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REVIEW

The regenerative capacity of neonatal tissues

Angela M. Montero and Alice H. Huang*

ABSTRACT

It is well established that humans and other mammals are minimally regenerative compared with organisms such as zebrafish, salamander or amphibians. In recent years, however, the identification of regenerative potential in neonatal mouse tissues that normally heal poorly in adults has transformed our understanding of regenerative capacity in mammals. In this Review, we survey the mammalian tissues for which regenerative or improved neonatal healing has been established, including the heart, cochlear hair cells, the brain and spinal cord, and dense connective tissues. We also highlight common and/or tissue-specific mechanisms of neonatal regeneration, which involve cells, signaling pathways, extracellular matrix, immune cells and other factors. The identification of such common features across neonatal tissues may direct therapeutic strategies that will be broadly applicable to multiple adult tissues.

KEY WORDS: Mouse regeneration, Neonatal healing, Neonatal regeneration

Introduction

Regeneration is the biological process of restoring damaged cells and tissues to full function. Whereas invertebrates (such as planaria and hydra) and lower-order vertebrates (such as zebrafish and axolotl) possess the remarkable capacity to regenerate entire body parts and organs, it is well appreciated that regenerative healing in humans and other mammals is restricted to a small number of tissues (Hantash et al., 2008). For most mammalian tissues, injury and insult results in fibrotic healing that is characterized by disorganized scar formation and loss of endogenous structure and cell types, resulting in permanently impaired function. Even for regenerative tissues such as muscle and bone, successful regeneration depends on the severity of the original injury, as critical-sized defects also lead to non-regenerative fibrotic healing. For example, volumetric muscle loss injuries created by >20% muscle excision do not regenerate (Wu et al., 2012; Garg et al., 2015). Inducing functional regeneration of mammalian tissues has therefore been the focus of considerable research.

In recent years, our understanding of mammalian tissues as either fibrotic or regenerative has been challenged by the discovery that some tissues that heal by scarring in adult mammals are able to undergo regeneration after injury during the neonatal stage after birth. The first example to be discovered, and the most well-known model of neonatal regeneration, is the mouse heart. In a landmark study (Porrello et al., 2011b), neonatal hearts injured within a short postnatal window (1 week after birth) showed full regeneration of cardiac structure and function. Since this initial discovery, an increasing number of tissues have been shown to possess similar regenerative capacity; although these tissues share several common cell and molecular characteristics (e.g. mitotic capacity of differentiated cells is a common mechanism of neonatal regeneration), there are also differences between neonatal regeneration across tissues, and differences in the signaling pathways that regulate tissue-specific regeneration.

In this Review, we focus on several mammalian tissues with regenerative, improved healing or unique healing capacity during the neonatal stage of life. For each tissue, we discuss the common and distinctive cell and molecular mechanisms of neonatal regeneration. Because there are few studies in fetal sheep and other mammals, we focus on the neonatal mouse, as studies in this model comprise the majority of the existing literature. Although we devote a large section to heart regeneration, owing to the extensive body of literature on the neonatal heart, we also discuss recent research on the regeneration of cochlear hair cells, dense connective musculoskeletal tissues and the brain/spinal cord (Fig. 1). The mammalian digit tip, however, will not be discussed; although it was once considered a tissue that regenerated only during early life, research now suggests that digit phalangeal position rather than age is the limiting factor in regenerative capacity, and that digit tip regeneration is observed in both neonates and adults (Neufeld and Zhao, 1995; Fernando et al., 2011; Johnston et al., 2016). Although aging may slow the healing process, loss of healing does not occur (Fernando et al., 2011). Additionally, there is clinical evidence of digit tip regeneration in adult humans (Douglas, 1972; Illingworth, 1974; Lee et al., 1995; Masaki and Kawamoto, 2021).

Neonatal heart regeneration

The heart is a complex organ composed of four chambers that function together as a pump to circulate blood throughout the body (Fig. 2). Heart function is enabled by the coordinated contraction of cardiomyocytes that reside within the myocardium – the muscle layer of heart tissue. In addition to cardiomyocytes, heart tissue contains multiple other cell types, including cardiac fibroblasts, endothelial cells and vascular smooth muscle cells. As we discuss below, a number of studies have examined how these cells, together with molecular factors, contribute to neonatal heart regeneration.

Resident cardiomyocytes drive neonatal regeneration

Complete regeneration of the neonatal mammalian heart was first shown in 2011 (Porrello et al., 2011b). In this study, ventricular resection of 1-day old (postnatal day 1, P1) hearts led to regeneration through proliferation of resident cardiomyocytes that replace the lost myocardium. Full structural and functional regeneration was observed with normal contractile function at 2 months post-injury. Follow-up studies extended these findings to other models of cardiac injury, including myocardial infarction (MI) at P1 (Haubner et al., 2012; Porrello et al., 2013). Despite extensive cell death at the infarction site, neonatal hearts underwent complete regeneration following MI, exhibiting neovascularization and normal functional marker expression at 3 months. Lineage tracing and BrdU incorporation demonstrated cardiomyocyte proliferation

Department of Orthopedic Surgery, Columbia University, New York, NY 10032, USA.

*Author for correspondence (ah364@columbia.edu)

A.H.H., 0000-0002-5037-6829

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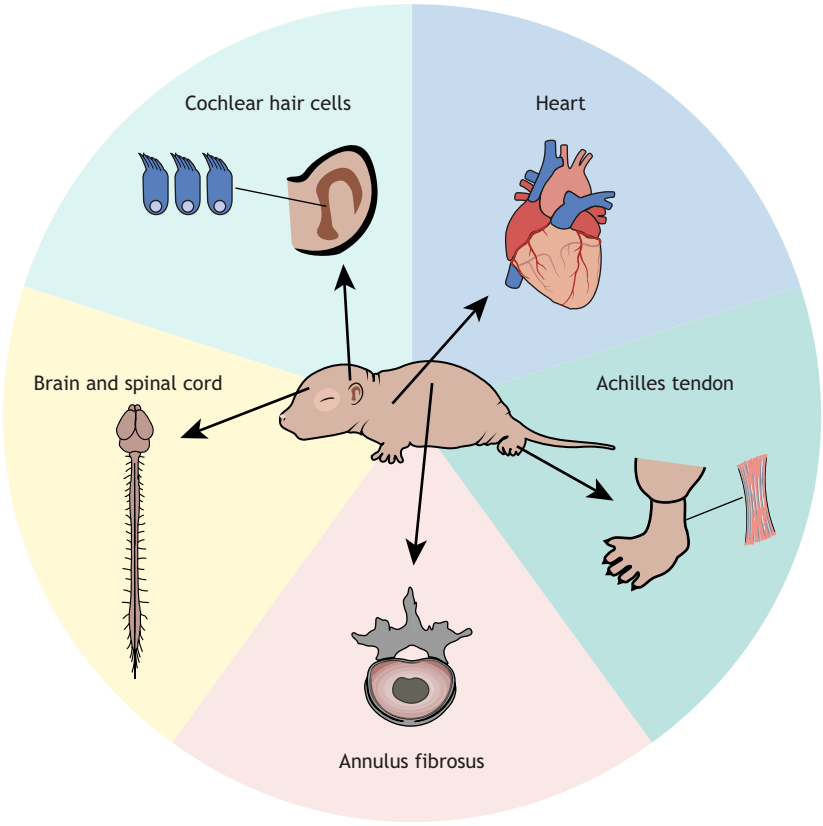

Fig. 1. Regenerative or improved healing is observed in select tissues in neonatal mice. The recent literature shows that neonatal mice have the capacity to regenerate or display improve healing of multiple tissues that either fail to heal or heal by scar formation in adult mice. These tissues include the neonatal heart, cochlear hair cells, the Achilles tendon, the annulus fibrosus of the intervertebral disc, and the brain and spinal cord. Although not all of these neonatal tissues regenerate fully, all show distinctive mechanisms of healing compared with their adult counterparts.

as the cellular mechanism of regeneration, as observed in resection injury. This capacity for neonatal mammalian heart regeneration has been further demonstrated in humans following myocardial infarction (Haubner et al., 2016).

These early studies defined the neonatal regenerative window as 7 days post-birth, as MI and resection injuries performed on P7 mice resulted in scar-mediated healing and failed regeneration (Porrello et al., 2011b; Haubner et al., 2012; Porrello et al., 2013). This is thought to be related to the rapid loss of intrinsic mitotic activity in resident cardiomyocytes at P7 (Porrello et al., 2011b). Indeed, although mature cardiomyocytes undergo limited self-renewal, these cells are relatively quiescent in adult stages (Senyo et al., 2013). RNA-sequencing on uninjured mouse hearts at P10 and P1 revealed significant differences in the transcription of cell cycle regulators and DNA replication machinery, consistent with the link to mitotic potential (Haubner et al., 2012). In addition, transcriptional and cell cycle regulators such as GATA4, MEIS1 and D-type cyclins, all of which are required for normal heart development and cardiomyocyte proliferation, are also necessary for neonatal regeneration. (Pasumarthi et al., 2005; Mahmoud et al., 2013; Yu et al., 2016; Cardoso et al., 2020). Although most studies compared P0 and/or P11 with P7 and older mice, a recent study suggests that the regenerative window may close as early as P2 after birth (Notari et al., 2018).

The role of neonatal extracellular matrix components and matrix stiffness in heart regeneration

The extracellular matrix (ECM) has emerged as a key regulator of neonatal heart regeneration. Differential gene expression comparing P1 versus P7 and P1 versus P2 hearts identified ECM components among the most differentially expressed terms (Haubner et al., 2012; Notari et al., 2018), suggesting that the ECM microenvironment may underlie regenerative potential. Consistent with this hypothesis, decellularized neonatal mouse hearts and regenerative zebrafish hearts are both capable of inducing adult cardiomyocyte proliferation (Chen et al., 2016; Bassat et al., 2017). Furthermore, delivery of zebrafish-derived ECM is sufficient to improve cardiac function in the adult mouse after MI (Chen et al., 2016). Although the ECM is composed of multiple components, a groundbreaking study identified agrin (a heparan sulfate proteoglycan) in the neonatal cardiac ECM and demonstrated its ability to reduce scar formation and improve juvenile and adult function after MI (Bassat et al., 2017). Agrin is also necessary for regeneration; loss of agrin from neonatal ECM results in impaired regeneration (Bassat et al., 2017). Agrin may act through Dag1 to regulate Yap signaling (a Hippo pathway effector; discussed below), which may account for its regenerative effects (Bassat et al., 2017; Morikawa et al., 2017). Another matrix component linked with cardiac regeneration is the glycoprotein follistatin-like 1 (FSTL1). Interestingly, epicardial FSTL1 but not cardiomyocyte-derived FSTL1 is capable of stimulating cardiomyocyte proliferation and improving functional healing following MI in mouse and pig models, likely due to differences in post-translational modifications between these glycoproteins (Wei et al., 2015; Magadum et al., 2018).

In addition to specific ECM components, matrix stiffness and the local mechanical environment are key drivers of regenerative potential (Notari et al., 2018). Specifically, Notari and colleagues demonstrated loss of regenerative potential far earlier than previous studies (P2 rather than P7). As changes in cardiomyocyte mitotic ability and Agrin were not yet apparent at P2, the transition from a regenerative P1 state to a non-regenerative P2 state was attributed to increasing stiffness of the heart due to collagen crosslinking. Using pharmacological inhibition of collagen and

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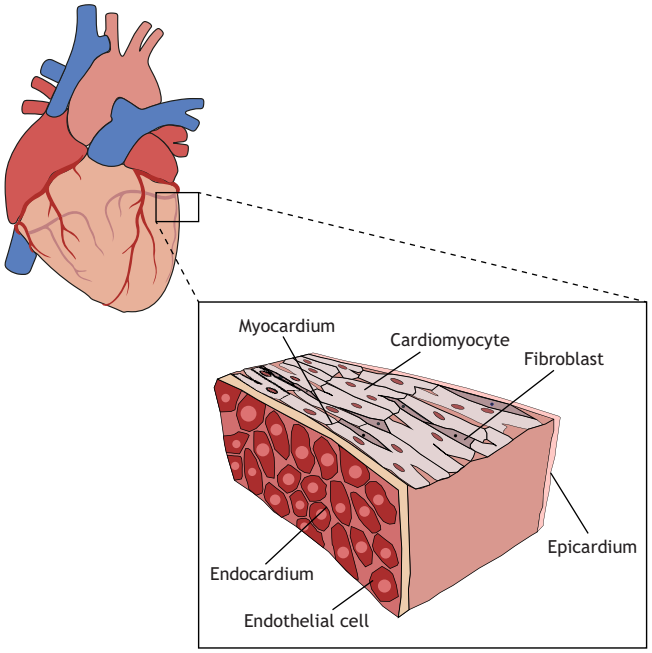

Fig. 2. Heart tissue is composed of specialized layers and cell types. Cardiomyocytes within the heart myocardium are the primary source of regenerative cells during neonatal cardiac healing. Other cell types within the heart include endothelial cells and cardiac fibroblasts; however, their potential functions during neonatal regeneration have not been studied.

elastin crosslinking, cardiac regeneration was rescued in P3 mice resulting in reduction in fibrosis by day 21 post-injury. Importantly, no change in proliferative ability of cardiomyocytes was observed with treatment, suggesting lost mitotic capacity did not sufficiently explain loss of regenerative competence (Notari et al., 2018). Other research has linked postnatal changes in the pressure load on the heart to cardiomyocyte withdrawal and regenerative loss (Nguyen et al., 2020). Calcineurin signaling-mediated regulation of the nuclearization of HOXB13, a transcription factor dephosphorylated by calcineurin and upregulated postnatally, has been implicated in the switch from hyperplastic growth to hypertrophic growth in the postnatal heart. Conditional deletion of *Hoxb13* and *Meis1*, which encodes an associated transcription factor also linked to loss of regenerative capacity in the adult heart (Mahmoud et al., 2013), in cardiomyocytes 1 week after myocardial infarction led to increased proliferation and improved systolic function (Nguyen et al., 2020).

The Hippo and Wnt signaling pathways are critical to neonatal heart regeneration

One key signaling pathway associated with neonatal cardiac regeneration is the Hippo pathway. Hippo signaling is a critical pathway in heart development and organ size regulation (Heallen et al., 2011; Xin et al., 2011). In the context of neonatal cardiac regeneration, the Hippo pathway transcription factor YAP1 is required for successful regeneration following artery ligation (Xin et al., 2013). Agrin-dependent regeneration is also mediated by downstream YAP nuclear localization and signaling (Bassat et al., 2017; Morikawa et al., 2017). Other studies further link the pro-proliferative and pro-regenerative effects of cardiac-associated microRNAs with the regulation of Hippo signaling (Tian et al., 2015; Torrini et al., 2019).

Wnt signaling has also been implicated in neonatal cardiac regeneration. Age-dependent changes in Wnt signaling, such as maturation-associated decreases in β-catenin, have been linked to loss of regenerative potential (Quaife-Ryan et al., 2017, 2020). In neonatal mice, constitutively active Wnt signaling enhances proliferation while inhibition of Wnt impairs regeneration. However, increased Wnt signaling in adult mice does not induce a pro-proliferative or pro-regenerative transcriptional program but rather enhances apoptotic and inflammatory gene signaling. These data suggest that the intrinsic response of cardiomyocytes to Wnt signaling rather than alterations in Wnt signaling may drive the loss of regenerative potential. Although the interaction between Yap and Wnt signaling via β-catenin is crucial for regulating cardiomyocyte proliferation and heart size in development (Heallen et al., 2011), it is not clear whether this crosstalk also occurs during neonatal regeneration.

Additional mechanisms of neonatal heart regeneration

MicroRNAs are small, single-stranded non-coding RNA molecules that regulate gene expression (O'Brien et al., 2018). Overexpression of microRNAs such as *miR-199*, *miR-590* and *miR-302-367* has been shown to enhance cardiomyocyte proliferation (Eulalio et al., 2012; Aguirre et al., 2014; Tian et al., 2015; Torrini et al., 2019; Abbas et al., 2020). After adult cardiac injury, these microRNAs improve functional regeneration and reduce scarring (Eulalio et al., 2012; Tian et al., 2015). Interestingly, the mechanisms of action of several microRNAs relevant to heart regeneration are linked to Hippo pathway signaling (Tian et al., 2015; Torrini et al., 2019). For example, overexpression of *miR 302-367* reduces expression of the Hippo pathway components *Mst1*, *Lats2* and *Mob1* resulting in increased nuclearization of YAP (Tian et al., 2015). Similarly, *miR-199* and *miR-590* overexpression results in activation of Yap signaling (Torrini et al., 2019). These findings are in line with other reports showing that activation of Yap signaling enhances cardiomyocyte proliferation and regenerative potential of the adult heart (Heallen et al., 2011; Xin et al., 2013; Bassat et al., 2017). Other microRNAs, such as the *miR-15* family of microRNAs, increase with cardiac maturation, resulting in mitotic arrest of aged cardiomyocytes (Porrello et al., 2011a; Hullinger et al., 2012; Porrello et al., 2013). Accordingly, inhibition of *miR-15* increases proliferation and improves systolic function after ligation-reperfusion injury (Hullinger et al., 2012; Porrello et al., 2013).

In addition to microRNAs, other mechanisms such as changes in metabolism have been studied in the context of regeneration. The reduction in cardiomyocyte mitotic capacity during the first postnatal week coincides with a switch in metabolic energy source from glucose to fatty acids (Puente et al., 2014; Fajardo et al., 2021). Research has shown that introduction to an oxygen-rich postnatal environment shifts cells towards fatty acid oxidation and, as a result, increases reactive oxygen species (ROS) and oxidative DNA damage, thereby inducing cardiomyocyte cell cycle arrest (Puente et al., 2014). Increasing glucose metabolism over oxidative phosphorylation by forcing Glut1 overexpression in neonatal mice reduces fibrosis after cryoinjury (Fajardo et al., 2021). Gradual hypoxia to inhibit oxidative metabolism can reactivate cardiomyocyte proliferation in aged mice and induce a regenerative response following MI (Nakada et al., 2017). Most recently, research has attributed cardiomyocyte arrest to the buildup of succinate (Bae et al., 2021), which is oxidized postnatally and results in a burst of ROS production (Chouchani et al., 2014; Zhang et al., 2018). Treatment with malonate to inhibit succinate dehydrogenase and thereby block succinate build up is capable of inducing cardiomyocyte proliferation and functional regeneration in adult hearts after myocardial infarction (Bae et al., 2021). Most

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importantly for its translational potential, the same group showed that delayed treatment with malonate initiated 1 week post-myocardial infarction in the adult heart is still capable of promoting a regenerative response, marked by reduced fibrosis and improved cardiac function.

Alterations in hormonal regulation also coincide with lost regenerative potential. For example, inhibition of thyroid hormones increases proliferation and leads to improved systolic functions and increased proliferation after ischemia reperfusion injury (Hirose et al., 2019). The immune system is also a crucial driver of cardiac regeneration, and immune cells such as macrophages are essential for angiogenesis during neonatal regeneration. As the immune response to regeneration is highly complex and involves multiple immune cell types and cytokines, we direct the interested reader to an excellent and comprehensive review of neonatal and adult immune differences in cardiac repair (Sattler and Rosenthal, 2016).

Crosstalk between cardiomyocytes and other cardiac cell types has emerged as an important aspect of neonatal regeneration. In certain cases, e.g. for FSTL1, differences in cell source can lead to different outcomes – in this case cardioprotective (cardiomyocyte-derived) versus proliferative (epicardial-derived) effects. (Wei et al., 2015; Magadum et al., 2018). More recently, research has shown that cardiac endothelial cells engage in hypoxia signaling through prolyl hydroxylases and thereby regulate cardiomyocyte proliferation (Fan et al., 2019; Dai et al., 2021). These are just a few examples to demonstrate the importance of cellular crosstalk in heart regeneration; a more detailed discussion of this topic can be found in an excellent recent review (Wagner and Dimmeler, 2020).

Finally, innervation and nerve-related growth factors and signaling networks have been shown to play a role in regeneration in neonatal mice and zebrafish (D'Uva et al., 2015; Mahmoud et al., 2015; White et al., 2015; Chen et al., 2016). Neuregulin 1 (NRG1) in particular was initially believed to improve healing after adult injury by activating cardiomyocyte proliferation (Bersell et al., 2009). However, more recent studies have demonstrated that NRG1 does not activate adult cardiomyocyte proliferation regardless of injury (Reuter et al., 2014). Rather, loss of *Erbb2* expression, a receptor through which NRG1 signals that declines in expression within the first postnatal week, is sufficient to reduce cardiomyocyte proliferation in neonates (D'Uva et al., 2015). Transient expression of *Erbb2* after juvenile or adult injury results in reactivation of cardiomyocyte proliferation, reduced fibrosis and improved functional healing (D'Uva et al., 2015). The precise role of nerve crosstalk and interactions with cardiac cell populations remains an emerging area of research.

Injury models and controversies

Commonly used neonatal cardiac injury models include resection, MI and cryoinjury. However, the extent of regeneration following cryoinjury is more variable compared with other models, with multiple papers citing sustained fibrosis months after injury and others demonstrating limited or absent proliferation (Jesty et al., 2012; Strungs et al., 2013; Darehzereshki et al., 2015; Mizutani et al., 2016; Zhao et al., 2021). Furthermore, transmural cryoinjuries that affect the entire heart wall do not regenerate in the neonate (Darehzereshki et al., 2015). Controversy has also arisen over the extent of regeneration after apical resection and MI. A study using apical resection on P1 mice identified extensive scaring at day 21 post-injury and did not observe proliferation of cardiomyocytes or neomyogenesis (Andersen et al., 2014). Notably, this injury model resected a much larger proportion of the heart than previous studies, which may account for the increased scarring observed. In response to this paper, a systematic review of different resection sizes and techniques was performed, demonstrating that, regardless of the extent of injury, neonatal cardiomyocytes proliferate and regenerate damaged myocardium, but that some fibrosis may be seen at later timepoints depending on the size of the resection (Bryant et al., 2015). This study also showed that certain surgical techniques result in proliferation of cardiomyocytes and scarring, even in sham controls, which can obscure results. Other research identified limited healing following MI, showing that a residual small infarct is visible at day 28 post-injury and results in aneurysm in some pups (Darehzereshki et al., 2015).

Despite these discrepancies and controversies, there is consensus that new myocardium is formed through proliferation of existing cardiomyocytes after both infarction and resection, but injury size and technique may impact the extent of regeneration (Lam and Sadek, 2018). Injury type, technique and severity are therefore crucial considerations in neonatal regeneration models, and these observations from the cardiac field may be widely applicable to other tissues as well.

Cochlear hair cells

The cochlea is located within the inner ear and is responsible for transmission of auditory signals. Within the cochlea, a specialized sensory epithelium termed the organ of Corti houses two types of hair cells (inner and outer) that express *Myo7a* (which encodes myosin VIIa) and are arranged in rows, with each cell displaying a stereocilia bundle on its apical surface (McPherson, 2018) (Fig. 3). The deflection of stereocilia by mechanical stimuli depolarizes hair cells, which activate afferent spiral ganglion neurons, thereby conveying information to central auditory structures to enable hearing (McPherson, 2018). In adult mammals, injury to cochlear hair cells leads to permanent loss and sustained hearing impairment (Brigande and Heller, 2009).

The utricle is another organ located within the inner ear and is responsible for balance and orientation sensing. Like the cochlea, the utricle relies on hair cells with aligned stereocilia to detect motion and orientation (McPherson, 2018). The utricle undergoes a similar loss of regenerative capacity with age, although notably some low levels of self-renewal are maintained (Forge et al., 1993; Kawamoto et al., 2009; Burns et al., 2012; Golub et al., 2012; Wu et al., 2016; Jen et al., 2019; Sayyid et al., 2019; González-Garrido

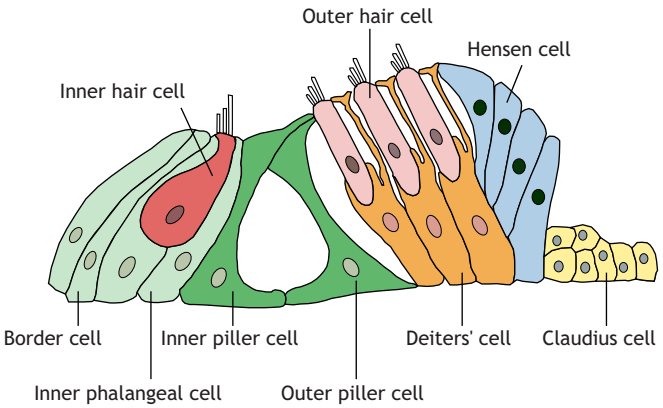

Fig. 3. Supporting cell types within the organ of Corti. Although hair cells of the cochlea do not regenerate, neonatal regeneration is possible via transdifferentiation of supporting cell types housed within the organ of Corti. These cells include inner phalangeal cells, inner piller cells, outer piller cells, Deiters' cells, Clausius cells and Hensen cells.

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et al., 2021). For this review, we focus primarily on cochlear hair cells; however, many of the relevant signaling pathways and factors within cochlear regeneration are also applicable to the utricle.

Hair cell regeneration is driven by transdifferentiation of supporting cell populations

In contrast to adult mice, neonatal mice are capable of regenerating *Myo7a*$^{+}$ hair cells after genetic ablation or ototoxic damage. Unlike the neonatal heart, which regenerates via proliferation of existing cardiomyocytes, neonatal hair cells do not possess intrinsic mitotic potential for regeneration. Instead, neonatal cochlear hair cell replacement is driven by transdifferentiation of neighboring supporting cells (Monzack and Cunningham, 2013) (Fig. 3). These *Sox2*-expressing non-sensory cells, which serve various roles in ion homeostasis and release supporting proteins/factors, are a heterogenous mixture of distinctive cell types with differential regenerative capacities. They include greater epithelial ridge cells, border cells, inner phalangeal cells, inner pillar cells, outer piller cells, Deiters' cells, Hensen cells and Claudius cells.

The isolation of different cochlear cell populations showed that *Sox2*-expressing supporting cells differentiate into hair cells in culture, whereas other supporting cell types proliferate but do not differentiate *in vitro* (Sinkkonen et al., 2011). Subsequent studies showed that, within the *Sox2* population, a subpopulation of *Lgr5/Sox2* supporting cells appear to have enhanced regenerative potential (Chai et al., 2012; Shi et al., 2012; Bramhall et al., 2014; Cox et al., 2014; Li et al., 2015; Ni et al., 2016b). These *Lgr5*$^{+}$ cells form hair cells at higher rates than non-specified supporting cell populations (Chai et al., 2012; Shi et al., 2012, 2013; Li et al., 2015). In the ear, *Lgr5*-expressing cells encompass inner pillar cells, third row Deiters cells, some cells of the greater epithelial ridge and some inner border cells (Shi et al., 2012). Earlier reports suggested that most hair cells are derived from pillar and Deiters cells (Sinkkonen et al., 2011). Inner pillar cells can be identified as *Lgr6*-expressing cells within the *Lgr5*-expressing population (Zhang et al., 2020). These cells may have enhanced differentiation capacity as they produce more hair cells in culture; however, they have not been extensively studied *in vivo*. In addition to these populations, *Axin2*-expressing cells located along the tympanic border have also been identified as a potential progenitor source capable of generating hair cells *in vitro* and *in vivo* (Jan et al., 2013).

In neonatal mice, supporting cell-mediated regeneration of hair cells occurs through two mechanisms: (1) direct transdifferentiation of neighboring supporting cells; and (2) mitotic regeneration of supporting cells. In direct transdifferentiation, supporting cells that surround hair cells in the cochlea differentiate into hair cells without undergoing cell division. In mitotic regeneration, supporting cells first divide and one or more daughter cells then acquire a hair cell fate. The primary role for supporting cells in neonatal cochlear hair cell regeneration was first demonstrated in neonatal mouse cochlear explants and cell culture experiments (Kelley et al., 1995; White et al., 2006; Sinkkonen et al., 2011; Chai et al., 2012). Early studies showed that the organ of Corti isolated from mouse embryos and neonates regenerated by transdifferentiation after laser beam irradiation (Kelley et al., 1995). Whereas neonatal and mature hair cells were both post-mitotic and quiescent, supporting cells adopted a progenitor-like phenotype when isolated (Sinkkonen et al., 2011; Chai et al., 2012; Shi et al., 2012). Similar to the heart, supporting cell-mediated regeneration is also restricted to a narrow window after birth, as P14 supporting cells are unable to re-enter the cell cycle or produce new hair cells *in vitro*, in contrast to P2 supporting cells (White et al., 2006; Sinkkonen et al., 2011).

These *in vitro* findings were later confirmed *in vivo* using two models of genetic ablation to target hair cells at P1: *Pou4f3*^{DTR} and *Atoh1-CreER*™*; Rosa26*$^{DTA/+}$ (Cox et al., 2014). Lineage tracing showed that *Lgr5*-lineage supporting cells formed new *Myo7a*-expressing hair cells, with some cells triple positive for *Lgr5lin*, *Myo7a* and the proliferation marker EdU, suggesting mitotic activation and regeneration of supporting cells after injury. Consistent with studies of neonatal heart regeneration, P7 mice also do not regenerate hair cells *in vivo*. Interestingly, newly generated hair cells after P1 injury were primarily located in the apical turn of the cochlea, a finding that had also been observed in explant models (Kelley et al., 1995; Cox et al., 2014; McGovern et al., 2018). This is likely due to the differential maturation states of apical versus basal cells; as the maturation of cochlear hair cells and supporting cells occurs in a basal to apical gradient, cells of the apex are considered less mature compared with cells of the basal turn during the early postnatal period, as evidenced by delayed morphological and cytoskeletal maturation (Waguespack et al., 2007; Lelli et al., 2009). Defining the regenerative window simply by postnatal day may therefore be overly simplistic.

In addition to genetic injury models, many studies have used aminoglycosides to induce hair cell ablation and examine regeneration (Hu et al., 2016; Ni et al., 2016b). Gentamicin and neomycin, both of which are aminoglycosides, penetrate hair cells through mechanoelectrical transduction (MET) channels on the tips of stereocilia and induce oxidative stress leading to necrosis or apoptosis (Lin et al., 2018; Qi et al., 2018). Unlike genetic injury models, however, aminoglycoside treatment primarily targets basal hair cells, sparing hair cells in the apical turn. This is attributed to the fact that apical hair cells are less mature and therefore do not have functional MET channels (Lin et al., 2018); this key difference in the selectivity of induced cell death highlights the importance of the injury model in interpreting neonatal regeneration potential. Following gentamycin treatment, proliferation is induced but regeneration of new hair cells does not occur or occurs to a lesser extent compared with genetic ablation injury (Hu et al., 2016; Ni et al., 2016b). Some studies suggest this is due to absence of Wnt signaling activation following aminoglycoside injury (Hu et al., 2016). However, selective targeting of the more mature regions of the cochlea may bias these results.

Despite their proper morphology and gene expression patterns, newly regenerated hair cells fail to mature, and the majority are lost by 15 days post-injury. Functional recovery of hearing also does not occur after injury, demonstrating key limitations of this mode of regeneration in contrast to neonatal cardiac regeneration. One potential explanation for this partial regeneration may be due to the reduced number of supporting cells present after injury, as supporting cells are necessary for hair cell survival (Mellado Lagarde et al., 2013). Thus, successful functional neonatal hair cell regeneration likely will require regeneration of both hair cells and supporting cells. Biasing the regenerative process toward mitotic regeneration may therefore overcome these limitations as both cell types are generated.

The Wnt and Notch signaling pathways are key components of the neonatal hair cell regenerative response

The main hair cell regeneration signaling pathways identified to date include the Wnt and Notch pathways. The importance of Wnt was suggested by the presence of *Lgr5*$^{+}$ and *Axin2*$^{+}$ supporting cell types that give rise to regenerated hair cells. In general,

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Wnt signaling induces proliferation of supporting cells, leading to new hair cell formation (Chai et al., 2012; Shi et al., 2012; Bramhall et al., 2014). By contrast, genetic or pharmacological inhibition of Wnt signaling results in impaired regeneration following injury *in vitro* and *in vivo* (Bramhall et al., 2014; Hu et al., 2016).

In addition to Wnt signaling, Notch signaling has emerged as a major pathway in hair cell regeneration. During regeneration, Notch target genes are downregulated, and pharmacological or genetic inhibition of Notch signaling increases formation of new hair cells derived from *Lgr5*$^{+}$ supporting cells (Bramhall et al., 2014; Li et al., 2015; Hu et al., 2016; Ni et al., 2016a,b). Furthermore, overexpression of the Notch1 intracellular domain (N1ICD), which activates the Notch pathway, in cochlear supporting cells results in a 92% reduction in the number of regenerated hair cells, suggesting that Notch signaling suppression occurs naturally after neonatal injury and is crucial to neonatal regenerative capacity (McGovern et al., 2018). The impact of Notch inhibition depends on Wnt signaling, as deletion of β-catenin abrogates the increase in hair cells following Notch inhibition (Bramhall et al., 2014; Hu et al., 2016).

Although Notch inhibition was repeatedly shown to induce proliferation and hair cell formation in the neonatal cochlea, its role in failed adult regeneration is less clear. Notch is naturally downregulated within the cochlea during maturation, but regeneration does not occur beyond the neonatal window (Murata et al., 2006; Maass et al., 2015). These results suggest that Notch inhibition would not be effective for adult regeneration and is also not sufficient to sustain regenerative potential in older mice.

The mechanoregulation of neonatal hair cell regeneration

Several studies suggest that neonatal hair cell regeneration may also be mechanically regulated. For example, it has been shown that cytoskeletal F-actin thickening at the junctions between hair cells and supporting cells occurs with postnatal maturation and is associated with loss of regenerative potential (Burns et al., 2008; Burns and Corwin, 2014). Another potential regulator of the local mechanical environment includes cell-cell attachments mediated by E-cadherin. With maturation, E-cadherin expression at supporting cell junctions increases (Burns et al., 2013; Burns and Corwin, 2014; Kozlowski et al., 2020). Studies in mouse utricle explants showed that internalization of E-cadherin (by culture with γ-secretase inhibitors, GSIs) results in proliferation of supporting cells and induction of hair cell markers (such as *Atoh1*, myosins VI and VIIA, and visible hair cell bundles) (Collado et al., 2011). As GSIs also inhibit Notch signaling, however, these effects may be due to Notch inhibition (Collado et al., 2011). As in other studies that targeted Notch directly, the effectiveness of GSI treatment on supporting cell proliferation and differentiation decreases with maturation and ceases by P16. This supports other findings that Notch inhibition postnatally is not sufficient to induce regeneration in mature mice.

The activation of Yap and Taz signaling, which is known to play a role in mechanosensing, has also been linked with regeneration of sensory hair cells. In the cochlea, viral gene delivery of constitutively active YAP after hair cell ablation in P6 mice initiates cell cycle re-entry, as shown by an increase in EdU^{+} *Sox2*$^{+}$ supporting cells in the organ of Corti (Gnedeva et al., 2020). Consistent with these results, additional studies showed that enhancing YAP localization to the nucleus results in proliferation of vestibular hair cells and regeneration of hair cells after injury (Rudolf et al., 2020; Kastan et al., 2021). However, whether these activities of Yap and Taz are linked to mechanoregulation has not been directly assessed.

Other molecular and transcriptional regulators of neonatal hair cell regeneration

A number of transcription factors have also been identified as crucial to hair cell formation or capable of regulating supporting cell regenerative potential. *Atoh1* encodes a bHLH transcription factor that is expressed in all hair cells and required for hair cell development and differentiation (Zhang et al., 2020). Ectopic expression of *Atoh1* is sufficient to induce hair cell formation from supporting cells of the neonatal cochlea but not from the adult cochlea (Zheng and Gao, 2000; Izumikawa et al., 2005; Kelly et al., 2012; Liu et al., 2012; Yang et al., 2013; Atkinson et al., 2014; Liu et al., 2014). *Atoh1* expression has also been linked to Notch and Wnt signaling; combined overexpression of *Atoh1* with Notch inhibition and Wnt manipulation increases mitotically forming hair cells (Ni et al., 2016a). A number of studies also showed that combined overexpression of *Atoh1* with other crucial hair cell transcription factors further improves regeneration. For example, overexpression of *Atoh1* and *Gfi1* (a transcription factor important for hair cell development and survival), results in a significant increase in new hair cells both in cochlear explants and in a *Pou4f3*^{DTR} mouse injury model (Lee et al., 2020). Forced expression of *Atoh1* and *Ikzf2* in adult cochlear supporting cells also results in formation of outer hair cells expressing prestin, a key marker that is typically absent from regenerated hair cells (Sun et al., 2021). Ectopic expression of *Pou4f3*, both alone and in combination with *Atoh1*, is also sufficient to induce conversion of supporting cells to hair cells after acoustic damage in adult cochlea (Walters et al., 2017). A recent study identified *Hic1* expression in the postnatal cochlea as a repressor of *Atoh1* that may modulate Wnt responsive gene expression and account for loss of regeneration with age (Abdul-Aziz et al., 2021). Knockdown of *Hic1* induces *Atoh1* expression and promotes hair cell differentiation in neonatal cochlear organoids (Abdul-Aziz et al., 2021), but the potential of *Hic1* in regeneration of the mature cochlea is still unknown.

In addition to *Atoh1*, *p27*^{Kip1} (*Cdkn1b*; a cyclin-dependent kinase inhibitor) has been identified as a regulatory factor in hair cell regeneration that may be responsible for hair cell quiescence (Chen and Segil, 1999; White et al., 2006). *p27*^{Kip1} is expressed in supporting cells and downregulated in neonatal supporting cells during differentiation to hair cells (White et al., 2006; Li et al., 2015). Suppression of *p27*^{Kip1} in P14 supporting cells results in increased mitotic activity, whereas deletion of *p27*^{Kip1} in hair cells of postnatal mice results in new hair cell generation, suggesting that age-dependent changes in supporting cell proliferative and regenerative capacity are, in part, due to changes in the ability to downregulate *p27*^{Kip1} (White et al., 2006). Additional studies showed that *p27*^{Kip1} deletion combined with *Atoh1* overexpression increases supporting cell conversion to new hair cells in a mature mouse after noise damage. Furthermore, *p27*^{Kip1} deletion results in upregulation of *Gata3*, which is a co-factor for *Atoh1* and is lost with age. The importance of *Gata3* was confirmed by experiments showing that co-activation of *Gata3* and *Atoh1* promotes the differentiation of supporting cells to hair cells in adult mice (Walters et al., 2017). Importantly, this study showed that p27 inhibition does not result in an increase in proliferation, and that deletion of cell cycle regulators downstream of p27 does not increase the conversion of SCs, suggesting a cell cycle-independent role for p27 in inhibiting the conversion of SCs to HCs.

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Dense connective tissues

Dense connective tissues are load-bearing tissues composed of fibrous extracellular matrix. These tissues include tendons and ligaments that connect muscle to bone or bone to bone, the articular cartilage lining the joint surfaces, as well as fibrocartilage structures [such as the menisci of the knee or the annulus fibrosus (outer layer) of the intervertebral disc (Benjamin and Ralphs, 2000, 2004)]. The primary functions of these tissues are to transfer or provide support to forces that arise from movement and to maintain stability of the musculoskeletal system. Simplistically, tendons/ligaments and articular cartilage withstand tensile and compressive forces, respectively, whereas the fibrocartilage tissues tend to operate under more-complex loading environments comprising compressive, shear and hoop tensile forces. The organization of the collagen matrix (and the type of collagen) in these tissues is therefore optimized for their specific loading environments. The regeneration of these tissues is defined by restoration of these matrix structural components, and by their organization and mechanical function.

Unlike muscles or bones, which regenerate well in adults, dense connective tissues do not undergo regenerative healing in response to traumatic injury or age-related degeneration. Rather, limited healing leads to the formation of disorganized scar tissue that fails to restore native mechanical function, leading to re-injury or persistent pain (McNulty and Guilak, 2008; Thomopoulos et al., 2015; Torre et al., 2019a). Moreover, initial damage to joint tissues such as the anterior cruciate ligament or menisci of the knee often introduces instability to the joint that is not resolved with surgical repair. Injury of these tissues is thus a predictor of subsequent degeneration of the articular cartilage and osteoarthritis (Roos et al., 1995; Berthiaume et al., 2005; Friel and Chu, 2013).

In recent years, there is emerging evidence to suggest that neonatal regeneration (or improved healing) of dense connective tissues may be possible. Studies of tendons and annulus fibrosus tissues show that neonatal healing is mediated by proliferation of differentiated cells, similar to neonatal regeneration of cardiomyocytes in the heart (Howell et al., 2017; Torre et al., 2019b). For both tendon and annulus fibrosus tissues, proliferation of differentiated cells is highest in the first week after birth and drops off rapidly after 2 weeks (Dahia et al., 2009; Grinstein et al., 2019). After full transection injury of the Achilles tendon at P5, lineage tracing with *Scleraxis-CreERT2* (*ScxCreERT2*) showed proliferation and subsequent migration of *Scx*-lineage neonatal tenocytes into the injury space, leading to restoration of hindlimb gait and tendon tensile properties (Howell et al., 2017). A similar injury in adult mice (at 4-6 months) resulted in minimal proliferation of tenocytes, no migration and lost function. Annulus fibrosus cells also proliferate and differentiate after needle puncture injury at P5; however, the time course of healing in this tissue is much extended compared with that in tendons (Torre et al., 2019b). Although *Scx*-lineage tendon cells expressing differentiated markers are detected in the injury space by 14 days post-injury, *Scx*-lineage annulus fibrosus cells do not differentiate until day 56.

Although a robust hypercellular scar is formed in adult tendons, adult annulus fibrosus tissues show minimal cells or healing (Torre et al., 2018; Torre et al., 2019b). This may be due to the harsher loading environment of the intervertebral disc compared with that of tendon, which may be exacerbated by permanent loss of the inner nucleus pulposus (the inner portion of the disc) after puncture. Unlike the annulus fibrosus component, which shows regenerative capacity in neonates, the nucleus pulposus is herniated immediately after puncture and does not regenerate in either neonates or adults (Torre et al., 2018). Despite this loss, overall disc mechanical function and disc height (a measurement of degeneration) show full restoration after neonatal injury, in contrast to impaired function after adult puncture (Torre et al., 2018).

One surprising feature of both tendon and annulus fibrosus neonatal healing is the limited regeneration of structural organization despite functional recovery. Although collagen fibrils are highly aligned after neonatal tendon injury and composed largely of type I collagen components with no cartilaginous features, transmission electron microscopy analyses suggest limited fibril maturation (Howell et al., 2017). During normal postnatal growth, tendon collagen fibrils transform from a homogeneous field of small diameter fibrils to a heterogeneous composition of very large and small fibrils (Ezura et al., 2000). Although mid-range fibrils are observed in the neonatal regenerate, large diameter fibrils are missing (Howell et al., 2017). Similarly, the distinctive lamellar organization of the annulus fibrosus is not restored after neonatal puncture (Torre et al., 2018) and, although the puncture defect is filled by dense and aligned tissue, the tissue is oriented perpendicular to the lamellar layers (Torre et al., 2018).

The regulators of neonatal tendon and annulus fibrosus regeneration are only beginning to be elucidated. One key pathway is the TGFβ pathway, which is required for neonate tenocyte migration and functional restoration, but not for tenocyte proliferation (Kaji et al., 2020). This was demonstrated using small molecule inhibition during the first 14 days post-injury and tenocyte-specific deletion of the TGFβ type 2 receptor, *Tgfbr2* (Kaji et al., 2020). In addition to TGFβ signaling, immune cells are crucial for neonatal tendon regeneration, as depletion of either macrophages or regulatory T cells results in poor recruitment of tenogenic cells, poor formation of regenerated tissue and impaired functional healing (Arvind et al., 2021 preprint; Howell et al., 2021). Whether the requirement for these immune cells is solely due to their immune-related activities (inducing or resolving inflammation, for example) or whether these cells may directly activate or interact with tenocytes remains to be determined.

Regeneration of the neonatal brain and spinal cord

Neonatal brain injuries are commonly caused by hypoxia or ischemia after birth. These injuries are not regenerative and often lead to sustained disability or even death. However, there has been some evidence to suggest that the regenerative capacity of the mammalian neonatal brain is enhanced relative to that of the adult and may be more susceptible to interventions that yield a regenerative healing response (Jinnou et al., 2018; Jinnou, 2021).

After neonatal brain injury, neural progenitors from the subventricular zone (SVZ) migrate along scaffolds to the injury site to initiate healing (Jinnou, 2021). The neonatal brain contains a greater number of neural stem cells (neuroblasts) within this niche compared with the adult, and the proliferative capacity of neuroblasts after injury steadily declines with age (Covey et al., 2010). Although the exact timeline of this decline varies between hypoxia and cryoinjury models, declining proliferative capacity is consistent regardless of injury type. Other studies have shown that, after neonatal injury (by cryoinjury or hypoxia), embryonic radial glial cells (which produce neuroblasts and provide fibers for neuroblast migration), are transiently retained in the SVZ and enhance the number of neuroblasts and mature neurons that migrate to the injury site (Jinnou et al., 2018). Although this enhanced capacity does not result in regeneration or restoration of functional properties, interventions applying an N-cadherin scaffold to enhance migration efficacy of cells along radial glial fibers can

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improve function and restore left-right asymmetry (Jinnou et al., 2018); the same improvement was not observed after adult injury and intervention, suggesting that neonatal mammals have an enhanced regenerative potential that may be tapped to induce true regenerative healing.

Several studies have also examined regeneration of the mouse neonatal spinal cord. After spinal cord trauma, axonal connections between neurons of the brain and spinal cord are disrupted. Adult injury typically heals by formation of a cellularly heterogenous scar marked by an absence of axonal projections (He and Jin, 2016; Li et al., 2020). In amphibians and fish, glial cells bridge the injury site, allowing axons to regenerate (Zukor et al., 2011; Mokalled et al., 2016), and a recent paper suggests this mechanism may also occur in neonatal mice (Li et al., 2020). Two weeks after crush injury of P2 spines, serotonergic and corticospinal tract axons are visible across the spinal cord injury site in P2 mice but absent when injuries are induced in older animals. Scarring is also reduced in both P2 and P7 injuries with little ECM deposition compared with adult. Microglia are required for successful neonatal regeneration, acting through secretion of fibronectin across the injury. In line with this finding, inhibition of microglia (pharmacological or genetic) or inhibition of fibronectin impairs wound healing and neonatal axon regrowth (Li et al., 2020). Importantly, these studies did not confirm that the axons present after neonatal injury are in fact regenerated, so they may instead be distant uninjured axons that migrate to the injury site. Regardless, the neonatal environment appears to allow for axonal growth across spinal injury, a characteristic that is lost with age. In zebrafish, CTGFa is required for the proliferation and bridge formation of glial cells, enabling regeneration (Mokalled et al., 2016); however, whether this requirement is conserved in mouse has not yet been determined.

Discussion

The ability of neonatal mice to regenerate tissues that normally heal by scarring (or not at all) in adult mice presents exciting possibilities for adult regeneration but also some challenges. One major challenge is to assess whether findings in the neonate can ever be harnessed in the adult context. For example, signaling pathways that activate regenerative programs in the neonate may not induce similar responses in the adult if adult cells are no longer competent to respond to these pathways appropriately. It may be possible to overcome limitations in adult cells by reprogramming host cells to re-activate neonatal programs or potentially replace adult cells via delivery of newly differentiated cells from alternative cell sources, such as pluripotent stem cells. Although many current cell replacement strategies aim to engineer adult features into iPSC-derived cells, it may be that a less mature differentiated cell type can induce better regeneration. In addition to intrinsic cell potential, the complex neonatal environment after injury also includes other resident cells, the extracellular matrix, immune cells and other factors that may complicate interpretation. A full understanding of how these factors and their interactions impact regeneration is crucial for the development of successful therapeutics. For example, delivery of a regenerative cell type into the adult may also require appropriate tuning of the surrounding matrix or immune response in parallel, in order to best support a regenerative local environment.

Although we have focused here on neonatal mouse models of regeneration, it should be noted that neonatal regeneration is also observed in larger mammals, such as pigs (Ye et al., 2018; Zhu et al., 2018; Zhao et al., 2020). Although these larger mammals generally follow similar patterns of regeneration, some aspects vary, including the length of the regenerative window and the extent of regeneration. Large mammalian models have also been used to uncover novel aspects of regeneration. For example, it was shown that apical resection injury at P1 in the porcine heart extends the window of heart regeneration, and enables a regenerative response at P28 when myocardial infarction is induced, with restored function and structure of the heart (Zhao et al., 2020).

Although it is evident that tissue-specific features are observed in reported tissues with neonatal regenerative or improved healing potential (in particular, specific signaling pathways, microRNAs or transcription factors), the mitotic capacity of a regenerative cell type is a key feature of all of these tissues. In several cases (e.g. the heart, tendon and annulus fibrosus), regeneration is mediated by the injured differentiated cell type, whereas in others a supporting (cochlear hair cells and spinal cord) or progenitor (brain) cell type is required (Fig. 4). Understanding the mitotic and differentiation capacities of various cell types, and the factors that dictate these, during regeneration thus remains another challenge for the field. It also remains unclear why the neonatal stage retains regenerative potential and why it is lost upon adult maturation. One possibility is that the neonatal stage represents a continuation of embryonic development, as the cells contribute to tissue growth. Increasing physiological demands during postnatal stages (e.g. increased mechanical loading as mobility increases) may trigger the shift from cell proliferation to matrix deposition (as is the case for tissues such as heart or dense connective tissues). Insult to neonatal tissues, then, may more easily trigger developmental programs toward regeneration.

Finally, as the vast majority of neonatal regeneration studies have been carried out in the heart, it is still unknown whether other neonatal tissues and organs can regenerate more broadly and whether the processes identified in the neonatal heart will apply to other tissues. Recent studies suggest that the unique immune environment of the neonate may be one crucial determinant of regeneration, but the contribution of specific immune cell populations and their interactions with resident cell types remains to be uncovered. Differences in mechanical and/or matrix environments underlying regeneration may also be broadly applicable across tissues, even if the specific matrix components or stiffness ranges that are required are tissue dependent (Fig. 4). It is well established, for example, that mechanical feedback loops from

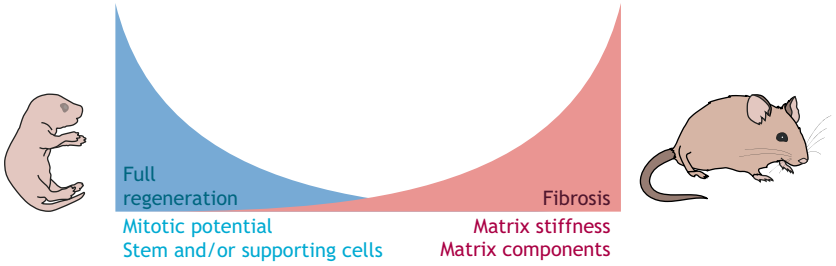

Fig. 4. Factors associated with loss of regenerative potential during adult maturation. We propose that features associated with adult maturation (including loss of cells and cell potential, and a changing extracellular matrix) may be universal features underlying loss of neonatal regenerative potential.

the matrix to cell types such as myofibroblasts exacerbate scarring (Hinz, 2007; Huang et al., 2012). Matrix stiffness can also regulate tissue-specific differentiation. Determining and characterizing such commonalities and differences across neonatal tissue types will no doubt inform global strategies that may be effective for multiple tissues.

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Competing interests
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