdTomato* animals leads to genetic tracing of peripheral glial cells. We observed presence of recombined cells in NBs and nerve endings found throughout the skin (Fig. 3.2 a).

Upon full-thickness excision wounding, however, we noted the presence of many *Plp-CreER^{T2}*-traced (tdTomato^{+}) single cells scattered within the wound bed (Fig. 3.2 b). In the non-wounded skin expression of Sox10, a marker for the peripheral glial lineage (Kuhlbrodt et al. 1998), is confined to NBs surrounded by a prominent laminin-positive perineurium (Fig. 3.2 c). Interestingly, some NBs located adjacent to the wound displayed laminin expression

suggesting disrupted perineurium (Fig. 3.2 d) and were associated with single Sox10-positive (Sox10^{+}) cells, which seemed to disseminate from the disrupted nerves (Fig. 3.2 e).

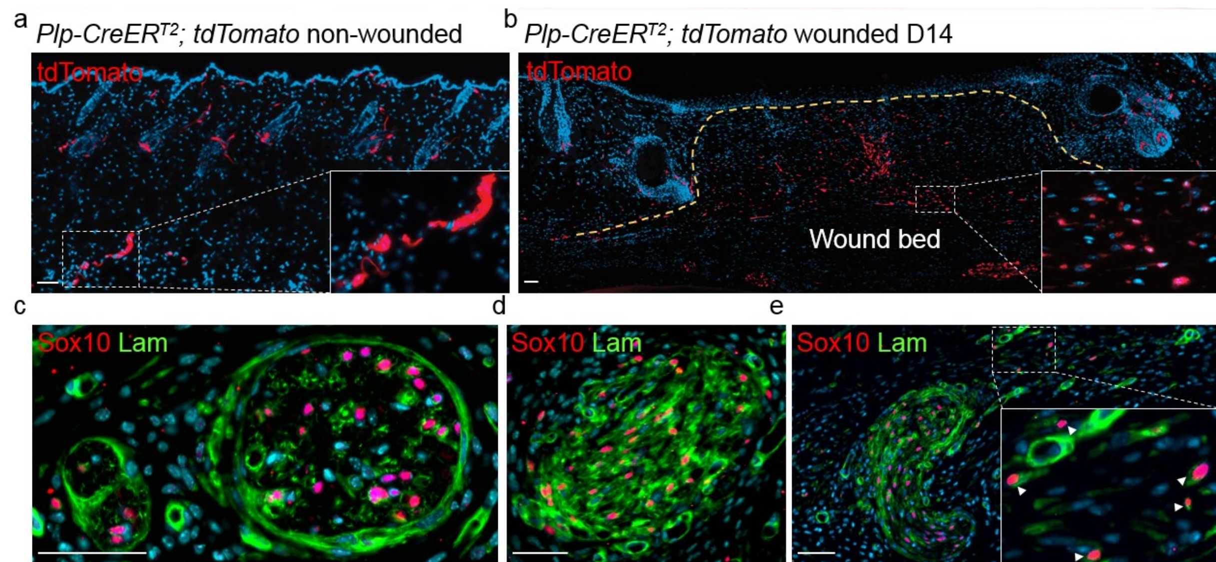

Figure 3.2. **Glial lineage tracing of intact and wounded skin in *Plp-CreER^{T2}; tdTomato* Tamoxifen (TM)-injected animals.** (**a**) In non-wounded skin single traced cells are not scattered in the dermis, but are rather associated with nerve bundles (NBs). Boxed area in the dermis is magnified in the inset showing labelled NBs. (**b**) Glial lineage tracing of *Plp-CreER^{T2}; tdTomato* skin at D14 post-injury. Inset displays magnified boxed area of wound bed, arrows denote the presence of multiple individual traced cells populating the wound. (**c-e**) Immunofluorescence staining of skin NB for the transcription factor Sox10 and the extracellular matrix protein Laminin (Lam) in intact (**c**) and D7 injured skin (**d,e**) shows disruption of perineurium. Arrows in the inset (**e**) denote single Sox10^{+} cells proximal to disrupted NB. Scale bars, 50µm.

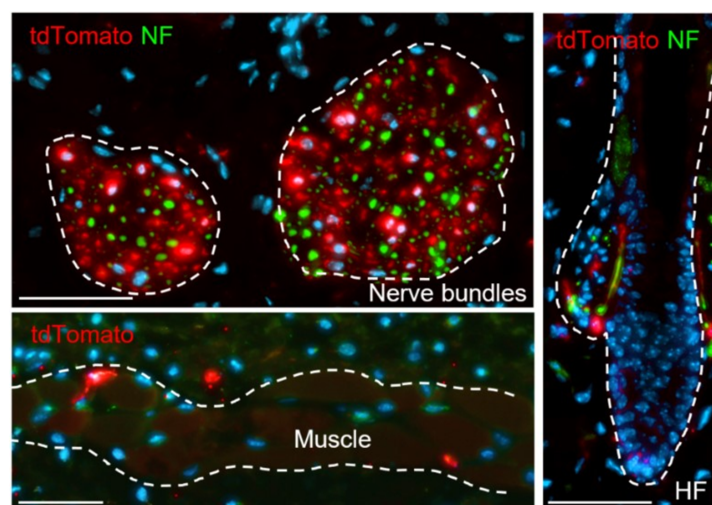

Figure 3.3. ***Plp-CreER^{T2}*** **glial lineage tracing in intact skin.** tdTomato reporter expression in nerve bundles, between muscle fibres and around hair follicles (HF). Scale bars, 50µm.

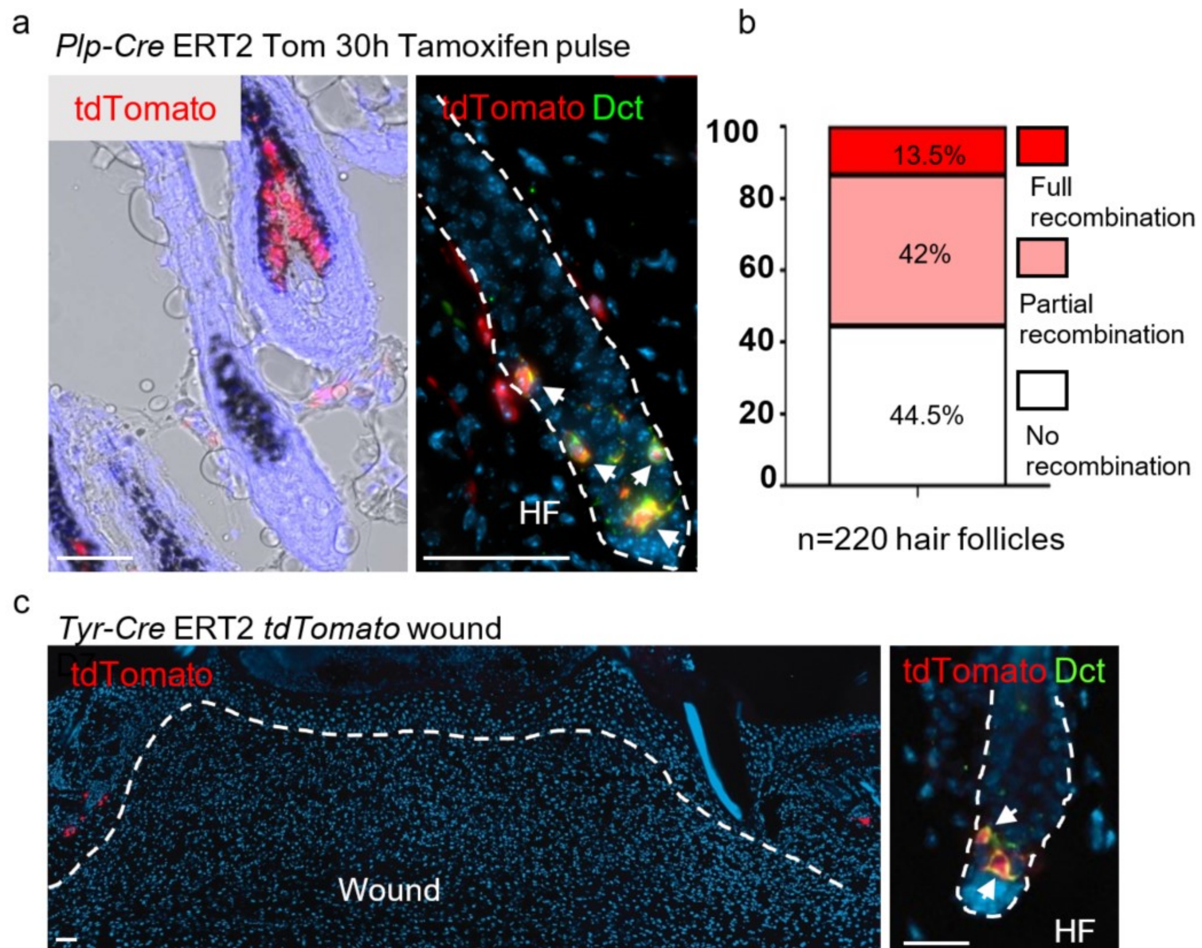

Figure 3.4. **Absence of melanocytic lineage contribution in the wound bed.** (**a**) *Plp-CreER^{T2}* driven recombination of anagen murine skin shows only partial tdTomato reporter expression in HFs, 30h post Tamoxifen injection. Brightfield image of a frozen section on the left and co-labelling of tdTomato (red) and Dct (green) on the right. Arrows denote tdTomato-Dct double positive cells. (**b**) Quantification of percentage of hair follicles displaying no, partial or complete *Plp-CreER^{T2}* mediated recombination of their respective melanocytic population, 30h post-TM injection. (220 HFs counted). (**c**) Immunolabeling for tdTomato reporter of *Tyr-CreER^{T2}* traced cell population (melanocyte) in D7 wound bed and adjacent hair follicle shows no detectable presence of traced melanocytes in the granulation tissue upon injury. Sections were counter stained with Hoechst 33258 (blue). Scale bars, 50µm.

Apart from recombination in NBs, nerve terminals around hair follicles, as well as in the nerve endings between muscle fibres (Fig. 3.3), we observed *Plp-CreER^{T2}*-recombined cells within hair follicles. These cells were pigmented and also expressed dopachrome tautomerase (Dct),

a marker for melanocytes (Fig. 3.4 a). Around 55.5% of hair follicles contained recombined cells in melanocyte compartment when quantified after 30h pulse of TM in anagen skin (Fig. 3.4 b).

Because *Plp-CreER*^{T2}*,* not only marks peripheral glia, but labels also a subpopulation of melanocytes within the skin (Hari et al. 2012), we aimed to assess the contribution of melanocytic cells to formation of the wound tissue. To do so, we performed wounding experiments in *Tyr-CreER*^{T2}*; tdTomato* animals, in which the melanocytic lineage can be traced *in vivo* (Bosenberg et al. 2006; Shakhova et al. 2012). However, unlike in *Plp-CreER*^{T2} model, we were unable to detect any tdTomato^{+} cells in the wound bed of these animals, suggesting that the melanocytic lineage does not contribute to the cell population traced by *Plp-CreER*^{T2} upon injury (Fig. 3.4 c).

Within the wound bed we observed certain NBs with distorted morphology with some Sox10^{+} and glial traced cells seemingly leaving the nerve, but it was difficult to make conclusion on origin of these cells based on 5 µm thick histological sections. To confirm that NBs indeed serve as a source for cells homing to the wound bed in injured mouse skin, we made use of a separate Cre line, *Dhh-Cre*, which efficiently labels the glial lineage in the peripheral nervous system (Jaegle et al. 2003; Joseph 2004).

To visualize the glial lineage before and after skin injury, we employed confocal 3D-imaging techniques combined with a fructose-based tissue clearing protocol of *Dhh-Cre; tdTomato* animals (Ke et al. 2013). Using this method in intact skin we observed a network of tdTomato^{+} NBs, while the stroma in-between the nerve fibres was almost devoid of labelled cells (Fig. 3.5 a). In contrast, the wounded skin displayed many single cells apparently stemming from the injured nerves at the wound edge, already 6 days post injury (D6) and by day 14 (D14), the entire wound bed was densely populated by *Dhh-Cre*-traced cells (Fig. 3.5 b,c).

Similar to the Sox10^{+} cell population observed in Plp-CreERT2-tdTomato mice (Fig. 3.2 c-e), cells traced by *Dhh-Cre* appeared to emigrate from NBs upon injury (Fig. 3.5 b, 3.6 a,b). Of note, some, but not all traced cells, were positive for glial acidic fibrillary protein (GFAP), confirming their glial identity. Interestingly, the majority of NB-derived cells found in the wound both on D7 (90.2%) and D14 (63.9%) were not associated with axons, as shown by double labelling for Sox10 and neurofilament (NF) staining (Fig. 3.6 c). Taken together, data from separate tracing methods show that cells derived from peripheral nerves disseminate into

the healing tissue upon wounding of the skin and by D14 mostly remain dissociated from the axons.

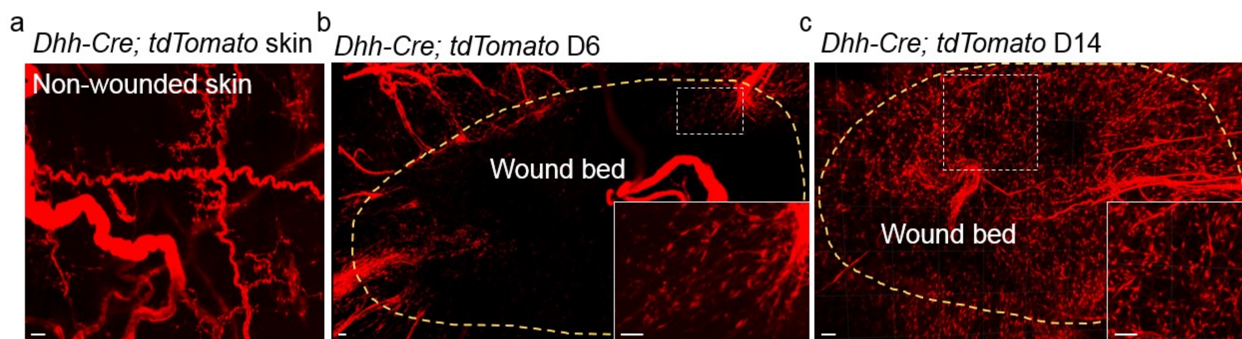

Figure 3.5. **3D imaging of the glial lineage in *Dhh-Cre; tdTomato* animals.** (**a-c**) Cleared intact and wounded mouse skin at D6 and D14 after injury. Individual traced cells detaching from NB are visible at D6 (**b**). Skin is populated by *Dhh-Cre* traced cells at D14 (**c**). Insets show magnification of boxed area in the wound (**b**,**c**). Scale bars, 50µm.

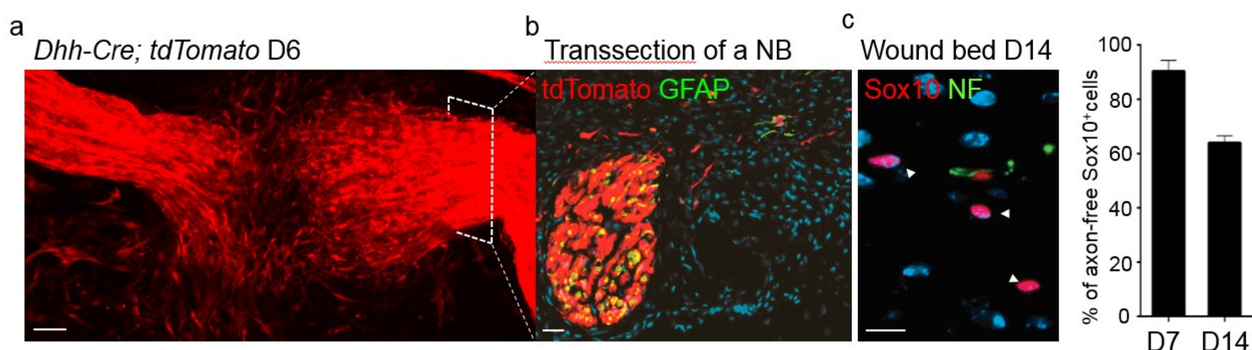

Figure 3.6. **Glial cell migration from NB and their association with the axons.** (**a,b**) Damaged sprouting nerve was subsequently sectioned and immunolabeled for the lineage tracer tdTomato and the glial marker GFAP. (**c**) D7 and D14 wounded skin samples were immunolabeled to quantify the amount of glial cells (Sox10^{+}) detached from Neurofilament (NF) positive axons. Arrows denote glial cells with no adjacent NF staining and considered axon free. Quantification shows the vast majority of Sox10^{+} cells are not associated with axons at D7 and D14 (number of animals (N)=4, number of wounds (n)=8 (D7); N=4 animals, n=7 wounds (D14). Scale bars, 50µm (a,b), 10 µm (c).

3.2 Wounding induces peripheral glial de-differentiation and proliferation

Schwann cells in peripheral nerves possess remarkable plasticity and in response to nerve injury have been shown to be able to activate or de-differentiate, proliferate and later re-differentiate to support axon sprouting (Jessen et al. 2015).

To clarify the identity of nerve-associated cells populating skin wounds, we performed several immunohistological stainings for markers known to be associated with different Schwann cell states. Based on these experiments, we identified two classes of NBs located adjacent to or within the wound, which we term 'quiescent' and 'activated', respectively (Fig. 3.7 a). 'Quiescent' NBs were characterized by intact perineurial laminin expression and a uniform morphology, similar to that seen in non-wounded skin (Fig. 3.2 a). They expressed high levels of myelin basic protein (MBP), and axonal NF, but showed weak expression of the low affinity nerve growth factor receptor (p75) (Fig. 3.7 a,a').

In contrast, 'activated' NBs were often associated with a distorted NB morphology, displayed increased levels of p75 protein expression and stained only weakly for MBP and NF. These features resembled the processes of myelin degradation and axon retraction in those NBs, a phenomena seen in Wallerian degeneration. Further, single cells positive for p75 were seen next to activated NBs, seemingly emigrating from the nerve bundle (Fig. 3.7 a'').

We wanted to further characterize these different NBs using glial markers and markers of Schwann cell activation. In activated NBs we found lower expression levels of glial fibrillary acidic protein (GFAP) (Fig. 3.8a). Only activated NBs and cells dissociating from them were positive for c-Jun (Fig. 3.8 b) and pERK (Fig. 3.8 c) – markers associated with Schwann cell plasticity and de-differentiation (Harrisingh et al. 2004; Napoli et al. 2012; Arthur-Farraj et al. 2012). Importantly, cells within activated NBs displayed increased proliferation rate (quantified as percentage of $Ki67^{+}$ cells) compared to quiescent nerves, which were mostly composed of terminally differentiated non-proliferative cells (Fig. 3.8 d,e).

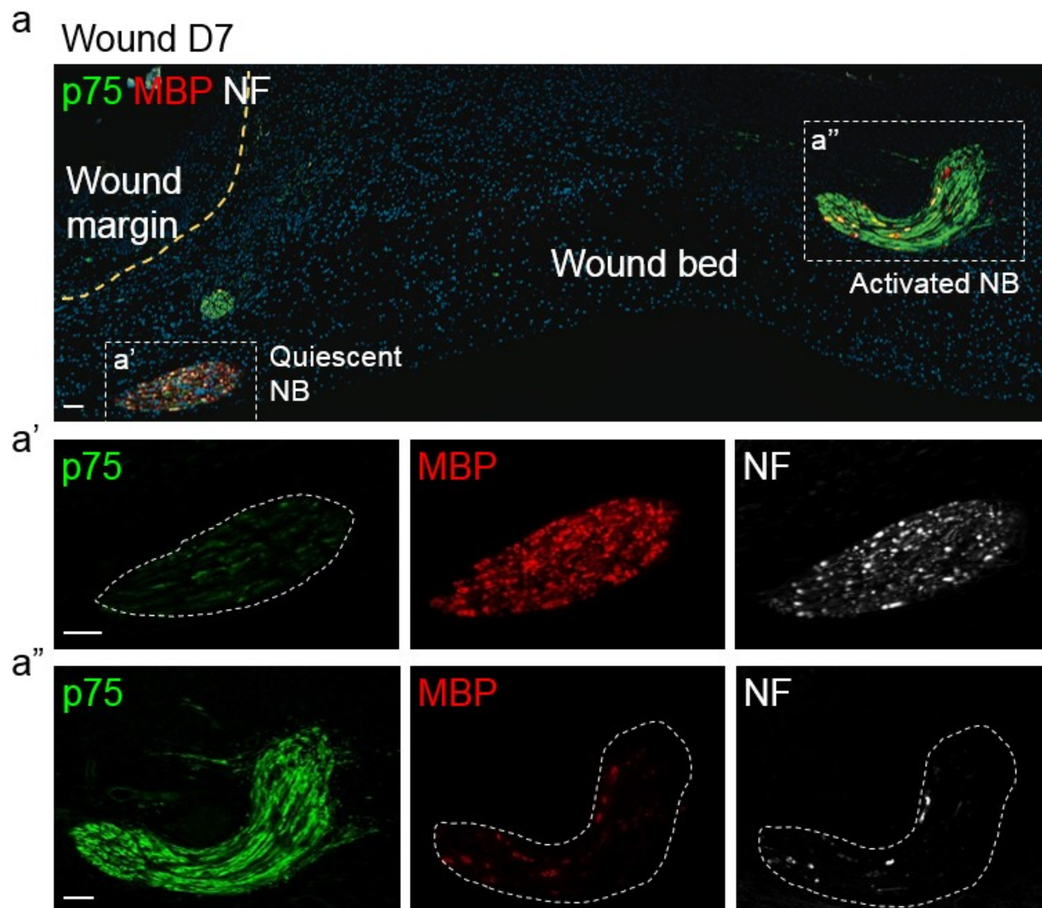

Figure 3.7. **Skin injury induces peripheral glial de-differentiation in 'activated' NBs.** (**a**) p75 (green), MBP (red) and NF (white) triple immunolabeling of wound sections show increase of de-differentiation marker p75 and loss of differentiation marker MBP as well as axon retraction as shown by loss of NF in certain NBs that we termed 'activated'. Single channel insets show expression of p75, MBP and NF in activated and quiescent NBs in injured skin (**a'**, **a''**). Scale bars, 50µm.

These data demonstrate that wounding of murine skin results in de-differentiation and active proliferation of peripheral glial cell population and suggest that this process allows peripheral glia to dissociate and emigrate from injured nerves.

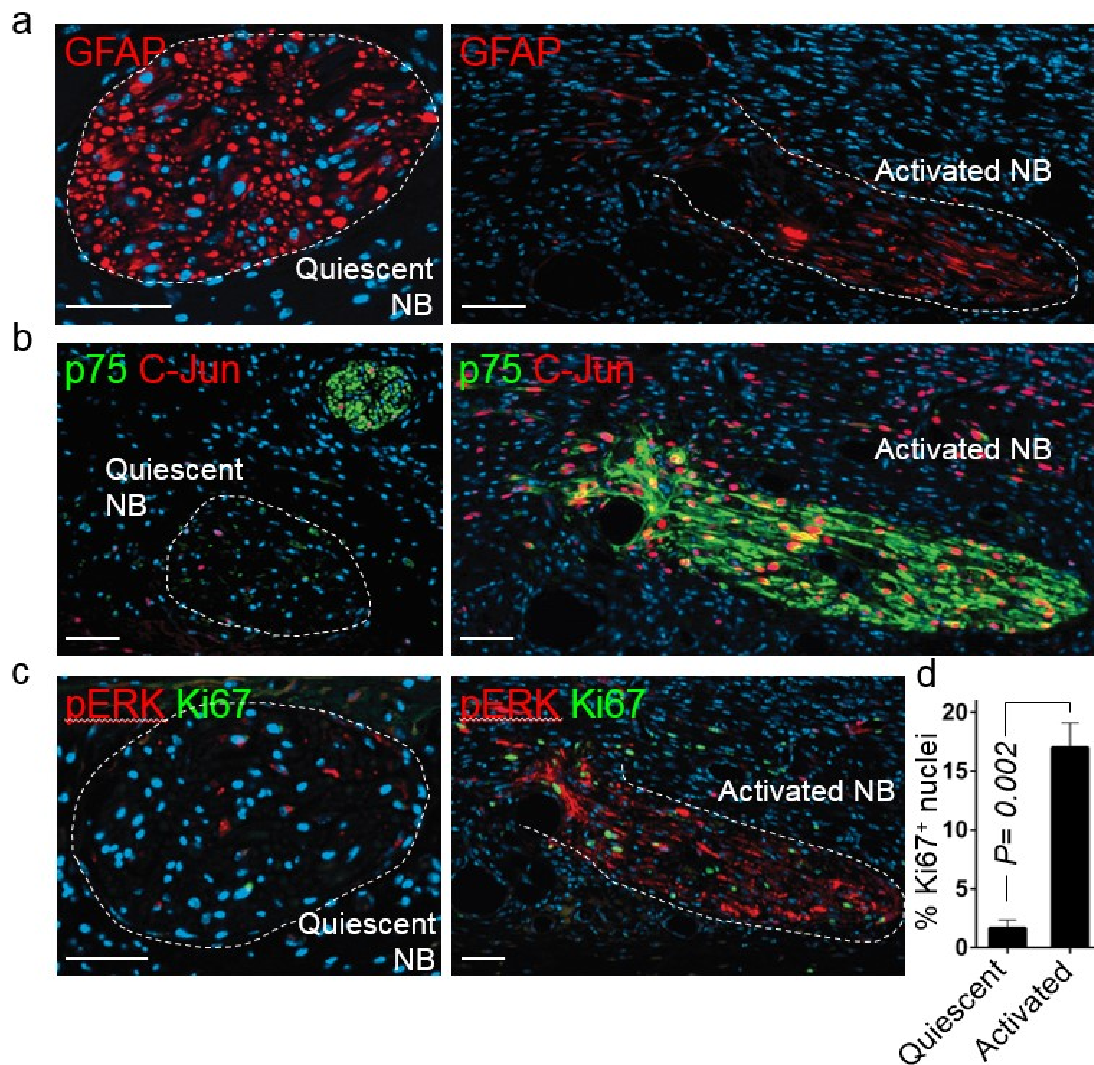

Figure 3.8. **Skin injury induced glial de-differentiation and proliferation.** (**a**) Immunolabeling of GFAP (red) of wound sections show a decreased marker expression in activated NB compared to quiescent NB. (**b**) Double immunolabeling of p75 (green) and c-Jun (red) shows upregulation of both markers in activated versus quiescent NB in injured skin (red). (**c**) Immunolabeling of pERK (marker of activated MAPK signaling pathway) and Ki67 (marker for proliferative or cycling state) in quiescent versus activated NB in injured skin. (**d**) Quantification of the percentage of nuclei (Hoescht^{+}) positive for Ki67 marker show increased proliferation in activated versus quiescent NBs of injured skin. Data are represented as mean±SEM of 8 quiescent NBs from 4 wounds (2 animals) and 14 activated NBs from 6 wounds (4 animals). Scale bars, 50µm. All samples are D7 post injury.

Following the fate of these nerve-derived cells at later time points post injury, to see if they would adopt other neural crest-related cell phenotypes, we found virtually no genetically traced cells of glial origin positive for either melanocytic or myofibroblast markers up to 14 days after injury, except for a minor fraction of traced cells labelled with α-SMA^{+} at D7 after injury (Fig. 3.9).

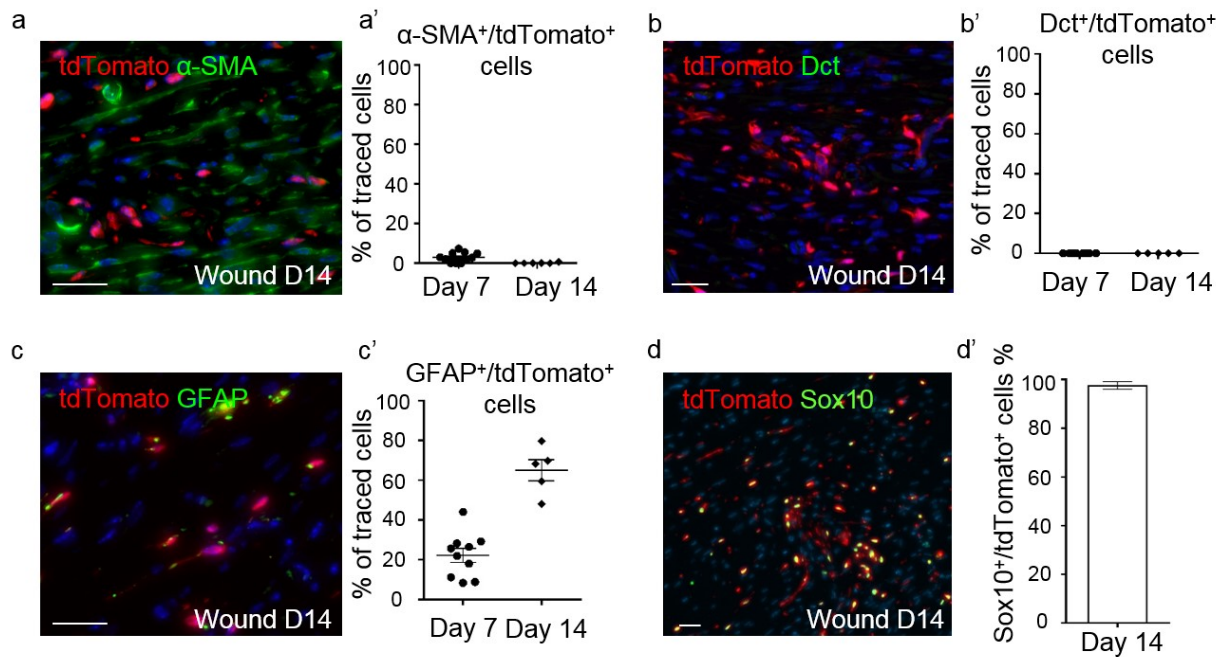

Figure 3.9. **Glial lineage restriction of Plp-CreERT2 traced cells upon injury.** *Plp-CreER^{T2}* traced cells do not differentiate into neural crest derivatives other than glia upon injury. (**a**) Granulation tissue immunofluorescence staining of *Plp-CreER^{T2}* traced cells using tdTomato labelling (red) and of myofibroblasts marked by alpha smooth muscle actin (α-SMA) (green). Quantification of the percentage of α-SMA$^+$ per tdTomato$^+$ cells in the granulation tissue of injured skin at D7 (N=3 animals, n=11 wounds) and D14 (N=2, n=6). (**b**) Granulation tissue immunofluorescence staining of *Plp-CreER^{T2}* traced cells using tdTomato labelling (red) and of melanocytes marked by Dopachrome tautomerase (Dct) (green). (**b'**) Quantification of the percentage of Dct$^+$ per tdTomato$^+$ cells in the granulation tissue of injured skin at D7 (N=3, n=8) and D14 (N=2, n=5) (**c**) Granulation tissue immunofluorescence staining of *Plp-CreER^{T2}* traced cells using tdTomato labelling (red) and of glia marked by glial fibrillary acidic protein (GFAP) (green). (**c'**) Quantification of the percentage of GFAP$^+$ per tdTomato$^+$ cells in the granulation tissue of injured skin at D7 (N=3, n=10) and D14 (N=2, n=6). (**d**) Granulation tissue immunofluorescence staining of *Plp-CreER^{T2}* traced cells using tdTomato labelling (red) and of Sox10$^+$ cells (green). (**d'**) Quantification of the percentage of Sox10$^+$ per tdTomato$^+$ cells in the granulation tissue of injured skin at D14 (N=2, n=6). Data are represented as mean±SEM. Scale bars, 25µm.

This suggests that injury to the skin induces glial reactivation and de-differentiation to a cell state reminiscent of adult neural crest stem cell-like cells (Shakhova & Sommer 2010; Toma et al. 2001; Fernandes et al. 2004; Wong et al. 2006). However, reactivated peripheral glial cells, which can disseminate into the wound bed, retain a certain lineage restriction under our experimental settings (Fig. 3.9). If this glial population does not contribute to the myofibroblast

pool, a cell population well known to benefit wound healing (Hinz et al. 2012), it raises the question of what the role of these cells during wound healing is.

3.3 Reactivated glia secrete a cocktail of factors implicated in wound healing

Given that glial cells do not contribute to wound healing structurally by transdifferentiation into a particular cell type important for wound repair, we hypothesized that these cells could still provide support by paracrine secretion of factors or microenvironment modulation of the healing surrounding tissue, as it has been previously shown in amphibian and mammalian regeneration models (Kumar & Brockes 2012; Johnston et al. 2016). To identify these factors, we performed RNA sequencing of *Dhh-Cre* traced nerve-derived tdTomato$^+$ cells isolated by fluorescence activated cell sorting (FACS) from uninjured control animals and 7 Days post injury (Fig. 3.10 a).

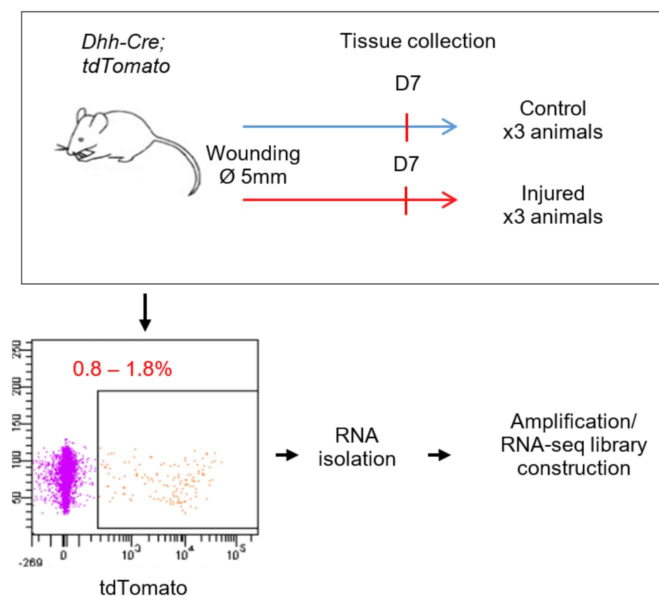

Figure 3.10. **Experimental design of skin sample collection for RNS sequencing.** Single cell suspensions were sorted for tdTomato expression using a flow cytometer from non-wounded skin and D7 wound tissue samples of *Dhh-Cre* animals (0.8 to 1.8% of total cell fraction in wound samples). Total RNA of gated cells were amplified and subsequently subjected to RNA seq using Illumina RNA seq platform.

Of the 1033 genes found to be differentially expressed, 526 were upregulated and 506 downregulated (Fig. 3.11 a,b) Among the genes downregulated in nerve-derived cells upon injury, we found several genes regarded as positive regulators of myelination pathway, such as

Egr2 and *Pou3f1/Oct6,* myelin genes and other genes – *Mbp, Mal,* and *Cdh1*, all previously reported to be repressed in Schwann cells upon injury and de-differentiation (Mirsky et al. 2008; Buser et al. 2009; Crawford et al. 2008). Further, we found supportive data among the genes significantly upregulated upon injury, including *Ngfr*, *Gap43, Thy1, Igfbp2, Mdk, Pdgfb, Runx2, Cxcr4,* which have been reported to be upregulated in Schwann cells upon injury in previous studies (Fontana et al. 2012; Mirsky et al. 2008; Ma et al. 2015; Le et al. 2005; Oya et al. 2002).

Consistent with previous reports, we observed similar regulation of genes implicated in axon growth and guidance, including upregulation of *Epha5*, *Sema4f* and downregulation of *Nfasc*, *Nrn1*, and *Atp1b2* (Barrette et al. 2010). We also found a set of genes previously described as important players in Schwann cell-mediated innate immune response, chemotaxis, and myelin phagocytosis such as *Lif, Cxcl5, Areg, Megf10, Mmp14*, which all followed a similar regulation pattern in our data set as previously described after sciatic nerve injury (Arthur-Farraj et al. 2012; Tofaris et al. 2002; Fontana et al. 2012).

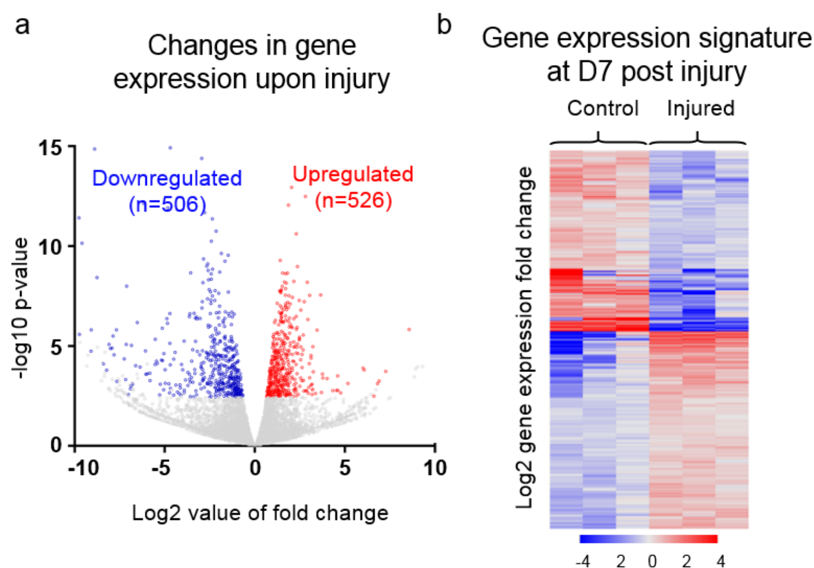

Figure 3.11. **Gene expression signature after injury.** (**a**) Volcano plot for the gene expression profile of *Dhh-Cre*-traced tdTomato^{+} cells of intact skin and D7 wounds. Coloured data points meet the thresholds of FC above 1.5 and under -1.5, pValue<0.05, FDR<0.05. (**b**) Unsupervised sample clustering heat map of gene expression with a significant differential expression.

Upon nerve injury, some of the crucial functions of denervated Schwann cells include the participation in the immune response and axon guidance. To do so, Schwann cells upregulate chemokines as well as growth factors in order to promote inflammation and axon sprouting

respectively. Because we hypothesized that our population of interest could exert its function through secretion of soluble factors, using Metacore analysis from GeneGO, we focused on the predicted localization of the gene products that were differentially expressed between non-wounded and wounded states. Intriguingly, extra cellular space and membrane come as top hits. While most of the annotated genes from downregulated gene list code for proteins important in cell-cell contacts and adhesion, products of the upregulated genes tend to be secreted and associated to the extracellular matrix and extracellular space (Fig. 3.12 a,b).

In fact, among the downregulated genes we detected a 2-fold enrichment in those coding for extracellular space/ECM-destined protein products – 14.6% compared to 7.2% observed among all expressed genes (n=16718 genes). In contrast, consistent with our hypothesis of a paracrine function of Schwann cells, upregulated gene fraction displays a 4.9-fold enrichment in extracellular space/ECM related genes upon injury – up to 35.5% (Fig. 3.12 c).

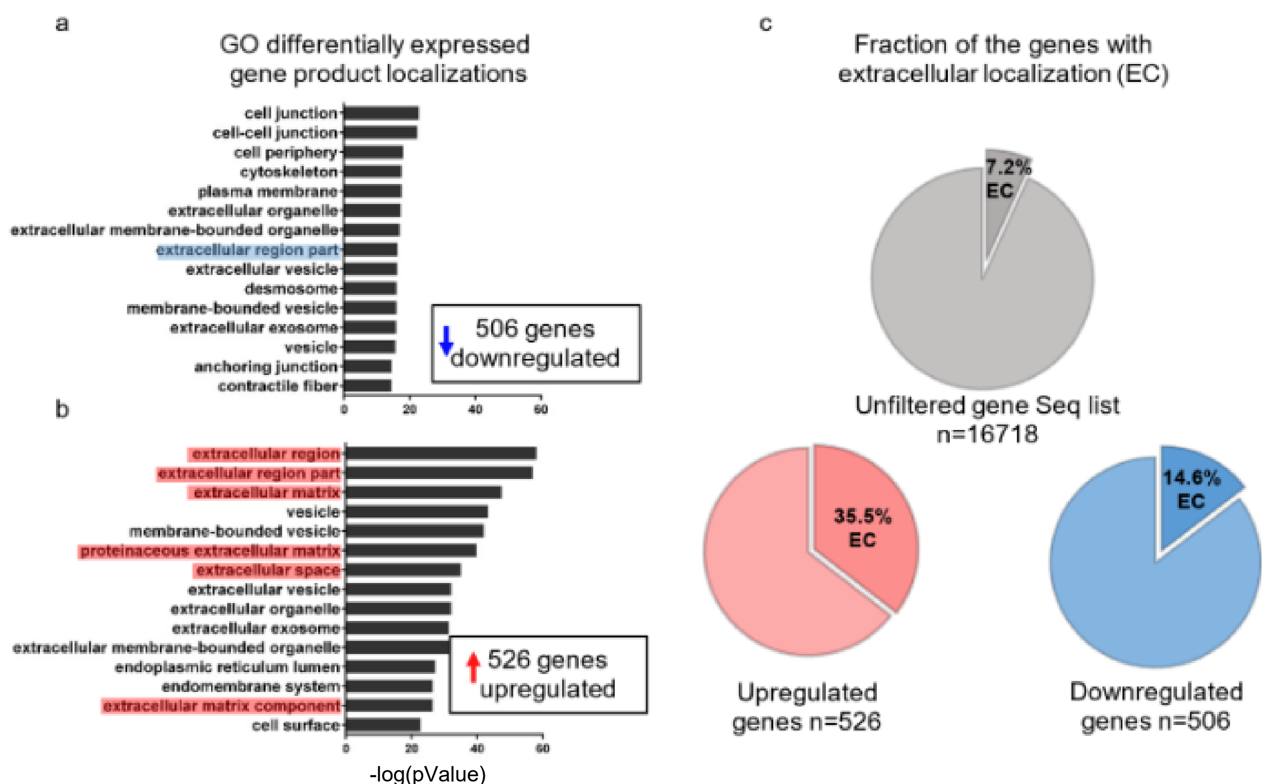

Figure 3.12. **Gene GO Metacore prediction of the gene product localization.** (**a,b**) Analysis of top localizations for the significantly downregulated (506 genes) and upregulated genes (526 genes) shows enrichment for excreted products. (**c**) Data representation for the percentage of gene product predicted to be destined to the extra cellular space including unfiltered expressed gene set.

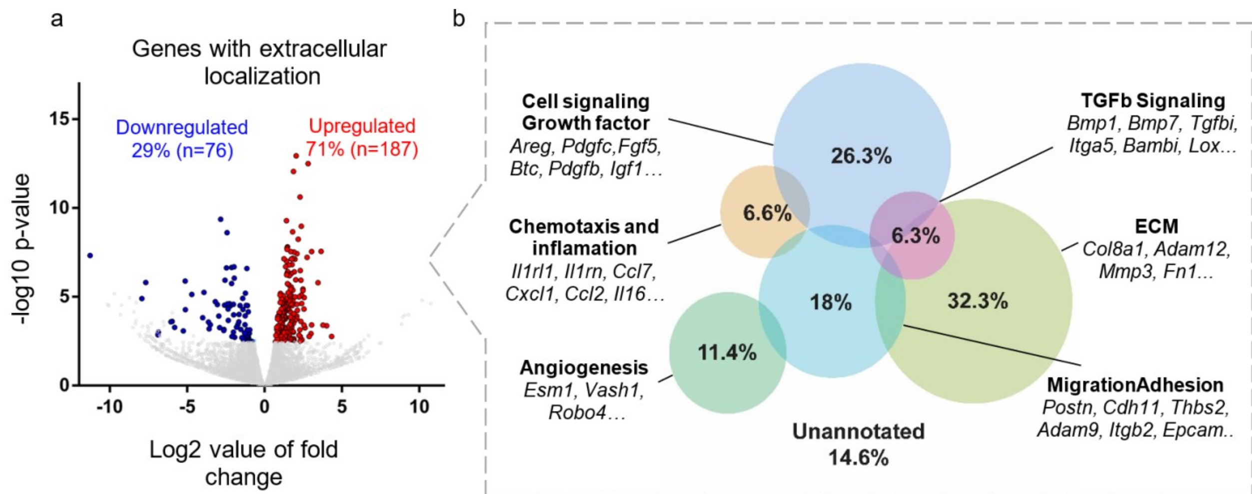

Figure 3.13. **Distribution of genes with EC localization and literature-based clustering of these genes based on their predicted function.** (**a**) Volcano plot of the expression value of the genes with predicted extracellular localization of their product. (**b**) Quantitative Venn diagram of the differentially expressed "secretome" and their associated biological function.

When considering all differentially expressed genes coding for proteins with extracellular localization (n=263), 71% of them were upregulated (n=187) and 29% downregulated (n=76) (Fig. 3.13 a). We clustered these 263 genes according to their function based on the previous reports in the literature and annotations given in GeneCards (www.genecards.og). Depending on their described role the upregulated genes we included following categories: (1) growth factors and cell signalling, (2) TGF-β signalling, (3) chemotaxis and inflammation, (4) migration and adhesion, (5) ECM remodelling, (6) angiogenesis. Interestingly, many genes from this list are implicated in different processes important for wound healing (Fig. 3.13 b and Supplementary Table S1-6).

3.4 Genetic depletion of injury-reactivated glia impairs wound healing

To assess the role of activated glial cells in an *in vivo* context and because they appear to secrete a diverse cocktail of factors, we decided to deplete them using genetic tools. Sox10 is an important survival and maintenance factor for the Schwann cell lineage during all stages of its differentiation (Britsch et al. 2001; Paratore et al. 2001).

In the skin, Sox10 is expressed in the melanocytic lineage (confined to hair follicles in murine back skin) and the glial lineage (Harris et al. 2013; Shakhova et al. 2012). To deplete nerve-derived cells in skin wounds and/or interfere with their migration into the wound, we bred *Plp-CreER*^{T2} mice with mice carrying floxed alleles of the *Sox10* gene to specifically delete *Sox10* in the glial linage (Fig. 3.14 a). Since effective depletion of SOX10 protein upon genetic ablation of the allele is a relatively slow process to achieve in adult mice (Bremer et al. 2011), we allowed three weeks post-TM injection before we performed excision wounds on both *Sox10* conditional knock-out (cKO) and control littermates. Tissue samples were collected for analysis on several time points as depicted in (Fig. 3.14 a).

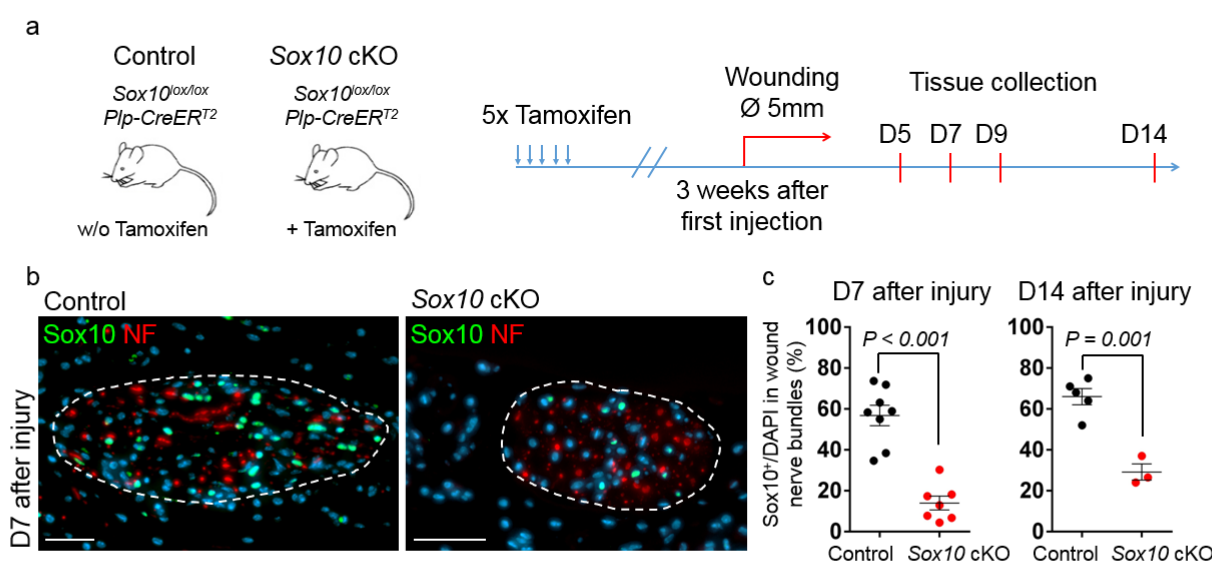

Figure 3.14 **Sox10 ablation in glial lineage – NB analysis.** (**a**) Scheme of the experimental strategy for *Plp-CreER*^{T2}-mediated ablation of *Sox10* in murine wound healing model. (**b**) Immunolabeling of Sox10 (green) and NF (red) in skin sections shows reduction of Sox10^{+} population in NBs upon *Sox10* cKO. (**c**) Quantification of the percentage of Sox10^{+} nuclei in NBs of injured skin of control and *Sox10* cKO animals at D7 and D14. Data are represented as mean±SEM of N=4, n=8 (control D7), N=3, n=5 (control D14); N=4, n=7 (*Sox10* cKO D7), N=3, n=3 (*Sox10* cKO D14) Scale bars, 50µm.

NBs of *Sox10* cKO animals visually appear similar to those of control animals and in 'quiescent' NBs preserve axons, as shown by NF staining (Fig. 3.14 b). To verify that we achieved depletion of glial cells in the nerves, we quantified Sox10^{+} cells within the NBs located close to the wound and found that 56.8±4.9% of all cells were Sox10^{+} in control versus 14.0±3.4% in cKO mice at D7 (Fig. 3.14 c).

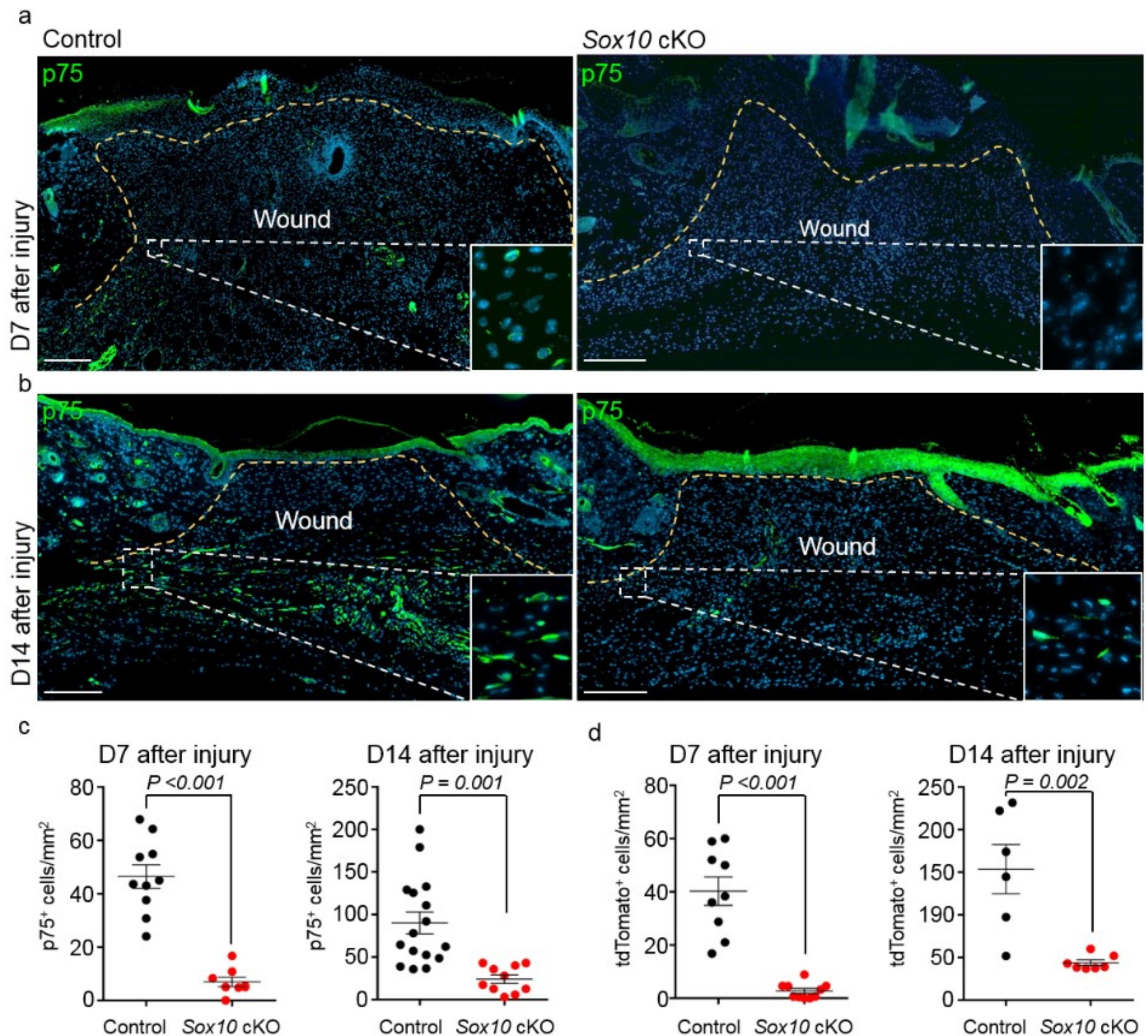

Figure 3.15 **Depletion of glial cell in the wound bed**. (**a**,**b**) Immunolabeling of p75 (green) in skin wound sections at D7 and D14 reveals a depletion of p75$^+$ population upon *Sox10* cKO. Boxed regions in the granulation tissue (delimited by the dotted lines) are shown at higher magnification in the insets. (**c**,**d**) Quantification of p75$^+$ and tdTomato$^+$ cells per mm^2 of granulation tissue of injured skin shows persistent reduction of both p75$^+$ and traced cells upon *Sox10* ablation at D7 and D14. Data are represented as mean±SEM of N=3, n=10 (control D7), N=8, n=16 (control D14); N=4, n=8 (*Sox10* cKO D7), N=5, n=10 (*Sox10* cKO D14) (**c**); N=4, n=9 (control D7), N=2, n=6 (control D14); N=3, n=10 (*Sox10* cKO D7), N=2, n=7 (*Sox10* cKO D14) (**d**) Scale bars, 200 µm.

To assess the number of reactivated glia in the wounds after Sox10 cKO we performed immunofluorescence analysis and quantification of p75$^+$ cells in the wound bed at two different time points (Fig. 3.15). The data showed a significant decrease of p75$^+$ cells in *Sox10* cKO animals on both D7 (46.5±4.4 vs. 7.0±1.8 p75$^+$ cells/mm^2 and 40.2±5.4 vs. 2.8±0.9 tdTomato$^+$

cells/mm^2 in Control vs. *Sox10* cKO respectively) and on D14 after wounding (90.2±12.8 vs. 24.1±5.0 p75$^+$ cells/mm^2 and 153.6±28.8 vs. 43.9±3.3 tdTomato$^+$ cells/mm^2 in Control vs. *Sox10* cKO respectively) (Fig. 3.15 c,d). Thus, *Sox10* cKO in the glial linage using *Plp-CreER^{T2}* efficiently decreased the number of peripheral nerve-derived cells in the wound. Similar results were obtained when traced cells where quantified (Fig. 4d).

To study possible effects caused by genetic depletion of nerve-derived cells in the wound bed, we performed macroscopic measurements and morphometric analysis of control and *Sox10* cKO wounds. Although the dynamics of wound contraction can be variable, as previously described (Ansell et al. 2014) we found a significant difference in the mean wound size at D7 (14.2±1,6% vs. 22.2±3.1% of initial size in control vs. *Sox10* cKO respectively) as well as at D9 (4.8±1.2% vs. 11.1±3.0% of initial size in control vs. *Sox10* cKO respectively) (Fig. 3.16 a,b).

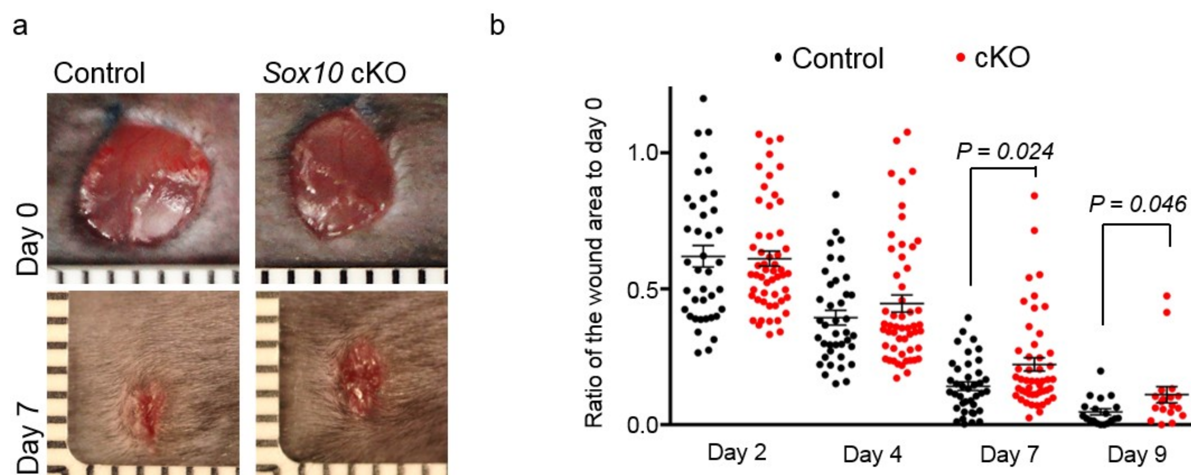

Figure 3.16. **Depletion of glial cells by genetic means leads to impaired wound closure as measured by macroscopic wound area analysis**. (**a**) Representative macroscopic illustration of wound healing in control and *Sox10* cKO animals at D0 and D7. (**b**) Longitudinal macroscopic quantification of individual wound areas at D2, D4, D7 and D9 in control and *Sox10* cKO animals in relation to their respective initial size at D0. Data show a significant delay in wound closure at D7 upon *Sox10* cKO. Data are represented as mean±SEM of N=10, n=39 (Control), N=13, n=49 (*Sox10* cKO).

Since the most significant difference in wound contraction is observed at D7, we carried out morphometric analyses of D7 wound sections to assess if other commonly measured wound closure parameters such as re-epithelialization and the area of hyperproliferative epidermis were changed. The principle of measurements and representative pictures of wounds at these

stage are shown (Fig. 3.17 a,b). Quantification of the distance that newly-formed epidermis has migrated to cover the wound revealed a delay in wound closure, in *Sox10* cKO animals – an average of 84% of the wound was covered by epidermis in control while only 66% of the wound was covered in *Sox10* cKO at the same time point (Fig. 3.17 c Likewise, the area of hyper-proliferative epidermis was significantly decreased (0.282±0.017 vs. 0.220±0.015 mm^2) in *Sox10* cKO wounds (Fig. 3.17 d). Importantly, in the absence of activated glia, the wound width was increased (1.99±0.08 vs. 2.46±0.08 mm), while the length of wound epithelium remained unchanged in *Sox10* cKO animals compared to controls, suggesting impaired contraction (Fig. 3.17 e,f). Taken together, this data suggest that reactivated glia is required for proper wound healing to take place.

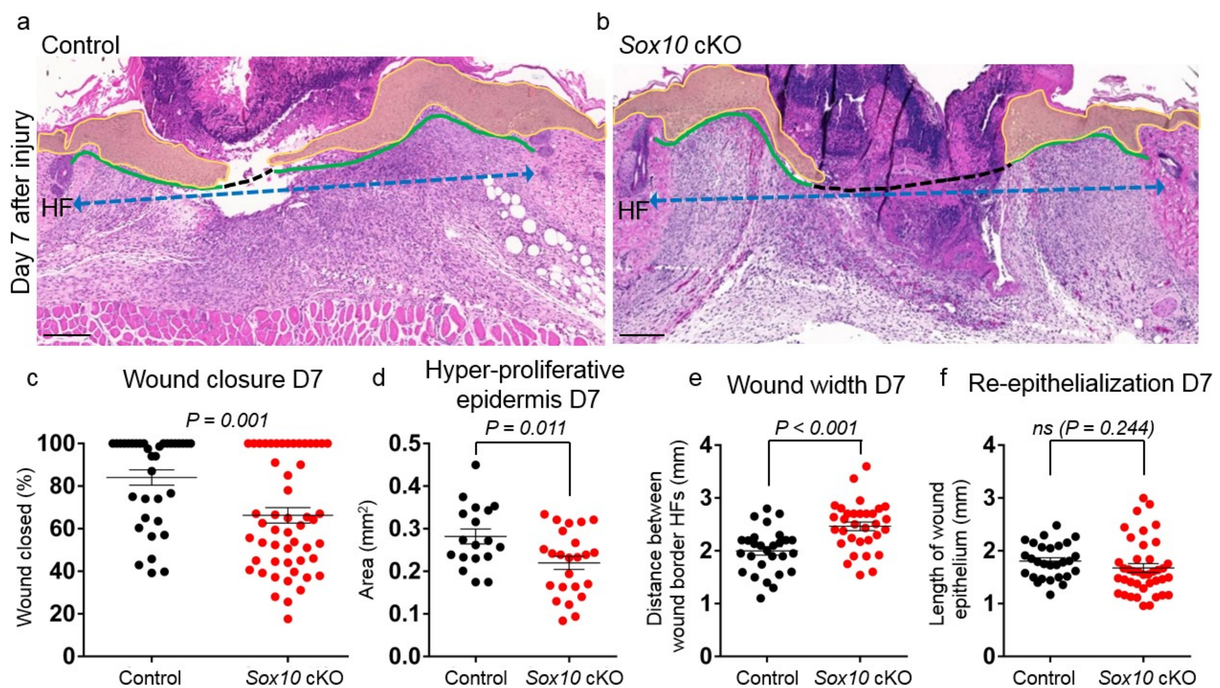

Figure 3.17. **Morphometric analysis of wound closure in control and Sox10 cKO animals. (a,b)** Haemotoxylin-Eosin stained sections of control and *Sox10* cKO D7 wounds used for morphometric analysis of percentage of wound closure (length of wound epithelium (green lines)/length of wound epithelium + length of gap between edges of wound epithelium (black dotted line)*100), area of hyper-proliferative epidermis (area highlighted in yellow), wound contraction (distance between wound border HFs (blue dotted line)) and re-epithelialization (length of wound epithelium (green lines)). Analysis of D7 wound sections for the percentage of (**c**) wound closure, (**d**) area of hyper-proliferative epidermis, (**e**) wound contraction, (**f**) re-epithelialization. Data are represented as mean±SEM of N=10, n=39 (control), N=13, n=49 (*Sox10* cKO) (**b**); N=9, n=35 (control), N=13, n=52 (*Sox10* cKO) (**c**); N=5, n=18 (control), N=6, n=24 (*Sox10* cKO) (**d**); N=7, n=28 (control), N=8 n=32 (*Sox10* cKO) (**e**); N=7, n=28 (control), N=10, n=40 (*Sox10* cKO) (**f**). Scale bars, 200µm.

3.5 Genetic depletion of peripheral nerve-derived cells/reactivated glia is not sufficient to impair neovascularization

Based on our expression profiling, upregulated genes coding for secreted factors such as Apln, Esm1, Mdk, and Vcan, have previously been shown to influence angiogenesis, suggested that depletion of the cells could lead to decreased neovascularization (Supplementary Table S1, S6). However, genetic depletion of nerve-derived cells in the wound did not significantly change the number of blood vessels (Fig. 3.18 b) and blood vessel density (Fig. 3.18 c) in the granulation tissue when compared to control wounds.

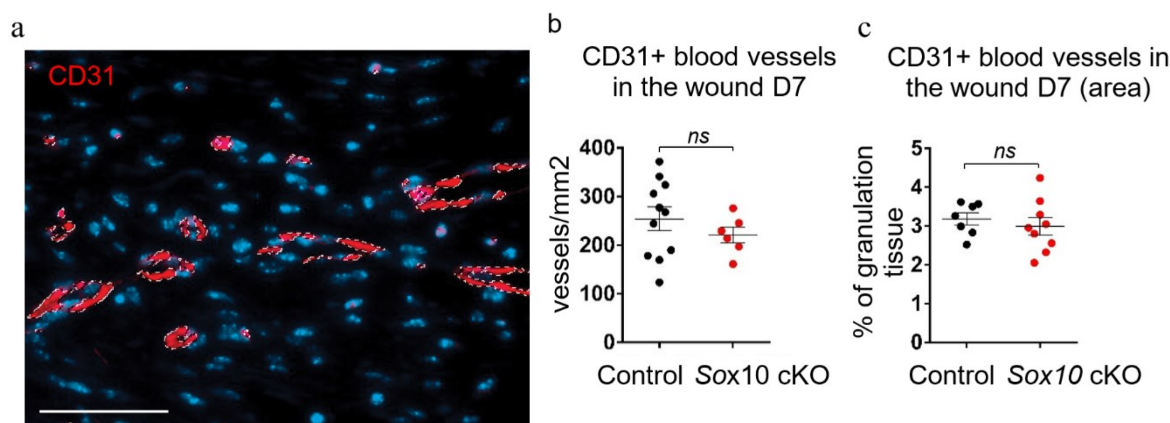

Figure 3.18. **Neovascularization is not affected upon loss of glial contribution to tissue repair**. Immunolabeling of CD31 (red) in granulation tissue at D7 (**a**) and related quantification of number of blood vessels/mm^2 (**b**) and percentage of CD31$^+$ area per total granulation tissue surface (**c**) in skin wounds at D7 did not display noticeable differences upon loss of *Sox10*. Data are represented as mean±SEM of N=3, n=11 (control), N=2, n=6 (*Sox10* cKO) (**b**); N=3, n=7 (control), N=6, n=9 (*Sox10* cKO) (**c**). Scale bar, 50µm.

3.6 Genetic depletion of peripheral reactivated glia is not sufficient to impair the immune response and collagen maturation

Further, we assessed the role of activated glia regarding immune cell chemotaxis, as the expression profiling revealed several genes coding for proteins known to be important for innate immune cell chemotaxis, such as *Cxcl5*, *Cxcl2*, *Lif* and *Trem2* among others (Supplementary Table S3). No detectable changes in the number of cells stained for pan-leukocyte marker CD45 or macrophage marker CD206 were observed upon manipulation of number of nerve-derived cells in the wound bed (Fig. 3.19).

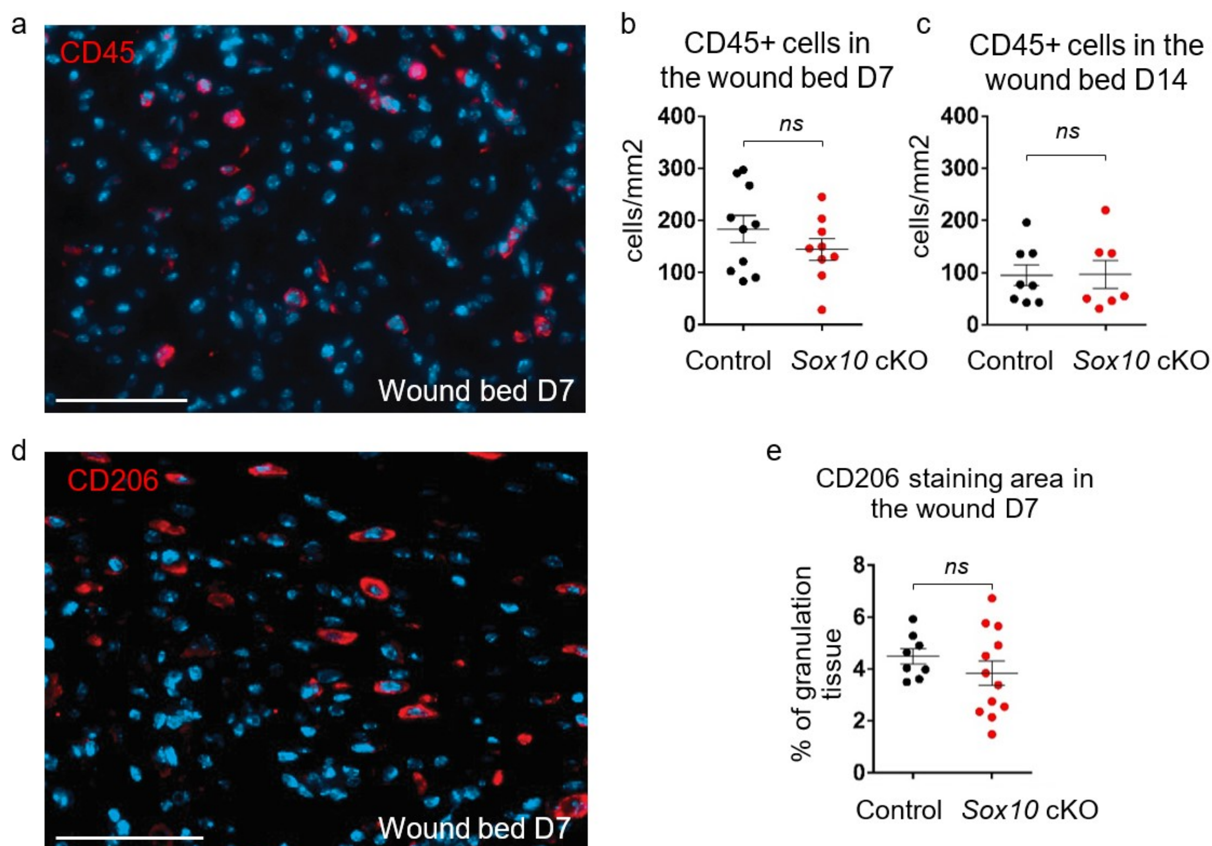

Figure 3.19. **Immune cell infiltration is not affected upon loss of glial contribution to tissue repair.** (**a**) Immunolabeling of CD45 (red) in granulation tissue at D7 and related quantification in skin wounds at D7 (**b**) and D14 (**c**) did not show any difference upon *Sox10* depletion. (**d**) Immunolabeling of CD206 (red) in granulation tissue at D7 and related quantification in skin wounds at D7 (**e**) showed no significant changes upon *Sox10* cKO in glial linage. Data are represented as mean±SEM of N=3, n=10 (control D7), N=5, n=8 (control D14); N=5, n=9 (*Sox10* cKO D7), N=4, n=7 (*Sox10* cKO D14) (**b,c**); N=5, n=8 (control), N=8, n=12 (*Sox10* cKO) (**e**) Scale bars, 50µm

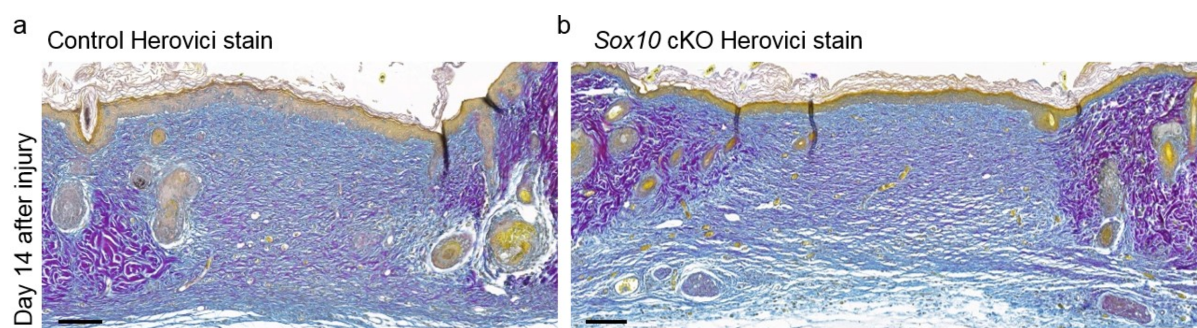

Figure 3.20. **Collagen maturation is not affected upon loss of glia in the wound.** Representative images of Herovici staining performed on D14 wound sections from control (a) and cKO animals (b). Scale bars, 100µm

Similarly, the above mentioned expression profile analysis underlined significant changes in the transcription of genes involved in ECM deposition (Supplementary Table S5). Hence, we investigated the state of collagen maturation of day 14 control and *Sox10* cKO wound sections using Herovici stainings. However, we could not detect obvious changes in young vs. mature collagen deposition between control and *Sox10* cKO animals with our experimental settings (Fig. 3.20).

3.7 Genetic depletion of peripheral nerve-derived cells/reactivated glia impairs the myofibroblast numbers through TGF-β signaling

In the wound granulation tissue, α-smooth muscle actin (α-SMA) is a marker for myofibroblasts, which, as previously mentioned, play an important role in wound healing by providing wound contraction and extracellular matrix protein (ECM) deposition (Klingberg et al. 2013). Our expression profiling analysis showed an upregulation of a number of genes important for TGF-β signalling regulation and myofibroblast formation, such as *Ltbp2*, *Mmp2*, *Bmp1*, *Loxl2*, *Plod1*, and *Plod2* (Supplementary Table S2). This prompted us to look at the area occupied by myofibroblasts post injury. Interestingly, the area of α-SMA expression was significantly reduced in *Sox10* cKO wounds at D7 (20.7±2.7% of the total granulation tissue area) compared to control (33.6±2.3%) (Fig. 3.21 a,b).

To investigate whether TGF-β signalling activation is indeed altered in skin wounds upon ablation of the glial cell population, we quantified the number of cells with prominent nuclear pSMAD2 localization in the granulation tissue of the wounds. pSMAD2 is a downstream marker of activated TGF-β signalling and for this quantification we considered all cells found in the α-SMA area, even if they were not expressing α-SMA, since TGF-β activation is prerequisite of fibroblast to myofibroblast conversion (Fig. 3.21 c). At D7, depletion of glial cell population led to decreased TGF-β signalling as shown by the decreased numbers of strongly-positive pSMAD2 nuclei in *Sox10* cKO wounds (11.29±0.8% vs. 24.1±1.8% in *Sox10* cKO and control respectively) (Fig. 3.21 d). This suggests that expansion of nerve-derived cells in the wound leads to a local increase in TGF-β signalling activation at early stages post injury.

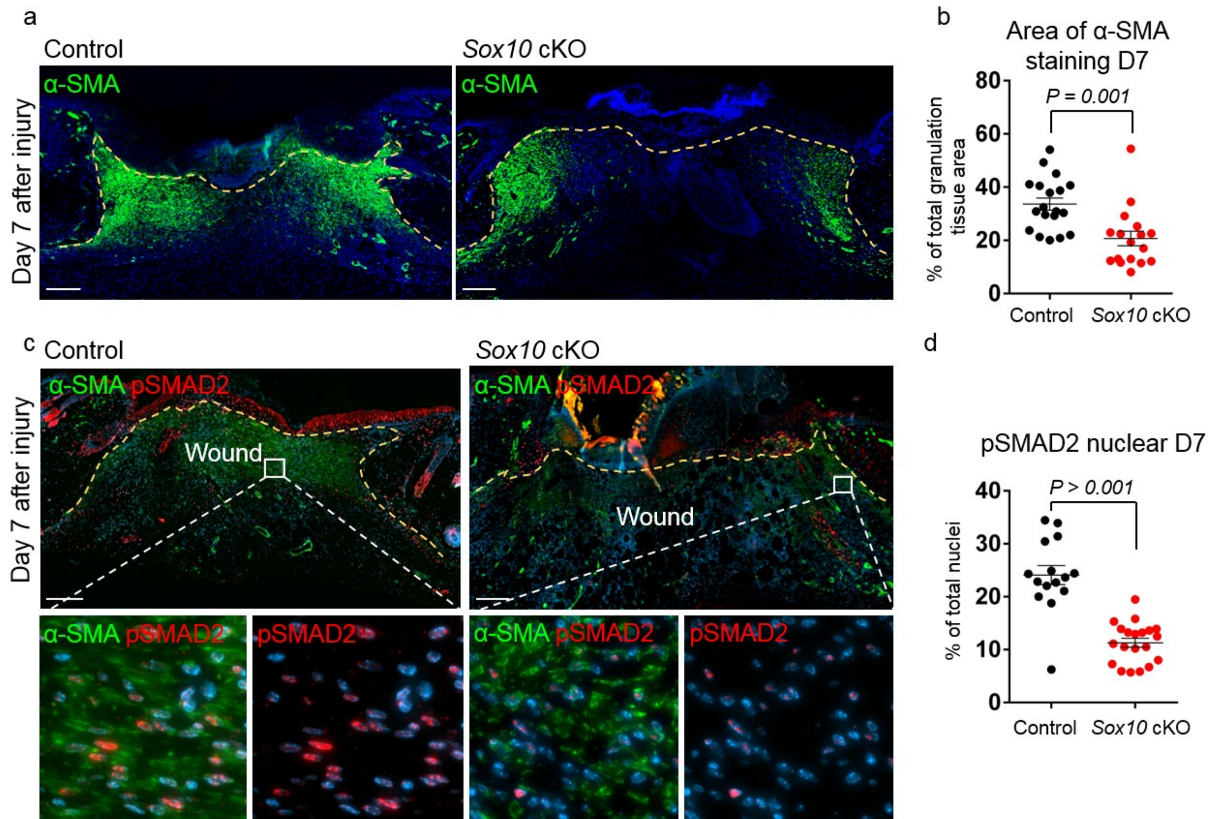

Figure 3.21 **Evaluation of myofibroblast population in control and cKO wounds. (a.b)** Quantification of the percentage of α-SMA⁺ area of the granulation tissue at D7 shows a reduction of the area occupied by Myofibroblasts upon *Sox10* cKO (N=5, n=19 in control; N=5, n=17 in *Sox10* cKO). (**c**) Immunolabeling of α-SMA (green) and pSMAD2 (red) in control and *Sox10* cKO skin wound sections at D7. Boxed regions in the dermis are shown in the insets at higher magnification as pSMAD2 single channel and merged with α-SMA channel. (**d**) Quantification of the percentage of highly pSMAD2⁺ cells per α-SMA⁺ area of the granulation tissue at D7 shows a reduction in canonical TGF-β signaling upon loss of *Sox10* (N=8, n=15 in control; N=10, n=20 in *Sox10* cKO). Data are represented as mean±SEM. Scale bars, 200µm.

In sum, conditional ablation of *Sox10* in peripheral nerves prior to injury is sufficient to prevent colonization of the wound tissue by nerve-derived cells and leads to delayed wound contraction and re-epithelialization. It decreases the formation of hyperproliferative epidermis as well as the area occupied by myofibroblasts, through reduced TGF-β signal activation.

3.9 Genetic expansion of peripheral nerve-derived cells/reactivated glia promotes myofibroblast formation during wound healing

Loss of the tumour suppressor gene *Pten* has previously been shown to result in proliferation of both myelinating and non-myelinating Schwann cells (Goebbels et al. 2010). Thus, *Pten* inactivation might represent a genetic tool to expand the number of nerve-derived cells in wounds. For this experiment, we crossed *Plp-CreER*^{T2} mice with mice carrying floxed alleles of the *Pten* gene. *Pten* was then depleted homozygously shortly prior to injury (Fig. 3.22a). In both quiescent and activated NBs of control animals, pAKT expression is not detectable by immunohistochemical methods. However, upon *Plp-CreER*^{T2}-mediated recombination, peripheral nerve cells prominently express pAKT, indicating efficient activation of PI3K/AKT signalling pathway upon *Pten* deletion (Fig. 3.22 b).

Remarkably, NBs of *Pten* cKO animals displayed overt expansion of nerve-derived cells in the wound (Fig. 3.22 c). Quantification revealed an approximately two-fold increase in the number of p75^{+} and lineage-traced tdTomato^{+} cells in the wound bed of *Pten* cKO mice (Fig. 3.22 d,e).

Normal wound healing in healthy mice appears to be naturally optimized and it has been notoriously hard to experimentally improve this process (Werner 2011; Gurtner et al. 2008). Indeed, the increase in the number of nerve-derived cells in the wound of *Pten* cKO mice did neither lead to a significant acceleration of the wound closure at D7 (86.3±5.0% covered by epidermis in control vs. 87.2±5.1% in *Pten* cKO) (Fig. 3.23 a) nor did it alter the area of hyperproliferative epidermis (0.250±0.02 vs. 0.278±0.03 mm2), wound width (2.12±0.09 vs. 1.90±0.10mm) or length of wound epithelium (1.81±0.06 vs. 1.68±0.08 mm), measured at the same time point (Fig. 3.23 b-d).

Interestingly, however, the area of α-SMA staining in the wound bed significantly increased upon *Pten* cKO, with myofibroblasts occupying around 47% of the total granulation tissue area in *Pten* cKO wounds, compared to 34% in control settings (Fig. 3.23 e,f). Previously we showed that depletion of nerve-associated cells results in reduced TGF-β signaling activation (Fig. 3.21 c,d). Hence we hypothesized that, in contrast, increased number of glial cells would lead to increased TGF-β signalling. Strikingly, at D5, *Pten* cKO wounds showed increased numbers of cells with strong nuclear staining for pSMAD2 compared to control (22.2±2.9% vs. 14.2±1.2% respectively) (Fig. 3.23 g).

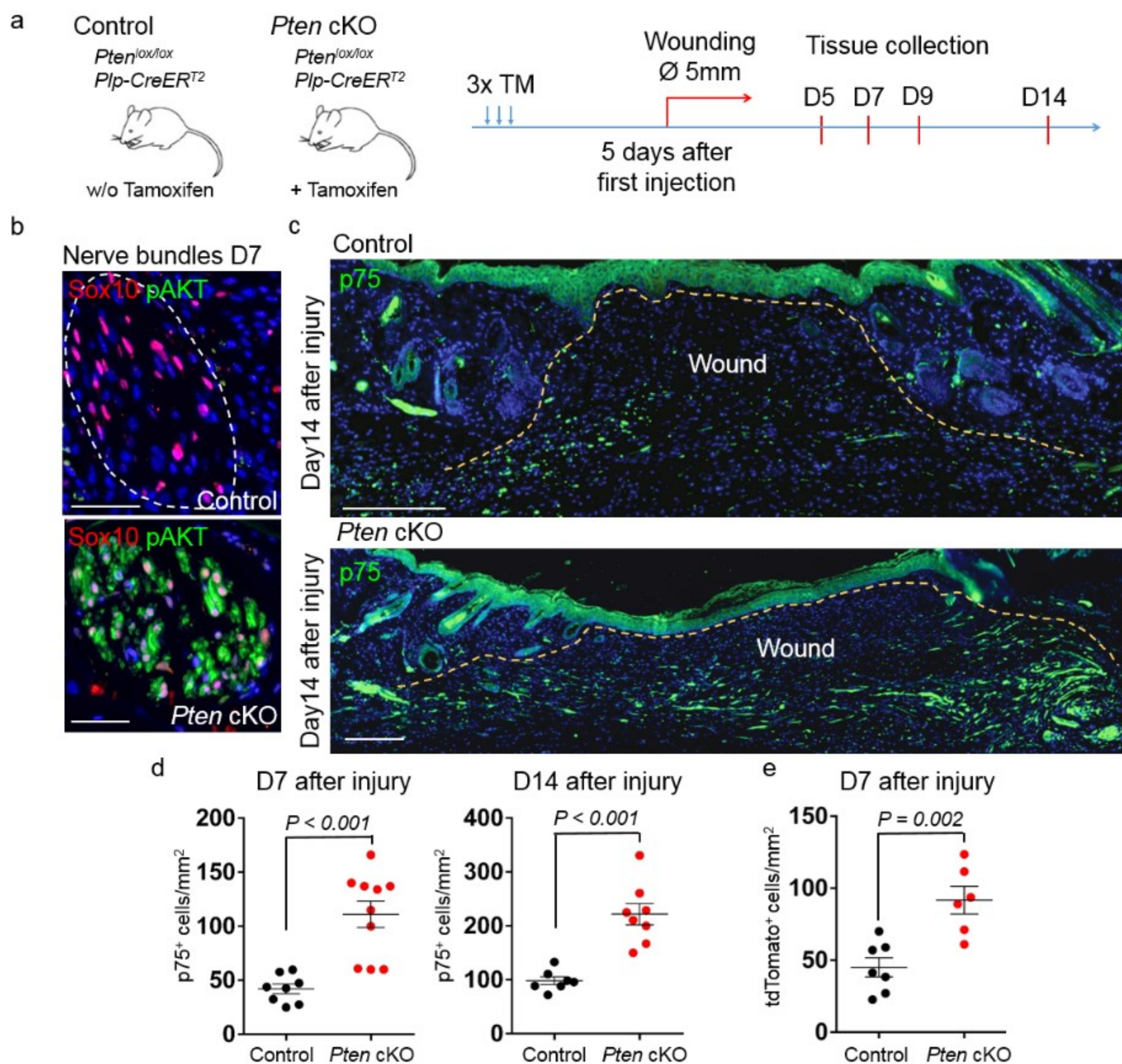

Figure 3.22 **Expansion of activated glial cells in the wound by genetic means.** (**a**) Scheme of the experimental strategy for *Plp-CreER*^{T2}-mediated glial gain of function in our murine wound healing model through *Pten* cKO. (**b**) Immunolabeling of pAKT (green) and Sox10 (red) in NB of skin wound sections at D7 shows a significant increase in PI3K signalling upon *Pten* cKO. (**c**) Immunolabeling of p75 (green) in control and *Pten* cKO skin wound sections at D14. (**d**) Quantification of p75$^+$ cells/mm^2 of granulation tissue at D7 and D14 (**e**) Quantification of tdTomato$^+$ traced cells at D7. Both quantifications show a significant increase of activated glial cells upon *Pten* cKO. Data are represented as mean±SEM of N=2, n=8 (control D7), N=2, n=8 (control D14); N=3, n=10 (*Pten* cKO D7), N=2, n=8 (*Pten* cKO D14) (**d**); N=4, n=7 (control), N=2, n=6 (*Pten* cKO) (**e**). Scale bars, 50µm (b), 200µm (c).

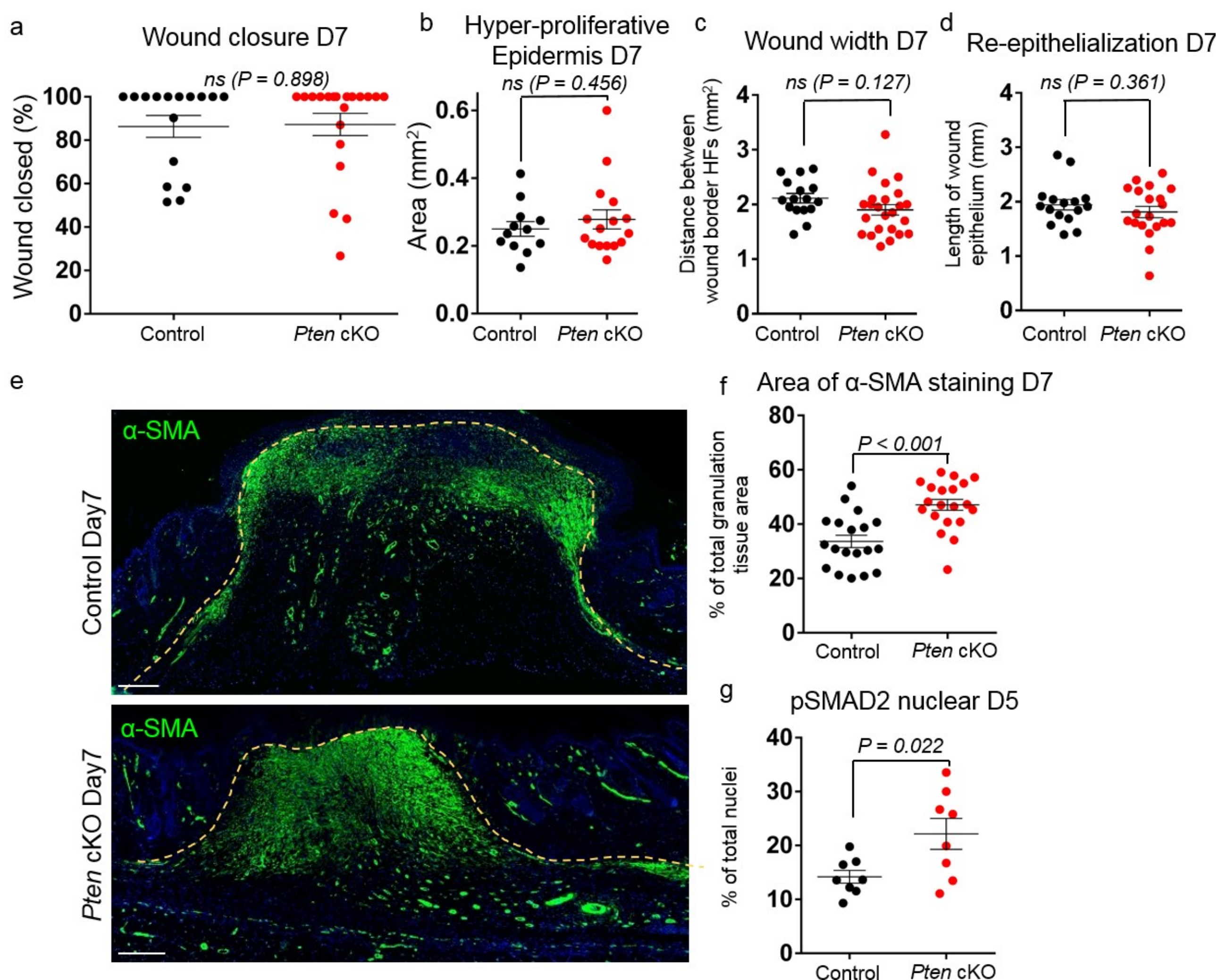

Figure 3.23 **Effect of genetic gain of function on wound healing dynamics and myofibroblast population.** Quantification of D7 wound sections for the percentage of (**a**) wound closure, (**b**) area of hyper-proliferative epidermis, (**c**) wound contraction, (**d**) re-epithelialization reveals no difference in these parameters. (**e**) Immunolabeling of α-SMA (green) in control and *Pten* cKO skin wound sections at D7. (**f,g**) Quantification of the percentage of α-SMA$^+$ area of the granulation tissue at D7 and of the percentage of pSMAD2$^+$ cells per α-SMA$^+$ area of the granulation tissue of skin wounds at D5 shows increase of both myofibroblast-occupied area and canonical TGF-β signalling in *Pten* cKO skin wounds. Data are represented as mean±SEM of N=4, n=16 (control), N=5, n=20 (*Pten* cKO) (**a**); N=3, n=12 (control), N=4, n=16 (*Pten* cKO) (**b**); N=4, n=16 (control), N=6, n=24 (*Pten* cKO) (**c**); N=4, n=16 (control), N=5, n=20 (*Pten* cKO) (**d**); N=5, n=19 (control), N=5, n=20 (*Pten* cKO) (**f**); N=3, n=8 (control), N=2, n=8 (*Pten* cKO) (**g**). Scale bars, 200µm.

Thus, loss of *Pten* in peripheral glia caused an expansion of activated nerve-derived cells in the regenerating skin, which was associated with increased α-SMA staining area in the wound and increased activation of TGF-β signalling consistent with the role of reactivated peripheral glia in regulating the number of myofibroblasts during wound healing.

In sum, conditional loss of *Pten* in peripheral glia prior to injury is sufficient to increase nerve derived cells in the healing tissue. These ectopic cells trigger increased TGF-β signalling and result in increased myofibroblasts differentiation, consistent with the inverse phenotype we observed upon loss of these cells in the same biological context.

3.10 Human dermis-derived cell characterization and differentiation potential

As mentioned in the introduction part, skin harbours several types of cells that exhibit stem cell properties and can be isolated and expanded *in vitro.* These different cells have been long considered as potential candidates for autologous cell therapies due to their accessibility and, bearing in mind, their broad differentiation potential could be potentially interesting for further elucidation of activated glia involvement in wound healing process. Our lab has focused on derivation and characterisation of adult human MSC from various sources including human skin dermis, bone marrow, heart and adipose tissue (Riekstina, Cakstina, et al. 2009).

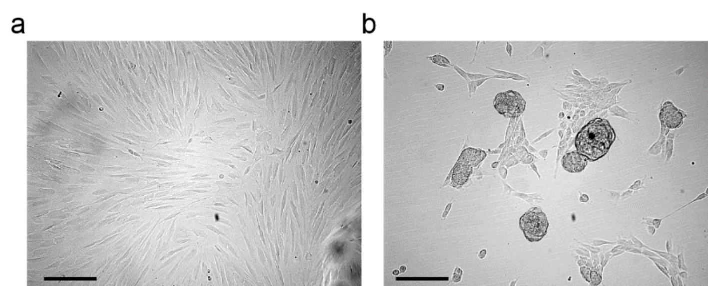

Figure 3.24 **Morphology of human skin dermis-derived cells in culture.** Morphology of MSCs adherently-grown in 10% FBS supplemented medium (**a**). Morphology of MSCs grown in serum-free medium (**b**). Scale bars, 100µm.

Cells that were derived from the dermis of human skin could be expanded adherently when cultured in 10% FBS supplemented medium and formed spheres if grown in serum-free medium with FGF-2, EGF and B27 supplements (FEB medium) (Fig. 3.24). Previously, low serum medium with FGF-2 and B27 supplementation was found to be the optimal propagation medium and boosted proliferation of MSCs in most of the formulations and culture samples

used (Riekstina et al. 2008). Some differences in response to FGF-2 suggested donor to donor variability and heterogeneity of primary cultures, which was also demonstrated by differences in cAMP production in response to various stimuli (Jekabsons et al. 2011). Interestingly, we also find that spheres can be derived from adherently growing population if 10% FBS medium is changed to a FEB medium.

Human dermis-derived cells similar to MSCs from other sources (Riekstina, Cakstina, et al. 2009) displayed expression pattern characteristic to MSCs (Dominici et al. 2006). Most of the freshly-isolated cells expressed surface markers CD73, CD90 and CD105 and lacked hematopoietic markers CD14, CD45 and HLA-DR (Fig. 3.25 a,b). Fraction of cells was positive for endothelial marker CD34, but after expansion in culture it was no longer present (Fig. 3.25 c). Immunocytochemical analysis demonstrated that other markers, notably, embryonic marker SSEA4 and neuroectodermal lineage marker Nestin were expressed in dermis-derived cells (Fig. 3.25 d).

Consistent with previous observations, we also found that certain media formulations induced human dermis-derived cell differentiation towards different mesenchymal lineages. Three weeks after the propagation medium was changed to a differentiation media we observed a change in morphology and deposition of extracellular calcium in Alizarin red stained MSCs (Fig. 3.26 b), accumulation of lipids in the cytoplasm of cells grown in adipogenic medium as demonstrated by Oil Red O stain (Fig. 3.26 c), and positive Alcian blue staining in dermis-derived cells that were grown as a clump in chondrogenic medium (Fig. 3.26 d). FGF-2 is an important factor for both MSCs proliferation and differentiation (Ng et al. 2008; Ahn et al. 2009; Lai et al. 2011). In our hands FGF-2 receptor inhibition resulted in increased adipogenic and osteogenic differentiation (Riekstina, Parfejevs, et al. 2009).

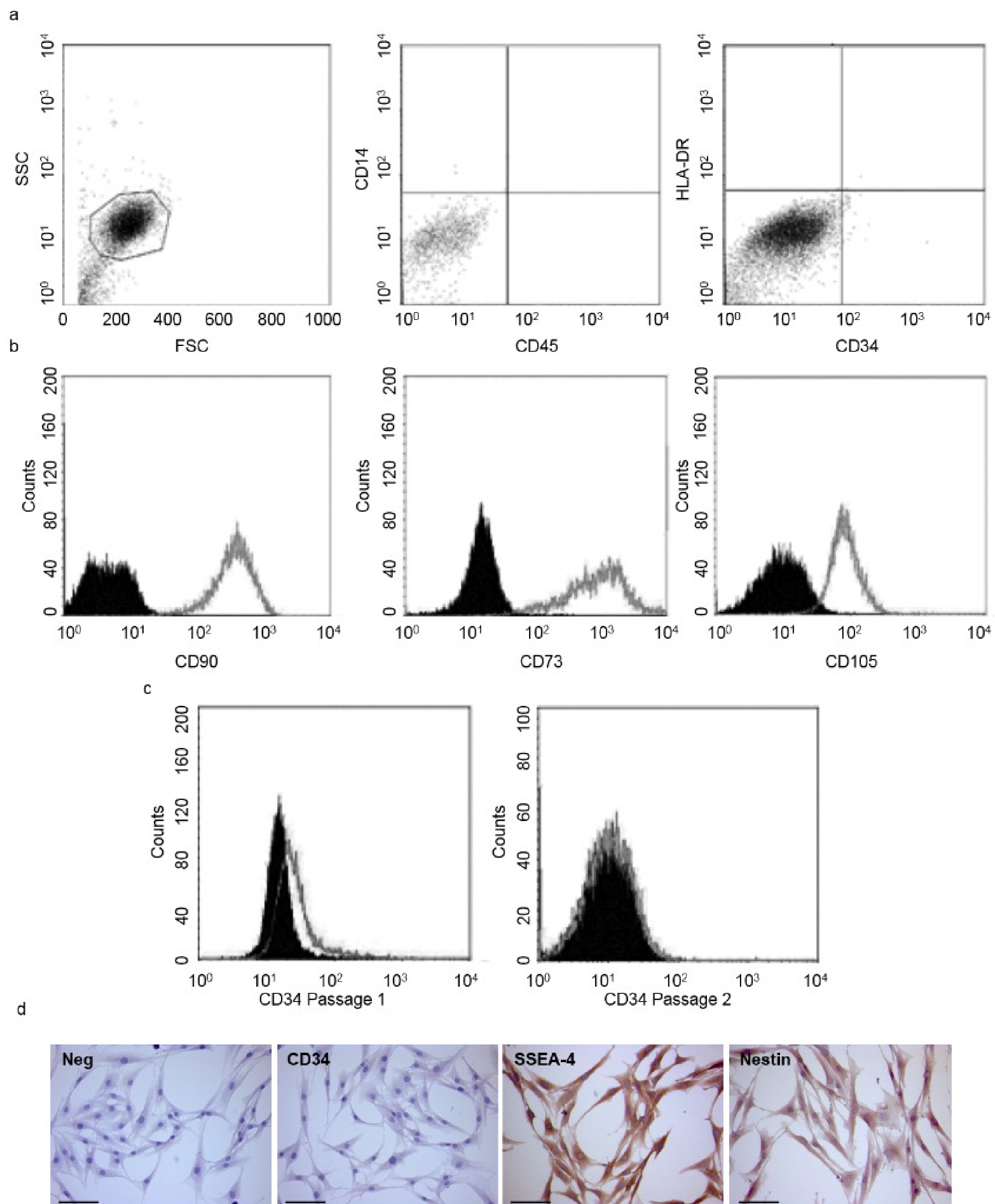

Figure 3.25 **Phenotypical characterization of human skin dermis-derived MSCs using FACS.** Expression of haematopoietic markers CD14, CD45 and HLA-DR and endothelial marker CD34 (**a**). Expression MSC markers CD90, CD73 and CD105 (**b**). Expression of CD34 marker in 1 and 2 passage MSCs (**c**) Immunocytochemistry for endothelial marker CD34, embryonic and MSC marker SSEA4 and neuroectodermal marker Nestin (**d**). Scale bars, 100 µm.

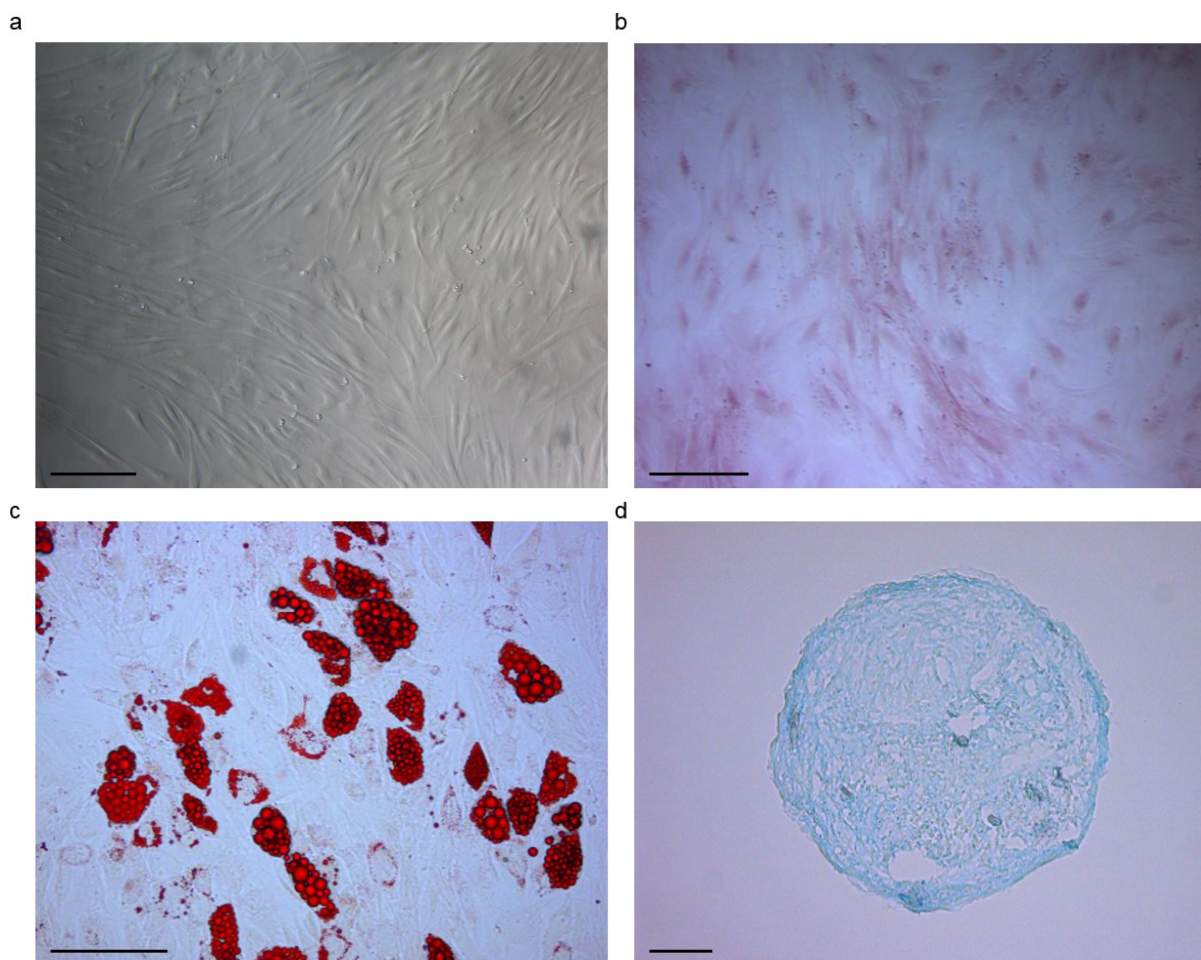

Figure 3.26 **Characterization of human skin dermis-derived MSCs – differentiation towards mesenchymal lineages.** Morphology of adherently-grown MSCs (**a**). Cells stained with Alizarin red after osteogenic differentiation (**b**). Adipogenic differentiation detected with Oil Red O staining (**c**). Cryosection of cells grown in chondrogenic differentiation medium and stained for Alcian blue (**d**). Scale bars, 100μm.

3.11 Human dermis-derived cell neuroectodermal differentiation

Based on the observation that human dermis-derived cells express markers characteristic to embryonic stem cells and display high neuroectodermal marker Nestin expression levels compared to MSC from other sources (Riekstina, Cakstina, et al. 2009) as well as on previous reports of SKP potential to differentiate towards cells of non-mesodermal lineages (Toma et al. 2001; Fernandes et al. 2004), we wanted to further investigate the potential of neuroectodermal differentiation of adherently-grown cultures of dermis-derived MSCs.

To do so we used different human dermis-derived cell cultures, which were previously characterized (see previous result section) and subjected them to neural progenitor expansion medium. In parallel, to compare differentiation outcomes we used commercially available immortalized neural progenitor cell line ReNcells CX (Millipore).

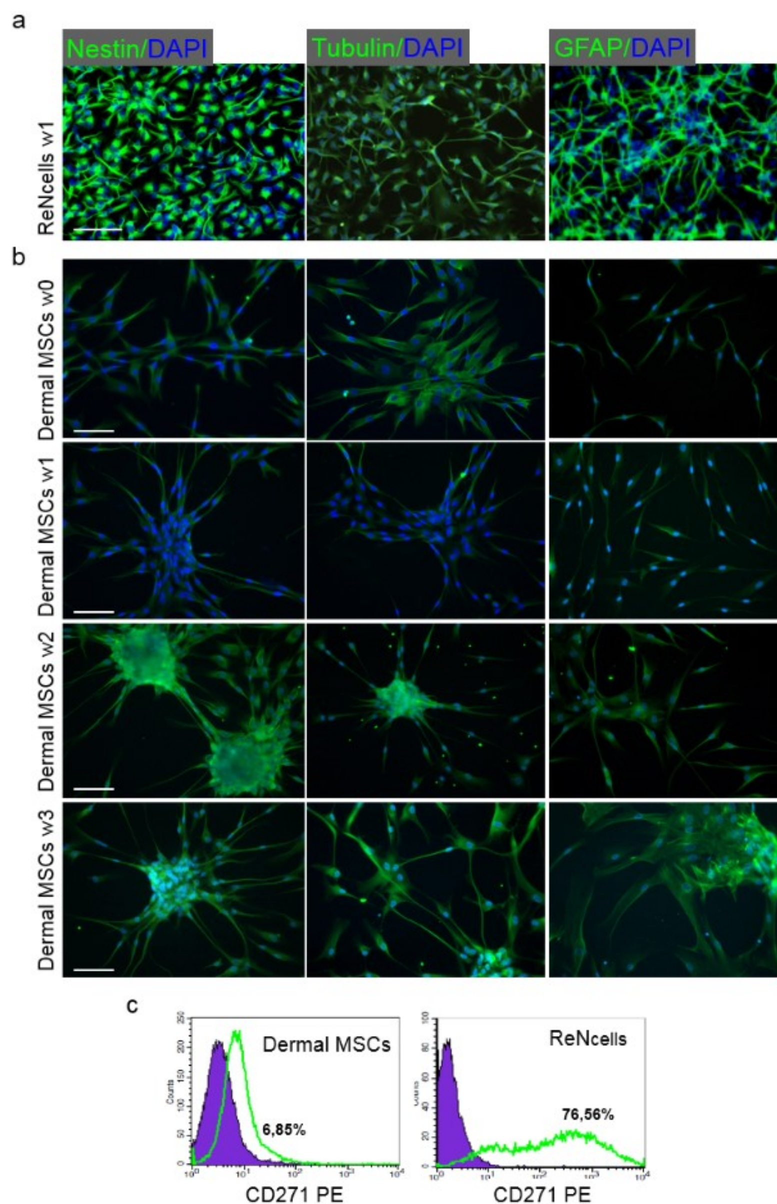

Figure 3.27 **Differentiation of human skin dermis-derived MSCs and marker expression comparison to ReNcells.** Immunolabeling of Nestin, Tubulin βIII and GFAP in ReNcells (**a)** Immunolabeling of Nestin, Tubulin βIII and GFAP in dermis-derived MSCs (**b**). FACS analysis of CD271 (p75) expression on the surface of dermal MSCs and ReNcells (**c**). Scale bars, 100µm.

Dermal cells were first grown in FBS containing media to allow cell adherence and switched to FEB medium (neural progenitor expansion medium) after 24 hours, while ReNcells were grown in in FEB medium on laminin coated surface. Immunocytochemical analysis suggested that Nestin, Tubulin βIII and GFAP were all expressed by ReNcells in these culture conditions (Fig. 3.27 a). We found that dermis-derived cells when switched to FEB growth medium tended to aggregate into sphere-like structures after 14 days in culture, and also stained positively for these three markers, with visually stronger expression in aggregated cells (Fig. 3.27 b). FACS analysis of these cultures revealed that majority of ReNcells (~76%) and a fraction of dermis derived cells (~7%) are positive for neural crest marker p75, which is in concert with previous reports of $p75^{+}$ cells present in the skin (Fig. 3.27 c) (Wong et al. 2006; Etxaniz et al. 2014).

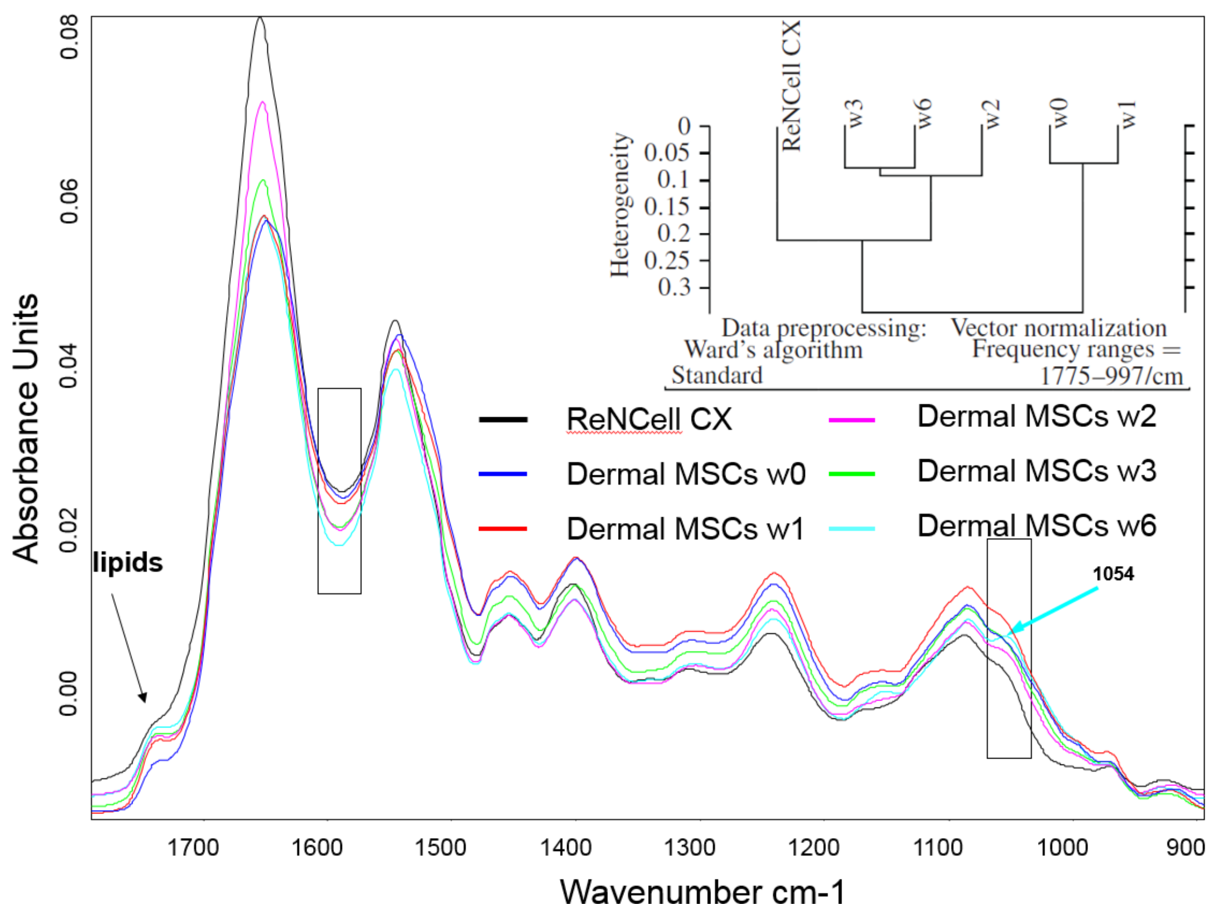

Figure 3.28 **FT-IR spectra comparison in dermal MSCs and ReNcells.** Normalized FT-IR spectra – analysis of lipid, protein, DNA and carbohydrate fractions at various time points in FEB medium and comparison with ReNcell spectrum.

To further characterize the cells subjected to FEB medium and identify possible biochemical changes, we collected the cells grown in FEB medium at various time points up to 6 weeks and

ReNcells and performed FT-IR spectroscopy. Quantitative analysis showed that during differentiation process the content of proteins decreased from 68% dry weight (dw) in w0 sample to 62% dw in 6th week sample. The minimum between Amid I and Amid II bands increased and this may indicate the changes in the overall protein content and α-, β-conformational states within S–MSCs. The content of lipids increased with the cell growth time from 3 to 9% dw. In the spectra of 6th week S-MSCs, a small band at 1054 cm^{-1} assigned to glycogen was well pronounced while not detected in the ReNcell spectrum (Fig. 3.28). IR spectroscopy detected qualitative and quantitative changes in different macromolecular fractions – lipids, proteins, and carbohydrates during differentiation in FEB medium. HCA showed that starting from week two, S-MSC spectra cluster with the reference cell line ReNcell CX, possibly indicating the divergence towards neural phenotype. In sum, FT-IR seems to be sensitive and fast method for the assessment of cell state and could be used in screening approaches (Parfejevs et al. 2012).

4. Discussion

Mammalian skin is a highly innervated organ (Hendrix et al. 2008; Roosterman et al. 2006) and full-thickness wound causes injury to many peripheral nerve endings within the skin. It is known that upon nerve injury peripheral glia gets activated and display plasticity often referred to as de-differentiation, since cells acquire less mature phenotype (Mirsky et al. 2008). It is also suggested that depending on the location at the injury site several distinct Schwann cell populations might be present (Jessen et al. 2015).

Recent reports, based on sequencing performed on the distal stump of the injured nerve, argue that cells formed after nerve injury are different from SCPs or immature Schwann cells and represent specialized repair cells that help to guide the axons and restore innervation. (Fontana et al. 2012; Arthur-Farraj et al. 2012). C-Jun upregulation, for example, is a key event in adult Schwann cell response to injury, however it is not observed at any point in development (Arthur-Farraj et al. 2012). In this work, we demonstrate a novel role of injury-activated peripheral glia in adult mammalian wound healing.

4.1 Innervation in regeneration and wound healing. Wound healing vs. regeneration

Dependence of regeneration process on tissue innervation has been long observed across the phylogeny (Kumar & Brockes 2012). Recently, more direct evidence was provided to support the importance of innervation for mammalian regeneration (Takeo et al. 2013; Rinkevich et al. 2014; Mahmoud et al. 2015). In these studies nerves were a source of growth factors providing trophic support for regeneration. Besides the role of neuronal projections, Schwann cells, another major neural crest-derived PNS component, have been directly implicated in amphibian limb (Kumar et al. 2007) and mammalian digit regeneration models (Johnston et al. 2016). Similar to the nature of axonal contribution, Schwann cells were found to secrete factors important for proliferation of blastemal cells. In the case of amphibian limb regeneration, it was a member of Anterior Gradient family proteins nAG and in digit tip regeneration model PDGF-AA and Oncostatin M. These studies provided one possible explanation for regeneration dependence on innervation.

At the same time not much is known about the role of innervation in normal and pathologic wound healing (Martin & Nunan 2015). Studies have shown that under normal circumstances hyperinnervation follows at the location of the injury (Harsum et al. 2001). Impairment of the peripheral nervous system, whether traumatic or pathologic, results in delayed tissue repair or failure to heal properly (Barker et al. 2006; Laverdet et al. 2015). While one of the main functions of innervation has been attributed to axonal sprouting of neurons and neuromediators/growth factors released by the axons in the wound bed upon injury (Donnerer et al. 1992; Ashrafi et al. 2016; Harsum et al. 2001; Chéret et al. 2013), recently, a cellular counterpart, supposedly stemming from the nerve – Sox2-traced population was implicated in the wound healing (Johnston et al. 2013).

Skin wound healing in adult mammals is different from regeneration and rarely leads to new appendage formation – instead, a scar is formed to replace the original tissue (Gurtner et al. 2008). It is very different from scarless healing observed in the wounds of foetal tissues that resembles regeneration (Larson et al. 2010). Adult wound healing is characterized by prominent inflammatory response, increased TGFβ signalling with predominance of TGFβ isoforms 1 and 2 over TGFβ3 and collagen type I deposition at the expense of collagen type III. Together with other factors these are thought to impede the activation of various skin stem cells in foetal wound healing and regeneration (Larson et al. 2010; A. Seifert et al. 2012).

In contrast, an exemplary scarless wound healing response reminiscent of regeneration is observed in spiny mouse (*Acomys percivali*) – rodent species found in central Africa. These mice are able to shed large parts of their skin to escape predators. When compared to wound healing observed in house mouse (*Mus musculus*), it displays faster re-epithelialization, almost no presence of myofibroblasts and complete reconstitution of skin appendages such as hair follicles and sebaceous glands. Wounding in these mice also leads to formation of Collagen III-rich matrix (A. W. Seifert et al. 2012).

4.2 Glial cell activation and identity

In this work we wanted to address the role of activated glia in cutaneous wound healing. We combined tissue clearing techniques with 3D-imaging to confirm that *Dhh-Cre* traced glial cells upon skin injury disseminate in the granulation tissue from apparently injured nerves.

However, one cannot exclude possible contribution from smaller non-injured fibres located next to the wound, as it has been shown that injury to myelinating efferent nerves can trigger cell proliferation in non-myelinated C fibres (Murinson et al. 2005). Further, our immuno-histological analysis suggests that glial cells switch to an "activated" state, start proliferating and express markers characteristic to repair Schwann cells formed in response to nerve injury (Mirsky et al. 2008; Jessen et al. 2015).

Markers of glia activation in the PNS, such as p75, pERK and c-Jun are upregulated in some nerve bundles in the wound and single cells emigrating from these nerve bundles. Our expression profile analysis of genetically traced glial population upon injury also reveal predicted change in several genes important in myelination. Performing sequencing on these cells we anticipated similarities to the previously published work, however, important difference is that in the previous studies gene profiling was done using the material obtained from the sciatic nerve, more precisely, from the nerve part distal to the injury, where Schwann cells exhibit their plasticity (Bosse et al. 2006; Barrette et al. 2010; Arthur-Farraj et al. 2012). In our case sequencing, however, was done on more general population of cells located close to the wound including those found in uninjured nerves and around both proximal and distal stumps of the of the nerve bundles transected in the wounding process.

It is difficult to say exactly which cell type is at the origin of the migrating activated glia in the adult. Persistence of a subset of NCSCs or SCPs into the adult nerves has not been demonstrated so far and it is plausible that activation of plastic myelinating or non-myelinating Schwann cells takes place. Neural crest-derived SCPs are multipotent in the embryo and apart from glia can give rise to endoneurial fibroblasts (Joseph 2004), melanocytes (Adameyko et al. 2009), parasympathetic neurons (Dyachuk et al. 2014; Espinosa-Medina et al. 2014) and mesenchymal cells (Kaukua et al. 2014).

In recent report on involvement of glial cells in adult regeneration $p75^{+}/S100^{+}$ cells scattered within digit blastema were called 'de-differentiated SCPs'(Johnston et al. 2016). However, this term is not fully correct for several reasons. First of all, it has been demonstrated that upon injury Schwann cells in the spinal nerves don't de-differentiate to SCP, but form a repair cell (Arthur-Farraj et al. 2012). Second, it has to be shown that these cells are phenotypically similar to SCPs and express markers like BFABP, PLP or Cadherin 19. And lastly, SCPs in development exist in close contact with axons and depend on survival factor neuregulin 1

(Meier et al. 1999; Jessen et al. 2015), whereas upon injury neural crest-derived cells clearly dissociate from the nerves.

4.3 Plasticity of activated glia

Intriguingly, also in the adult after environmental challenge PNS glia has been reported to give rise to melanocytes (Adameyko et al. 2009), neurons (Pardal et al. 2007; Laranjeira et al. 2011) and mesenchymal cells (Kaukua et al. 2014).Therefore, we had to consider the possibility that activated glia in the wound bed would structurally contribute to scar formation through transdifferentiation processes, as seen, for example in a model of tooth regeneration, where Schwann cells contribute to mesenchymal cell populations (Kaukua et al. 2014)

Lineage tracing combined with marker expression analyses in our hands did not provide sufficient evidence for injury-activated glia adopting alternative fates. In particular, traced cells did not generate melanocytes or myofibroblasts (apart from couple of aSMA positive cells detected at D7 (Fig. 3.9 a)), which are both cell types that can be produced by neural crest cells, the precursors of Schwann cells during development. Instead, in the undisturbed 3D context of the healing wound, injury-activated glia displayed lineage-restriction *in vivo* and continued to express peripheral glial lineage markers even after prolonged time points after injury. This is reminiscent of SCPs in the Mexican salamander axolotl that maintain Schwann cell identity after limb amputation while promoting regeneration (Kragl et al. 2009) and of astroglia in the mammalian CNS that are also activated in response to injury and contribute to tissue repair, but exclusively give rise to astrocytes unless genetically manipulated (Götz et al. 2015).

4.4 Secretome of injury-activated glia

Rather than a direct contribution of activated peripheral glia to cell types constituting the wound bed, our study demonstrates a cell non-autonomous effect on these cells during wound healing. Expression profiling of genetically traced peripheral glial cells either from unwounded or wounded skin demonstrated a significant enrichment for expression of various ligands and gene products destined to the extracellular space, representing a putative secretome of Schwann cells upon injury. This extracellular space, representing a putative secretome of Schwann cells

upon injury. This secretome includes gene products reported to affect multiple pathways, such as ECM remodelling, angiogenesis, chemotaxis of immune cells, or TGF-β signalling, which are all biological processes well known to influence wound healing (Gurtner et al. 2008). Interestingly, we don't find Oncostatin-M or PDGF-AA, factors reported to be derived from Schwann cell precursors in mammalian regeneration model (Johnston et al. 2016). Similarly, genes coding for these proteins were not observed among the upregulated transcripts in sequencing experiments performed on injured nerve distal stump (Barrette et al. 2010), suggesting that depending on the injury and tissue context activated Schwann cells could behave differently.

To test whether any of the processes revealed by sequencing could be significantly impacted by our cell population of interest, we genetically ablated injury-activated glia by preventing their expansion into the wound bed using lineage-specific conditional deletion of the transcription factor *Sox10*, a factor known to control neural crest cell survival and glial lineage specification (Finzsch et al. 2010). Preventing dissemination of glial cells into the wound bed delayed wound healing and, notably, interfered with wound contraction and proper myofibroblast formation, which was associated with decreased TGF-β signalling in a non-cell autonomous manner.

Myofibroblasts, a hallmark of adult wound healing, are activated through cooperation of mechanical tension and TGFβ1. In animals capable of regeneration and foetal wounds tissue repair happens with virtually no myofibroblast involvement, highlighting differences between wound healing and regeneration (Larson et al. 2010; A. W. Seifert et al. 2012). They participate in tissue contraction, contribute to scar formation by ECM deposition and may lead to hypertrophic scars and fibrosis if excessively activated (Hinz et al. 2012). Consistent with that, we found that in *Sox10* wound contraction is impaired.

Of note, besides the activin/inhibin ligand Inhba, our study did not provide evidence for specific TGF-β ligand expression by injury-activated glia. Rather, it pointed to modulation of ligand availability through ECM remodelling and TGF-β post translational modification, likely conferred by factors known to regulate these processes and present in the glia secretome, such as *Lox*, *Loxl2*, and *Plod2* and *Bmp1* (references in Supplementary Table S5). This is consistent with previous reports indicating that non-myelinating Schwann cells have the potential to secrete molecules regulating latent TGF-β activation (Yamazaki et al. 2011). Moreover, a link between innervation and myofibroblast presence in the wound has been previously proposed

(Liu et al. 1999). Although many other factors of the glia secretome characterized in our study have been functionally associated before with wound healing (see references in Supplementary Tables 1-5), their contribution to the paracrine effects by injury-activated glia remains to be elucidated.

Given the many distinct classes of factors present in the glia secretome, it is conceivable that injury-activated glia take part in processes of skin wound healing other than promoting myofibroblast formation. Indeed, loss of injury-activated glia also affected the area of newly formed, hyperproliferative epidermis. However, expansion of peripheral glia in the wound bed did not influence this process, presumably because it is naturally optimized in murine skin wound healing (Werner 2011). Nonetheless, our data speak for multiple effects of injury-activated glia and point to a therapeutic potential of these cells and/or of their secretome in human wound healing

4.5 Gain-of-function experiment

In our gain-of-function conditions *Pten* loss led to increased myofibroblast fraction compared to control. Interestingly, we don't detect differences in ECM deposition between these conditions as evaluated by Herovici collagen staining at day 14 after injury. Possible explanation is that myofibroblast activation in loss-of-function condition might later be corrected for by TGFβ1 contribution from immune cells, keratinocytes and autocrine stimulation (Hinz et al. 2012).

Expansion of glial cells in our hands does not result in faster wound closure, possibly because wound healing is a highly optimized process in healthy control animals (Werner 2011). *Pten* deficient glia proliferates more, but increased proliferation alone is not sufficient for activated state induction or maintenance (Jessen et al. 2015) and these cells are able to associate with axons on later stages and myelinate, in fact, prolonged *Pten* cKO in Plp-lineage has been used before to model hypermyelination conditions (Goebbels et al. 2010). Thus, if activation of myofibroblasts happens through the activated glia in this setting it might still be transient and not sufficient to induce fibrotic response. Conforming with that, recently wound healing dynamics was demonstrated to be similar in control and denervated splinted wounds, where wounds don't heal by contraction (Rinkevich et al. 2014).

4.6 Glial cells in injury repair and alternative sources of cells for further studies

Our work is in line with other recent reports that showed crucial roles of peripheral nerve-derived cells in tissue regeneration and repair, both in amphibians and mice (Kumar et al. 2007; Johnston et al. 2016; Johnston et al. 2013). Together with our study showing their influence on TGF-β-mediated myofibroblast formation and skin wound closure, peripheral glia emerge as important players in tissue regeneration. It remains to be shown, though, whether the nerve-derived cell populations reported in the literature to have regenerative potential represent distinct cell types or one dynamic cell population eliciting distinct paracrine responses to injury depending on the type of injury or the organ affected. Likewise, it will be highly relevant to address whether injury-activated glia are also implicated in other tissues for which regeneration was shown to depend on innervation (Kumar & Brockes 2012), including heart and skeletal muscle (Mahmoud et al. 2015; Pessina et al. 2014; Noah et al. 2002).

Considering importance of glial cells in peripheral nerve injury and emerging role of activated glial cells in repair and regeneration of other tissues, a reliable source of these cells for possible therapeutic applications, further research and identification of beneficial secreted molecules is desirable. In this and previous work we have characterized cells from human dermis. We have shown that these cells phenotypically resemble MSCs (Dominici et al. 2006) and possess similar differentiation potential. Moreover, previously we have demonstrated that apart from several embryonic markers found in MSCs from different tissue sources, human dermis-derived cell display the highest expression level of neuroectodermal stem cell marker Nestin (Riekstina, Cakstina, et al. 2009).

This finding was promising and made us investigate the neuroectodermal differentiation capacity of human dermis-derived cells. There are many different protocols aiming at generation of neuronal cells from MSCs, however, the yield of differentiated cells is low and observed differentiation towards neuronal phenotype has to be thoroughly evaluated. In some cases, for example, it was attributed to cell stress response to toxic phenotype inducers (Scuteri et al. 2011). Even if succeeded neuronal cell transplantation would pose a challenge. In our work we used neural progenitor expansion medium and observed that dermis-derived adherent cells tend to aggregate into neurospheres-resembling structure and display stronger expression of neuroectodermal and glial markers – Nestin, Tubulin βIII and GFAP.

It would be interesting to further differentiate these cells specifically towards Schwann cell linage and compare them to myelinating and activated Schwann cells found in tissues after injury. Different protocols exist for Schwann cell differentiation and differentiated cells from bone marrow skin and adipose tissue proved to be functional after transplantation into the injured nerve model animals (Dezawa et al. 2001; McKenzie et al. 2006; Liu et al. 2011).

Another aspect that has to be taken into account is dermis-derived cell heterogeneity. We have observed that after first tissue passages a marker for endothelial cells CD34 is present in some cells (Riekstina, Cakstina, et al. 2009; Riekstina, Parfejevs, et al. 2009). Analysis of p75 expression also revealed that skin-derived cell cultures contain around 7% of cells positive for this Neural crest marker. It is conceivable that observed neuroectodermal differentiation potential could be related to these cells and differentiation can be improved by sorting out distinct populations, for example, CD56 expressing cells, that in one study have been shown to be the origin of cells with neuronal potential in the skin (Etxaniz et al. 2014). However results from other studies suggest that non-neural crest cells found in the skin are able to differentiate to Schwann cells *in vitro* (Krause et al. 2014).

4.7 Potential future directions

Our preliminary data (not shown) suggest that human wounds and keloid scars also contain scattered $Sox10^+/p75^+$ cells. Thus, it would be interesting to check for the presence of activated glia in different wound types and to find possible correlations between, for example, different types of chronical wounds or, on the contrary, their involvement in hypertrophic wounds and keloid scars.

Several conditions such as diabetes, obesity, spinal cord injury and underlying sensory neuropathies are characterized with loss of innervation and delayed wound healing. One has to note that direct contribution of neuropathy can sometimes be difficult to uncouple from other parallel factors like diminished blood supply, impaired immune response or infection (Laverdet et al. 2015). In the case of sensory neuropathies lack of sensation and awareness could be responsible for excessive wounding and wound healing delay. However, substantial amount of evidence point towards important role of axon derived neuropeptides, such as CGRP and SP and growth factors such as NGF (Barker et al. 2006; Laverdet et al. 2015; Chéret et al. 2013).

It is not fully understood to what extent Schwann cells participate in neuropathic wound repair, but reports suggest diminished nerve regeneration and migration after injury in neuropathic tissues (Polydefkis et al. 2004; Cheng et al. 2013). Diabetes induced hyperglycaemia has been shown to inhibit Schwann cell proliferation and migration (Gumy et al. 2008), which might in part explain reduced nerve sprouting. Other important function that is affected is Schwann cell-mediated trophic support of blood-nerve interface (Mizisin 2014).

Conflicting data exist as to whether innervation plays a role in the formation of hypertrophic scars. There is evidence that elevated levels of innervation and SP can be detected in hypertrophic scars and is associated with increased inflammation (Scott et al. 2007; Zhang & Laato 2001). On the other hand, data from patients with spinal cord injury suggest that loss of innervation leads to nail hypertrophy, skin thickening and fibrosis and this effect could be correlated with the severity of paralysis (Stover et al. 1994). A link between innervation and presence of myofibroblasts in the wound has also been noted (Liu et al. 1999). So far there is no data regarding potential involvement of Schwann cells in formation of hypertrophic or keloid scars.

It would also be interesting to establish whether correlations exist between presence of glial cells and healing in acute wounds in different patient age groups. It is known that wound healing in young vs. older patients proceeds differently, which is often attributed to intrinsic and extrinsic changes in the skin (Gosain & DiPietro 2004). These changes in healthy elderly patients don't lead to healing failure, but they are observed at all stages of normal wound healing and, among other things, include increased platelet aggregation, impaired macrophage function, delayed angiogenesis and re-epithelialization and increased collagen maturation (Sgonc & Gruber 2013). One study linked these phenomena to hormonal differences between younger and older subjects. Microarray performed on human wounds suggested that majority of differently regulated genes between young and aged patient samples were estrogen-regulated (Hardman & Ashcroft 2008).

Other factors, however, are also likely to contribute to age-related differences in wound healing dynamics. It is perhaps not surprising that age-related changes in skin architecture also encompass cutaneous innervation. A study that investigated intraepidermal nerve density in the biopsies from distal limb areas demonstrated significant reduction in innervation densities with age (Chang et al. 2004). Several animal studies also showed that nerve regeneration as such is altered in older animals (Kang & Lichtman 2013b; Painter et al. 2014). Loss of muscle

innervation due to trauma, if not severe, can be repaired by regeneration of the axons that use existing endoneurial tubes to re-establish neuromuscular junctions. This ability is diminished with age because of slower clearance of debris by Schwann cells and macrophages. Axons despite growing at the same pace face more obstacles on their way which results in delayed re-innervation (Kang & Lichtman 2013a).

Other study focused on the recovery from sciatic nerve crush in young vs. old animals. Similarly, findings suggested that in older animals axon growth response is preserved, but Schwann cells and microenvironment are responsible for the phenotype. Grafting of nerve fragment from old and young animals demonstrated that recovery is not dependent on the age of recipient, but on the age of the graft and that changes are due to altered injury response of aged Schwann cells (e.g. failure to upregulate c-Jun) and failure to recruit macrophages (Painter et al. 2014). Both studies highlighted the key role of proper Schwann cell function for successful repair process in older experiment subjects.

Muscle tissue is also innervated. Using our experimental model we saw that some *Plp-Cre* traced cells were located in-between the fibres of *panniculus carnosus* muscle (Fig. 3.3) and upon wounding cells were emerging from the area where the muscle was cut. These cells might be potentially stemming from NMJ that contains Schwann cells that can be traced using *Plp* as a promoter and are important in NMJ formation, maintenance and regeneration (Ko & Robitaille 2015; Barik et al. 2016). Following injury or degenerative disease skeletal muscle can regenerate using muscle progenitor cells called satellite cells and other cells with myogenic potential (Tedesco et al. 2010).

Muscle denervation has been shown to increase muscle satellite cell susceptibility to apoptosis (Jejurikar et al. 2002) and on the big scale it causes muscle atrophy, which in turn leads to loss and dysfunction of muscle progenitors (Mitchell 2004). A recent report demonstrated the importance of innervation in cardiac muscle, where axons secrete growth factors important for proliferation of cardiomyocytes. Importantly, denervation caused regeneration defect could be rescued by growth factors neuregulin 1 and NGF (Mahmoud et al. 2015).

It remains to be elucidated whether Schwann cells play a direct role in muscle regeneration apart from supporting re-innervation and re-establishment of NMJ. A potential way to do this could be using murine wounding healing model and assessing regeneration of *panniculus carnosus*. *Panniculus carnosus* is a muscle with unique regeneration potential thought to

participate in wound contraction, able to fuse with HSCs (phenomena with yet unknown physiologic function) and being responsible for myogenic potential of murine dermis cell cultures (Brazelton et al. 2003; Naldaiz-Gastesi et al. 2016).

5. Conclusions

1. Using different genetic mouse models and imaging techniques we show that mouse skin is a densely innervated organ and neural crest-derived cells migrate from nerves to populate injured tissues
2. Upon injury nerve associated neural crest-derived cells acquire activated glia phenotype and are highly secretory cells
3. Specific *Sox10* gene deletion leads to ablation of activated glia, while loss of *Pten* causes expansion of glial cells in cutaneous wound tissues
4. Loss of activated glial cells leads to delayed wound closure through impaired wound contraction and affects myofibroblast numbers in the wound, while expansion increases the amount of myofibroblasts in the wound and TGF- β signaling activation
5. Human dermis-derived cells display differentiation potential towards mesenchymal cell lineages and can display expression of embryonic and neuroectodermal markers *in vitro*

Theses for defence:

- Nerve-derived cells of neural crest origin respond to skin injury, acquire activated glia phenotype, proliferate and populate granulation tissue
- Activated glial cells in the wounded tissue are highly secretory and express many genes relevant for the injury repair
- Activated glial cells participate in wound healing, promote TGF-β signalling activation, myofibroblast formation and wound contraction
- Adult skin dermis-derived cells are MSC-like, possess neuroectodermal differentiation capacity and represent potential source of cells for therapy

6. Scientific publications and conference presentations

Scientific publications:

U.Riekstina, I.Cakstina, V.Parfejevs, M.Hoogduijn, G.Jankovskis, I.Muiznieks, R.Muceniece, J.Ancans. Embryonic Stem Cell Marker Expression Pattern in Human Mesenchymal Stem Cells Derived from Bone Marrow, Adipose Tissue, Heart and Dermis. *Stem Cells Reviews and Reports*. 2009, 5(4):378-86

U.Riekstiņa, V.Parfejevs, R.Muceniece, I.Cakstina, M.Boroduskis, J.Ancans. FGF-2 effect on the self-renewal of human dermal stem cell population *in vitro*. *Acta Universitatis Latviensis*, Medicine, 2009, 750:117-129

V.Parfejevs, K.Jekabsons, M.Boroduskis, I.Cakstina, J.Ancans, R.Muceniece, U.Riekstina. Characterization of human skin-derived stem cell neurodifferentiation potential. *Acta Universitatis Latviensis*, Medicine, 2010, 755: 61-71

V.Parfejevs, M.Gavare, L.Cappiello, M.Grube, R.Muceniece, U.Riekstina. Evaluation of biochemical changes in skin-derived mesenchymal stem cells during in vitro neurodifferentiation by FT-IR analysis. *Spectroscopy: An International Journal*, Volume 27, 2012, Issue 5-6, 315-320

V.Parfejevs. J.Debbache, O.Shakhova, M.Glausch, M.Wegner, U.Suter, U.Riekstina, S.Werner, L.Sommer. Injury-activated glial cells promote wound healing of the adult skin. *Manuscript submitted and currently in revision*.

Poster presentations at the international conferences:

V. Parfejevs, M. Boroduskis, U. Riekstina, I. Cakstina, J. Kungs, L. Cappiello, J. Krustins, J. Ancans, Human skin derived MSC characterization and effects of DMSO on secretion of growth hormones and marker profile. **TERMIS EU Meeting,** Granada, Spain, June 7 – 10 2011.

V. Parfejevs, M. Gavare, L. Cappiello, M. Grube, R. Muceniece, U. Riekstina, Evaluation of biochemical changes in dermal stem cells during neurodifferentiation *in vitro*. **14th European Conference on the Spectroscopy of Biological Molecules.** Coimbra, Portugal, August 29 – September 3 2011.

V.Parfejevs, M. Boroduskis, I. Cakstina, L. Cappiello, J. Ancans, U. Riekstina. Changes of human skin-derived MSC phenotype by modelling neural stem cell niche conditions in vitro. **10th ISSCR Meeting**, Yokohama, Japan, June 10 – 13 2012. (Abstract F-2150).

V. Parfejevs, J.Debbache, M. Glausch, O. Shakhova, S. Werner, U.Riekstina, Lukas Sommer, Activation of neural crest stem cells and their role in a mouse model of cutaneous wound healing. **TERMIS-AP Meeting**, Daegu, Republic of Korea, September 24 – 27 2014.

Selected oral presentations:

"Role of Neural crest-derived cells in wound healing." Oral presentation at the **Institute of Anatomy Seminar series**. University of Zurich, Zurich, Switzerland, April 6 2016.

"Neural crest-derived cells promote wound healing of the adult mouse skin *in vivo*" Oral presentation at the **EMBO regeneration conference series**. Paestum, Italy, September 19, 2016.

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9. Supplementary information

Supplementary tables

Supplementary Table S1 Top upregulated growth factor and cell signalling related genes with reported functional relevance.

Tnn	3.007	Tenascin-W mediates neurite repulsion from hippocampal explants[1] and has anti-adhesion matrix modulating proerties[2,3].
Areg	2.826	EGFR ligand amphiregulin is a growth factor for keratinocytes important in wound healing[4,5]. TGFβ regulates amphiregulin to promote lung fibrosis[6]. Important in inflammatory wound response[7].
Tnc	2.742	Tenascin-C loss leads to corneal wound healing delay and decreased myofibroblast generation[8] and regulates myofibroblast recruitment after cardiac injury[9]. It is strongly expressed by Schwann cells during Wallerian degeneration[10].
Wisp1	2.546	Role in wound healing suggested[11].
Inhba	2.477	Subunit of activin – a crucial factor in wound healing[12]. Expressed in the granulation tissue[13].
Aspn	2.476	Antifibrotic. Found after spinal cord injury[14] Inhibits TGF-β/Smad signalling in osteoarthritis settings[15,16].
Tnfsf14	2.309	LIGHT plays role in the resolution of inflammation in wound healing[17], promotes collagen deposition and epidermal thickening and fibrosis in mouse skin[18]. It has been shown to be upregulated at the site of regeneration following nerve injury, but not reported in Schwann cells[19,20].
Btc	2.306	EGF family protein that regulates new hair follicle formation, hair cycle induction and angiogenesis after wounding[21]. Induces Schwann cell migration[22].
Fgf5	2.173	FGF5 expression is increased upon injury[23]. Promotes angiogenesis [24] and regulates hair growth cycle[25]. Found to be upregulated in the Schwann cells of the injured nerve[26].
Epha3	2.162	EphaA3 receptor is upregulated upon spinal cord injury [27]
Ltbp2	2.092	As an inhibitor of FGF-2 may modulate wound repair and contribute to fibrotic diseases[28]

Pdgfrl	2.080	Function in wound healing has not been reported so far.
Hilpda	1.965	Induced by hypoxia and HIF1[29], an important factor in wound healing[30].
Cd38	1.912	CD38 is found on several cell types in the brain including astrocytes and facilitates recovery from the brain injury[31].
Cpz	1.911	Carboxypeptidase Z (CPZ) – ECM protein that modulates Wnt signaling[32] Function in wound healing has not been reported so far.
Trf	1.897	Provided by PNS and necessary in amphibian limb regeneration blastemas[33]. Expressed in Schwann cells after injury[34].
Pdgfb	1.889	PDGF is important in wound healing[35] Topical application of PDGF BB enhances wound healing[36], however, conflicting data on the effects of PDGF BB exists[37].
Metrn	1.868	Neurotrophic factor Meteorin regulates glial cell differentiation and promotes axonal extension[38] and promotes gliogenesis after striatal injury[39].
Bgn	1.792	Biglycan is an ECM proteoglycan able to induce proto-myofibroblast phenotype and migration of fibroblasts[40].
Hgf	1.746	HGF promotes wound healing[41–43] and is implicated in PNS development and injury repair[44,45].
Gfra1	1.700	Found on Schwann cell membranes and can be secreted[46]. Important for injured motor neuron fate determination[47].
Igf1	1.646	IGF1 is important for keratinocyte migration and wound re-epithelializatin[48]. Promotes wound healing in oestrogen-deprived mice[49]. Can be produced by Schwann cells upon injury[50] and facilitates nerve regeneration[51].

Supplementary Table S2 Top upregulated TGF-β signalling related genes with reported functional relevance.

Postn	3.044	Activates TGFβ pathway[52]. Promotes wound healing[53,54] and myofibroblast differentiation in the wound[55]. Important for wound melanoma metastasis[56] and Schwann cell migration[57]. Astroglial periostin promotes axonal regeneration after spinal cord injury[58].
Adam12	2.628	Contributes to TGFβ signaling[59]. Secreted form promotes myogenesis[60], while anchored form interferes with re-epithelialization[61].
Fbn2	2.557	Found in wounds[62]. Needed for proper activation of latent TGF-β[61]. Sequesters BMP complexes in a latent state[63,64].
Wisp1	2.546	Role in wound healing suggested[11]. Inhibits Smad-2 mediated TGF-β1 signalling in osteoblasts[65].
Inhba	2.477	Subunit of activin – a crucial factor in wound healing[12] Expressed in the granulation tissue[13].
Aspn	2.476	Found after spinal cord injury[14] Inhibits TGF-β/Smad signalling in osteoarthritis settings[15,16].
Ltbp2	2.092	May bind and modulate FGF-2 effects in wound repair and contribute to fibrotic diseases[28]. Does not bind to latent TGFβ, but might indirectly regulate the activation of TGFβ by releasing LTBP-1 from microfibrils[66,67].
Plod1	1.697	Generates hydroxylysine residues from lysine residues in collagen peptides[68]. Mechanical state of the matrix can directly control the efficacy of TGF-β activation[69].
Cilp	1.638	Inhibition of TGF-β1 signaling[70]. TGF-β1 induces CILP suggesting functional feedback loop[71].
Lox	1.606	Profibrotic – increases ECM stiffness[72]. Binds to mature TGF-beta1 and enzymatically regulates its signalling in the bone[73].
Tgfbi	1.455	Is induced by TGFβ and plays role in wound healing[74]. Inhibition of TGFBIp expression leads to reduced myofibroblast differentiation[75].
Bmp1	1.431	BMP1-like proteinases are essential to the wound healing of skin[76] BMP1 controls TGF-β1 activation[77].

Fstl1	1.369	Failure of wound closure in the absence of Follistatin1[78]. Binds BMP4[79].
Fbn1	1.271	Fbn1 regulates TGF-β1 bioavailability[80].
Plod2	1.258	Important enzyme in fibrosis[81]. Expression is regulated by an alternative downstream TGFβ-1 activation mechanism[82]. Regulates ECM stiffness – myofibroblast differentiation[83].

Supplementary Table S3 Top upregulated chemotaxis and inflammation related genes with reported functional relevance.

Cxcl5	3.653	A potent neutrophil chemoattractant during allergic airway inflammation[84].
Il1rl1	3.461	The resulting soluble and receptor proteins have emerged as key regulators of the inflammatory process implicated in a large variety of human pathologies[85].
Trem2	3.048	TREM1 and TREM2 activate myeloid cells by signalling through the adaptor protein DAP12[86].
Tnfsf14	2.309	LIGHT plays role in the resolution of inflammation in wound healing[17], promotes collagen deposition and epidermal thickening and fibrosis in mouse skin[18]. It has been shown to be upregulated at the site of regeneration following nerve injury, but not reported in Schwann cells[19,20].
Pla2g7	2.229	PAF acetylhydrolase has the substrate specificity and lipoprotein association of the native enzyme, and blocks inflammation in vivo[87].
Ccl8	1.700	Monocyte chemotactic protein-2 activates CCR5[88].
Cfp	1.648	Properdin plays a key role in allergen-induced airway inflammation and represents a potential therapeutic target for human asthma[89].
Cd36	1.450	A scavenger receptor involved in immunity, metabolism, angiogenesis, and behavior[90].
Cxcl2	1.417	It promotes the recruitment of myeloid-derived suppressor cells[91].

Supplementary Table S4 Top upregulated migration and adhesion related genes with reported functional relevance.

Postn	3.044	Activates TGFβ pathway[52]. Promotes wound healing[53,54] and myofibroblast differentiation in the wound[55]. Important for wound melanoma metastasis[56] and Schwann cell migration[57]. Astroglial periostin promotes axonal regeneration after spinal cord injury [58].
Col8a1	2.817	Necessary for migration and proliferation of vascular smooth muscle cells and thus, has a potential role in the maintenance of vessel wall integrity and structure, in particular in atherogenesis[92].
Adam12	2.628	Anchored form interferes with re-epithelialization[60]. Marker that identifies a distinct proinflammatory subset of platelet-derived growth factor receptor-α-positive stromal cells that are activated upon acute injury in the muscle and dermis[93].
Emcn	2.561	Interferes with the assembly of focal adhesion complexes and inhibits interaction between cells and the extracellular matrix[94].
Thbs4	2.299	Role for the astrocyte-secreted protein Thbs4 in the migration of newly formed neurons within the RMS to the olfactory bulb[95].
Cdh11	2.225	Regulates collagen and elastin synthesis, both affecting the mechanical properties and contractile function of animal tissues[96].
Ccl6	2.224	Also known as MIP-1, it is abundant in acute wounds and serves as macrophage chemoattractant[97,98]. Leads to CCR1 mediated signalling[99].
Mfap4	2.125	May contribute to the elastic fiber assembly and/or maintenance[100].
Thbs2	1.922	Adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions. Ligand for CD36 mediating antiangiogenic properties[101].
Ccdc80	1.764	Promotes cell adhesion and matrix assembly[102].
Fbln2	1.687	Acts as an adapter that mediates the interaction between FBN1 and ELN[103].
Mfap5	1.216	Protein associated with elastic fiber microfibrils[104].
Spp1	1.179	Binds tightly to hydroxyapatite. Appears to form an integral part of the mineralized matrix[105].

Supplementary Table S5 Top upregulated ECM related genes with reported functional relevance.

Apln	3.778	Ectopic expression in keratinocytes induces enlarged but not leaky blood vessels in the dermis[106].
Col8a1	2.817	Necessary for migration and proliferation of vascular smooth muscle cells and thus, has a potential role in the maintenance of vessel wall integrity and structure, in particular in atherogenesis[92].
Adam12	2.628	Marker that identifies a distinct proinflammatory subset of platelet-derived growth factor receptor-α-positive stromal cells that are activated upon acute injury in the muscle and dermis[93].
Col1a1	2.616	Mutation in the gene cause Ehlers-Danlos syndrome, which is a connective tissue disorder characterized by hyperextensible skin, atrophic cutaneous scars due to tissue fragility and joint hyperlaxity[107]. Structural element of Type I Collagen, an ECM component important for wound matrix integrity at different stages of tissue repair[108].
Emcn	2.561	Interferes with the assembly of focal adhesion complexes and inhibits interaction between cells and the extracellular matrix[94]
Fbn2	2.557	Found in wounds[62]. Needed for proper activation of latent TGF-β[63]. Sequesters BMP complexes in a latent state[63,64].
Col3a1	2.543	Mutation in the gene cause Ehlers-Danlos syndrome 3 which is a connective tissue disorder characterized by hyperextensible skin, atrophic cutaneous scars due to tissue fragility and joint hyperlaxity. Is the major ligand of ADGRG1 in the developing brain and binding to ADGRG1 inhibits neuronal migration and activates the RhoA pathway by coupling ADGRG1 to GNA13 and possibly GNA12[109].
Eln	2.369	Essential determinant of arterial morphogenesis. Possess regulatory function during arterial development, controlling proliferation of smooth muscle and stabilizing arterial structure[110].
Col1a2	2.345	Structural element of Type I Collagen, an ECM component important for wound matrix integrity at different stages of tissue repair[108]. Loss of Col1a2 can cause Ehlers-Danlos syndrome[111].
Col5a2	2.343	Mutation in the gene cause Ehlers-Danlos syndrome classic type which is a connective tissue disorder characterized by hyperextensible skin, atrophic cutaneous scars due to tissue fragility and joint hyperlaxity[112].

Col6a5	2.090	Mutations in the gene reported to be associated with atopic dermatitis using a family-based association analysis[113].
Col5a1	2.020	Mutation in the gene cause Ehlers-Danlos syndrome classic type which is a connective tissue disorder characterized by hyperextensible skin, atrophic cutaneous scars due to tissue fragility and joint hyperlaxity[112].
Col6a1	1.964	COL6A1 may serve participate in the initiation and progression of cervical cancer. It correlates with poor prognosis in cervical cancer patients[114].
Serpine1	1.864	Its rapid interaction with PLAT may function as a major control point in the regulation of fibrinolysis[115].

Supplementary Table S6 Top upregulated angiogenesis related genes with reported functional relevance.

Esm1	4.015	May play role in new blood vessel formation, affects VEGF bioavailability[116].
Cxcl5	3.653	CXCL5 is required for increased intestinal angiogenesis during resection-induced adaptation[117].
Vash1	2.369	Vash1 inhibits neo-vascularization upon injury[118].
Sfrp2	2.302	Sfrp2 inhibits apoptosis in Hypertrophic scars[119].
Robo4	1.933	SLIT3/ROBO4 pathway is required for MSC-guided vascularization in engineered tissues[120].
Mdk	1.751	Mdk is associated with PGRN and promotes hepatocellular carcinoma angiogenesis[121]. Astrocyte secreted Mdk promotes CNS repair after injury[122].
Hgf	1.746	Enhances angiogenesis in skin wounds[41]. Scatter factor (hepatocyte growth factor) is a potent angiogenesis factor *in vivo*[123].
Fbln2	1.687	FBLN2 isoform has an important tumour-suppressive and anti-angiogenic role in nasopharyngeal carcinoma[124].
Vcan	1.678	Versican V2 isoform enhances angiogenesis by regulating endothelial cell activities[125].

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