Spatiotemporal dynamics of PDGFRβ expression in pericytes and glial scar formation in penetrating brain injuries in adults

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Running Title: Spatiotemporal dynamics of pericytes in injury

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ABSTRACT

Aims: Understanding the spatiotemporal dynamics of reactive cell types following brain injury is important for future therapeutic interventions. We have previously used penetrating cortical injuries following intracranial recordings as a brain repair model to study scar-forming nestin-expressing cells. We now explore the relationship between nestin-expressing cells, PDGFRβ$^+$ pericytes and Olig2$^+$ glia, including their proliferation and functional maturation.

Methods: In 32 cases, ranging from 3 to 461 days post injury (dpi), immunohistochemistry for PDGFRβ, nestin, GFAP, Olig2, MCM2, Aquaporin 4 (Aq4), Glutamine Synthetase (GS), and Connexin 43 (Cx43) were quantified for cell densities, labelling index (LI) and cellular co-expression at the injury site compared to control regions.

Results: PDGFRβ labelling highlighted both pericytes and multipolar parenchymal cells. PDGFRβ LI and PDGFRβ$^+$/MCM2$^+$ cells significantly increased in injury zones at 10-13 dpi with migration of pericytes away from vessels with increased co-localisation of PDGRFβ with nestin compared to control regions ($p < 0.005$). Olig2$^+$/MCM2$^+$ cell populations peaked at 13 dpi with significantly higher cell densities at injury sites than in control regions ($p < 0.01$) and decreasing with dpi ($p < 0.05$). Cx43 LI was reduced in acute injuries but increased with dpi ($p < 0.05$) showing significant cellular co-localisation with nestin and GFAP ($p<0.005$ and $p<0.0001$) but not PDGFRβ.

Conclusions: These findings indicate that PDGFRβ$^+$ and Olig2+ cells contribute to the proliferative fraction following penetrating brain injuries, with evidence of pericyte migration. Dynamic changes in Cx43 in glial cell types with dpi suggests functional alterations during temporal stages of brain repair.

Introduction

There is a fine balance in repair processes following brain injury between the beneficial effects that limit tissue damage and restore homeostasis and the long-term detrimental effects that can follow glial scar formation, such as impediment to axonal regeneration and increased risk of seizures [1, 2]. Understanding which endogenous progenitor cell types contribute to tissue reorganisation after brain injury, and the mechanisms that regulate their differentiation and functional maturation, are essential for any therapeutic interventions targeting this process.

Most studies of the spatio-temporal progression of glial and vascular alterations have been carried out using experimental animal models of brain injury [2-4] and are dependent on the type of model used and, together with inter-species differences, are not always translatable to human brain injury. Studies investigating brain repair in human tissues have largely been dependent on post-mortem material with its inherent limitations. We have previously overcome this by utilising surgical specimens with penetrating cortical injuries following intracranial recordings (ICR) carried out for the pre-operative investigation of epilepsy [5, 6]. As the time interval between initial electrode placement and subsequent tissue resection is precisely known (but variable between cases) and the injury is localised, this provides a unique spectrum from acute injuries to chronic scars, spanning from 3 days to over 1-year old injuries, to study spatiotemporal processes in human brain repair. Our previous studies identified that, in addition to microglia, nestin-expressing cells represent a highly proliferative cell type at the acute injury site, often elongated with a close relationship to vessels and remaining in chronic scars; a proportion of these nestin-expressing cells were immunopositive for GFAP [5]. In experimental injury models, functionally distinct subtypes of astroglia are recognised, regulating inflammatory responses and repair, including specific

'scar-forming astrocytes' with elongated processes [3, 7]. The source of proliferating and scar-forming glia in injuries is not fully established. Progenitor cell types in the adult brain, including oligodendroglial progenitor cells (OPCs), NG-2 cells as well as resident parenchymal nestin-expressing glia with proliferative capacity [8], are candidates. A further possibility, particularly in view of the observed intimate relationship of new capillaries and glia in the repair process, is that nestin-expressing cells are recruited from progenitors in the perivascular niche, including pericytes with capacity for neuroectodermal differentiation [9].

Pericytes form an essential part of the neurovascular unit, responsible for regulating the capillary blood flow through controlling capillary structure and diameter, promoting the development and maintenance of the blood-brain barrier, and controlling the proliferation, migration and stabilisation of endothelial cells [10-13]. Pericytes may be divided into different subtypes based on their function, morphology, location and expression of immunohistochemical markers including platelet-derived growth factor receptor beta (PDGFRβ), alpha smooth muscle actin (α-SMA), chondroitin sulfate proteoglycan (NG2), regulator of G protein signalling (RGS-5), and the potassium channel Kir6.1 [14-16]; they have also been reported to express nestin in neonatal and adult rat brain cultures [17, 18]. PDGFRβ expressing pericytes have been proposed to be an important source of endogenous progenitor cells [9, 13, 19, 20], capable of enhanced proliferation following injury in experimental models [10, 18, 21]. However, a recent study showed that $Tbx18^{+}$ pericytes did not behave as stem cells, including following brain injury and repair [22], highlighting an ongoing controversy in this area.

As reactive glial progenitors proliferate and differentiate following injury to form the chronic scar, the temporal expression of functional markers, for example those involved in glio-transmission, could be relevant to any local cellular dysfunction as well as epileptogenesis [23]. Cx43 is the main gap junction protein in astrocytes, establishing homeostatic cell

communication [24]. Cx43 hemichannels also mediate 'gliotransmission' of bioactive molecules [25, 26] and astroglial connexins and Aquaporin 4 are implicated in spreading depolarisation that can follow brain injury [27, 28]. Glutamate synthetase, while integral to normal glial glutamate metabolism and excitatory neuronal transmission, is deficient in glial progenitor types [29].

Using our series of ICR injury cases we hypothesised that (i) OPC, and PDGFRβ expressing pericytes contribute to the proliferative cell types following focal injury, (ii) their spatiotemporal distribution, morphology and dynamics are closely related to GFAP-negative nestin-expressing cells and (iii) that relative differences in markers of functional maturation between reactive glial populations over the time course of brain repair could be observed.

Materials and methods

The project has ethical approval and written informed consent was obtained from all patients.

Case selection

Surgical brain specimens from 32 patients, with pharmacoresistant focal epilepsy, who had placement of subdural and/or depth electrodes during pre-operative intracranial EEG recordings at various periods prior to resective surgery between 2002 to 2015, were included in the study. All samples were received through the Epilepsy Society Brain and Tissue Bank at the Department of Neuropathology, UCL Queen Square Institute of Neurology. The lesion age in each case was recorded as days post injury (dpi; interval between the placement of the electrodes and tissue resection) which ranged from 3 to 461 days. Cases were also arbitrarily grouped into four temporal stages of cellular injury and repair for qualitative analyses and to enable comparison with our previous studies [5]: acute (3-9 dpi; n=6), subacute (10-13 dpi; n=7), intermediate (28-70 dpi; n=5) and chronic lesions (107-461dpi; n=14). The clinical

details of the cases are presented in Table 1; 19 cases were male and the mean age at surgery was 36 years (range, 18 to 60 years). The injury types involved the superficial cortex including the sub-pial layer (following subdural grid placements; n=14) or were needle-like track penetrating injuries (from depth electrode insertion; n=19) (Table 1). Most tissue resections were from the frontal (n=17) and temporal (n=12) lobes. In 13 cases, no distinct pathology was identified in the resection (non-lesional); the remaining cases showed focal cortical dysplasia (FCD) type IIB (n=10), FCD IIID (n=1), hippocampal sclerosis (n=3), low-grade epilepsy-associated tumour (LEAT) (n=4) and a focal scar from traumatic brain injury (n=1). In all cases except one (case E22), the ICR lesion was *not* located in the same area as the main pathology.

Immunohistochemical and double-label immunofluorescence studies

Five-micron thick serial sections were cut from each block bearing the injury; one was stained with Haematoxylin and Eosin as a reference for the location of the ICR lesion as well as to delineate the anatomical boundaries of three zones: lesion core and margins (Zone 1 and 2) and control region. Further serial sections were used in single and double labelled chromogenic and immunofluorescence studies. The protocols and methods used were as published in previous studies [5, 8] (Supplementary methods). Antibodies used in single or double labelled studies included glial/glial progenitor markers, **nestin**, **GFAP**, **Olig2**; pericyte markers **PDGFRβ** [13, 21] and **SMA**; proliferative cell marker, **MCM2,** and functional glial markers, **Glutamine synthetase** (GS), **Aquaporin4** (Aq4) and astrocyte gap junction protein **Connexin 43** (Cx43) (Supplementary Table). It was not possible to include every case in all immunohistochemical and immunofluorescence studies as ICR lesions were small, and some were ‘cut-out’ in serial sections. However, for each study, a comparable number of cases from each of the four time periods were included (detailed in Table 1).

Quantitative analyses

The distribution and morphology of single and double labelled cell types were visualised using Nikon brightfield microscope, Zeiss Axio Imager Z2 motorized fluorescence microscope, and LSM700 confocal microscope (Zeiss, Oberkochen, Germany), and compared between differing zones, age of lesions, and the temporal stages of brain repair.

PDGFRβ, nestin, GFAP, and Cx43 double-labelled immunofluorescent sections: Each immunolabelled slide was tiled using a 20x objective and regions of interest drawn as described in previous studies [5] using Zen imaging software (Carl Zeiss Microscopy, Jena, Germany). **Zone 1** refers to the margin of the ICR lesion, the internal border formed by the medial edge of viable tissue to a radial depth of 350 μm, forming a circumferential region surrounding the injury site (Figure 1a, b). The precise shapes and size of zones drawn varied between cases, according to the contours of the ICR injury. **Zone 2** represented tissue of equal radial depth marginal to Zone 1 (qualitative evaluation only), and **Zone 3** was a control region of 2000 μm^2 located as far away from the ICR injury site as possible within normal-appearing tissue in the same tissue section. Care was taken to select a comparable region of control white matter or grey matter according to the ICR lesion site. Fluorescent signal thresholds were set for each fluorochrome using the Zen imaging software and were kept consistent across cases. For each set of double labelling studies, the labelling index (LI), rather than cell numbers, was measured since most markers labelled abundant cell processes in the brain parenchyma. In addition, the relative area of co-localisation (RAC; the area of co-labelling/total area of Zone 1 or Zone 3) and co-localisation coefficients were calculated including Pearson's and Mander's coefficients as measures of the degree of co-localisation, where 1 indicates perfect overlap of signal, -1 indicates a negative correlation and 0 indicates random overlap [30]. A proportion of cases were re-tested between two observers with good

reproducibly (intra-class correlation coefficient 0.995). For Olig2/MCM2 sections, the same procedure for acquisition of the zones was performed, but the number of double-labelled cells per area was manually counted.

MCM2/PDGFRβ double labelled chromogenic sections: Slides were scanned with a Leica SCN400F digital slide scanner (Leica Microsystems, Wetzlar, Germany) at 40x magnification, and immunolabelling in the same regions of interests as described above was analysed using the automated image analysis software, Definiens Tissue Studio software 3.6 (Definiens AG, Munich, Germany). Double-labelled cells were automatically counted as MCM2+ brown nuclear labelling (MCM2) when surrounded by PDGFRβ+ cytoplasmic purple labelling, and the data was expressed as the number of cells per area.

GS, Aq4 and Cx43 single labelling: Serial images were manually captured at 40x magnification from three regions of interest: zone 0 (injury core), zone 1 (organising edge of the lesion) and zone 3. Immunolabelling was thresholded using ImageProPlus (v6.3; MediaCybernetics, Bethesda, MD, USA), and the LI for each area calculated.

Statistical analyses were performed using SPSS for windows version 21 (IBM corporation, version 21), and significance between groups was taken at $P<0.05$. Tests included Kruskal–Wallis non-parametric tests with *post-hoc* correction for multiple comparisons and Wilcoxon signed rank to compare cases between different zones. Spearman's correlation and regression analysis were used to explore the relationships between labelling and dpi.

In vitro scratch assay

To investigate the response to injury after 24 hours, a scratch assay was performed on cells cultured from a gram of freshly resected brain tissue of two surgical patients with focal

epilepsy (Cases EC1, EC2; Table 1) and the proportion, morphology and spatial distribution of nestin-expressing cells incorporating EdU A was quantified (see supplemental methods).

RESULTS

Pericytes and glial cell reactions in ICR injuries

Qualitative analysis **Nestin/PDGFRβ:** In acute ICR lesions, multipolar nestin$^+$ cells and nestin$^+$ cells with elongated processes became prominent between capillaries in Zone 1 and 2 (Figure 2A-C); in Zone 3, nestin labelling was noted mainly in capillaries (Figure 2J). In subacute to intermediate ICR lesions, there was greater prominence of nestin$^+$ cells between new capillaries in both Zones 1 and 2 (Figure 1D, 2D-G). In chronic ICR lesions, dense meshworks of nestin$^+$ fibres persisted in Zone 1 around vessels at the scar site (Figure 2H, I) as previously described [5]. PDGFRβ in acute lesions showed focal expression in pericyte-like cells around capillaries in Zones 1 and 2 at 3 dpi (Figure 2A). By 8-9 dpi, increased numbers of PDGFRβ$^+$ bipolar cells and processes were present around capillaries (Figure 2 B, C arrows), and by 13 dpi forming lace-like meshworks extending away from capillaries, becoming more prominent in intermediate-aged lesions (Figure 1C-F 2D-F). In addition, in Zones 2 and 3, smaller multipolar PDGFRβ$^+$ glial-like cells not associated with capillaries were identified (Figure 2J-L). In chronic lesions and oldest scars, PDGFRβ expression at the scar site became limited to perivascular pericytes (Figure 2H). Co-localisation of nestin with PDGFRβ was noted around acute, subacute and intermediate lesions in a proportion of the elongated cells in proximity to capillaries in Zone 1 (Figure 2C, D). In addition, the lace-like processes extending between capillaries in Zone 1 (Figure 2D-F) and multipolar cells in Zone 2 and 3 also showed focal co-expression (Figure 2J-L). The small multipolar PDGFRβ cells, however, were not SMA positive, with PDGFRβ/SMA co-expression restricted to pericyte-

like cells (Figure 2M-P). GFAP/PDGFRβ: At low power, $GFAP^+$ cells and processes formed a clear band of labelling at the boundary of Zone 1 and 2 in subacute injuries, mainly peripheral to an inner region of $PDGFR\beta^+$ cells and new capillaries, forming distinct compartments at low power (Figure 1E). At higher magnification in Zone 1 of acute to chronic lesions, although many $GFAP^+$ reactive processes appeared separate from PDGFRβ (Figure 3B to E), intermingling and overlapping of processes with focal co-localisation was noted (Figure 3F, G). GFAP and PDGFRβ+ cells also appeared relatively distinct in Zone 3. **MCM2**: $PDGFR\beta^+/MCM2^+$ pericytes were prominent in subacute lesions in the immediate vicinity of capillaries and also in the elongated cells extending out from and between small capillaries (Figure 1C, 4A). Small $Olig2^+$ cells with nuclear MCM2 co-expression (Figure 4C, D), indicating replicative potential, were also prominent in the ICR lesion site compared to normal cortex. The overall morphology of $Olig2^+$ cells did not alter with dpi, although labelling was restricted to the nucleus.

Quantitative analysis: **Nestin, PDGFRβ, GFAP**. Nestin and PDGFRβ LI were significantly higher in Zone 1 (injury region) than Zone 3 (control region) in all cases ($p<0.001$), but GFAP LI was not different ($p=0.526$) (Table 2). There was a significant regression of nestin expression with dpi in Zone 1 ($p<0.04$; Figure 3A) but not in Zone 3 (Supplemental Figure 2A), nor for GFAP or PDGFRβ. There was a significant correlation between nestin and PDGFRβ LI for all cases in Zone 1 ($p<0.001$) but not with GFAP. There was a significantly greater relative area of co-localisation of nestin/PDGFRβ ($p=0.003$) and GFAP/PDGFRβ in Zone 1 than Zone 3 ($p = 0.02$) in all ICR lesions over the time stages (Table 2; Supplemental Figure 2B) and the Pearson correlation coefficient for co-localisation for nestin/PDGFRβ was significantly higher than for GFAP/PDGFRβ ($p=0.047$, Wilcoxon test). There was a trend for increased co-localisation between GFAP/PDGFRβ and decreased nestin/PDGFRβ with dpi, but this was not significant.

MCM2: There was a significant increase in MCM2/PDGFRβ$^+$ cell density in Zone 1 compared to Zone 3 ($p<0.005$); mean densities peaked in the subacute lesions in Zone 1 (Table 2) but there was no significant correlation of MCM2/PDGFRβ$^+$ cells with dpi in either zone. The density of Olig2/MCM2$^+$ cells was significantly higher in Zone 1 than Zone 3 ($p=0.006$); their number peaked at 13 dpi in subacute lesions with densities ten times higher than in normal regions (Table 2). By 28 dpi, the density of MCM2/Olig2$^+$ cells had markedly decreased and continued to fall in chronic lesions to levels comparable with normal regions, with a significant regression shown with dpi in Zone 1 (Figure 4E; exponential $p=0.028$). ***In vitro* scratch assay**: The responses of proliferative cell populations 24 hours following injury were further explored *in vitro,* since tissue samples with 1 dpi lesions were rarely available. 33% of the cells cultured from resected samples from two patients with focal epilepsy incorporated EdU A 24 hours after the scratch. 10% of proliferative EdU A incorporated cells also expressed nestin (Figure 4F). The majority of EdU A/nestin$^+$ cells showed unipolar or bipolar morphology and did not appear to be apoptotic. These cells were found up to 7000 μm away from the induced lesion site. More EdU A labelled cells seem to be located further away from the scratch border than at the lesion site (Figure 4G).

Functional glial maturation in ICR injuries

Qualitative **Cx43:** In the control regions (Zone 3), diffuse labelling of astrocyte-like cells was present in the cortex (Figure 5F) and in glial cells mainly around vessels in the white matter. In acute ICR injuries, loss of Cx43 was apparent in the core of the ICR lesions, forming a sharply demarcated boundary with the adjacent parenchyma (Figure 1F, 5A); immunoreactivity at later stages was noted in relation to capillary endothelium (Figure 5B).

By 8-9 dpi, Cx43 expression in the injury margin was observed in elongated cells around vessels and from subacute to intermediate lesions, Cx43 immunopositivity in the endothelium, pericyte cells and reactive multipolar cells became more evident (Figure 5C, D). In chronic lesions, the central necrotic core had contracted with intense labelling of $Cx43^+$ processes demarcating the ICR scar site (Figure 5E). **Aq4:** In control regions (Zone 3), dense labelling of glial processes in both cortex and white matter was apparent, particularly in foot processes around blood vessels (Figure 5J). In acute ICR lesions, the loss of Aq4 immunolabelling in the injury site formed a well-defined boundary, separating this area from the retained Aq4 immunolabelling in the adjacent viable tissue (Figure 5H), including labelling of reactive cells (Zone 1) (Figure 5I). **GS:** Expression was predominantly observed in the perinuclear cytoplasm of small glial cells in control regions (Figure 5M). GS^+ cells with reactive astrocytic morphology were present in subacute and intermediate ICR lesions Zone 1 (Figure 5K) and more intensely labelled GS^+ cells in older lesions (Figure 5L). **Cx43 with nestin/GFAP:** Striking membranous Cx43 expression in large, reactive nestin and GFAP positive cells was observed in acute and subacute ICR injuries in Zone 1 (Figure 6A, B). In both subacute (Figure 6D, E) and intermediate lesions (Figure 6 G, H) co-expression of both nestin and GFAP with Cx43 was noted in a proportion of processes at the organising margin, particularly around vessels. In chronic injuries, GFAP showed a more complete co-overlap with Cx43 (Figure 6K) than nestin (Figure 6J). In addition, large reactive $Cx43^+$ cells were noted, negative for both nestin (Figure 6G) and GFAP (Figure 6H). Cx43 labelling of endothelium was also noted. In chronic injuries, and Zone 3, the majority of GFAP reactive cells showed Cx43 labelling (Figure 6M). **Cx43/PDGFRβ**: In acute and subacute lesions, there was very occasional co-expression in pericytes around capillaries in the organising margin of Zone 1 (Figure 6C, F). Overall, there was less evidence for co-expression compared to nestin and GFAP, a more distinct pattern being $Cx43^+$ endothelium and

surrounding PDGFRβ-positive pericytes in organising vessels (Figure 1F, insert). Large $Cx43^+$ reactive cells were typically PDGFRβ negative (Figure 6I) and minimal co-localisation was observed in chronic scars (Figure 6L). In Zone 3, separate distribution of PDGFRβ and Cx43 was appreciated (Figure 6N); however, occasional co-localisation in small multipolar cells between vessels was observed (Figure 6N, inset).

Quantitative **Cx43:** Significant differences were noted in Cx43 LI for all injuries between injury core and control region (Zone 3) ($p<0.005$) (Table 2) with a significant linear increase in Cx43 in Zone 0 with dpi ($p=0.03$) (Figure 5G). **Aq4**: There was significantly lower Aq4 LI in the lesion core (Zone 0) in all injuries compared to Zone 3 ($p<0.001$) (Table 2) but no significant correlation of Aq4 LI with dpi. **GS**: There were no statistically significant differences in LI between zones or correlation with dpi (Table 2).

Double labelling quantitation: For Cx43/nestin, there was a significant increase in the relative area of co-localisation comparing between Zone 1 and 3 ($p=0.038$) (Table 2); this was not noted for Cx43/GFAP. There was a significant linear increase in relative area of co-localisation for both Cx43/nestin and Cx43/GFAP with dpi ($p=0.002$ and $p<0.0001$) (Figure 6O). No statistical differences were noted for Cx43/PDGFRβ co-localisation either between zones or with dpi (Figure 6O).

DISCUSSION

Using ICR penetrating injuries in human tissues, we investigated the spatiotemporal dynamics of $PDGFR\beta^+$ cells and its relationship with other glial cell types such as GFAP and nestin expressing cells in the formation of a glial scar. Several morphological types of $PDGFR\beta^+$ cells were observed in lesional and perilesional tissue. $PDGFR\beta^+$ pericyte cells as

well as Olig2$^+$ glia contribute to the proliferating cell fraction in acute and subacute injury sites, in addition to the nestin$^+$ cells and Iba1$^+$ cells as previously described [5] . PDGFRβ$^+$ pericyte cells also extended away from vessels in the active organising injuries, showed focal nestin expression, but diminished in chronic lesions. Furthermore, the relative differences and dynamic changes of Cx43 gap protein expression in glio-vascular and reactive cell types is of potential relevance to functional changes following penetrating brain injury. Together with data from our previous study [5] we can formulate the cellular spatiotemporal dynamics in brain repair in human tissues (Supplemental Figure 3).

Needle tracks following intracranial electrode insertion provide a specific model for penetrating brain injury, without superimposed rotational or ischaemic injury, and with the advantage that injuries of a precise age can be studied in optimally fixed surgical tissues. In our previous study, nestin $^+$ cells were prominent replicative cell types in ICR injuries with close relationship to vessels and remained in the chronic scar [5]. In the present study, using in vitro scratch assay on primary cell cultures, proliferative nestin expressing cells were seen as early as 24 hours after injury. Most of these cells have morphology similar to immature migrating cells. These findings are in line with observations in animal models of injury [7, 31, 32]. However, the source of the nestin$^+$ scar forming glial cells is unexplained and we speculated on their relationship to pericytes, which we further investigated in this current study.

PDGFRβ$^+$ pericyte migration and proliferation in injuries

PDGFRβ is widely recognised as a reliable marker of CNS pericytes under normal conditions [9, 13, 21]. Around the ICR injury, a significant increase in PDGFRβ$^+$ pericytes was observed compared to control regions, with a close spatial and temporal relationship to nestin$^+$ cells, sharing similarities in cell morphology and distribution, including elongated, bipolar cell

forms in proximity to new vessels. PDGFRB$^+$ pericyte cells also extended away from capillary walls at the injury margin. Pericyte migration or 'lifting' away from micro-vessels has been previously shown using electron microscopy in models of traumatic brain injury (TBI) at 2 days following injury [33], with loss of their contact with the blood vessels, invasion through the vascular basement membrane [34] and PDGFRβ$^+$ pericyte 'detachment' [21]. In tissues from patients with acute strokes, increased numbers of ramified pericyte cells bridging between vessels has also been reported [35]. Using MCM2 co-labelling, we also demonstrated increased replicative potential of PDGFRβ$^+$ pericytes as well as in the detached cells, that peaked at 8 to 13 dpi (subacute stage). In spinal cord and brain injury models, proliferation of type 1 pericytes was shown 14 days after injury [36] and proliferation of PDGFRβ$^+$ cells demonstrated with Ki67 labelling in ischaemic stroke lesions [35] as well as cortical models of TBI [37].

Evidence for the contribution of pericytes to glial scar formation

Experimental TBI studies mainly focus on the earlier stages of repair and have demonstrated a quantitative increase in PDGFRβ$^+$ cells and mRNA from 3-5 dpi [37] and 2 to 7 dpi [21]. The density of PDGFRβ$^+$ cells has been shown to diminish by 14 dpi [36] and by 3 months post injury, only perivascular pericytes were reported in the glial scar [21], but still with elevated numbers at 7 months compared to those before injury in another study [34]. In the current study with chronic injuries of over 15 months, PDGFRβ expression was restricted mainly to perivascular cells at the scar site. In contrast, although nestin labelling overall diminished with dpi, nestin$^+$ cells and processes remained, demarcating the chronic scar. A strong relationship between the LI of PDGFRβ and nestin across all stages of injuries was shown and co-labelling identified nestin$^+$/PDGFRβ$^-$, nestin$^-$/PDGFRβ$^+$ and nestin$^+$/PDGFRβ$^+$ cells in organising injuries. In our previous study, we reported increased co-localisation between nestin and GFAP with dpi [5] and in the current study we noted a trend for a

decreased relative area of co-localisation of PDGFRβ with nestin with dpi but an increase in co-expression with GFAP.

Although these temporal changes could reflect heterogeneous reactive cell populations during repair process (i.e. augmented nestin expression in PDGFRβ$^+$ pericytes or transient PDGFRβ$^+$ expression in nestin$^+$ glia), an alternative explanation is that following pericyte proliferation and migration, a subset differentiate to scar-forming glial cells [19, 34, 36]. In spinal cord injury models, ablation of NG2$^+$ pericyte cells reduced GFAP density in the glial scar [38]. In a further study, genetic ablation of pericytes led to failure to seal the scar, implicating pericytes as a probable source of scar-forming cells [34]. Recent studies have demonstrated that the application of a PDGFR inhibitor disrupted glial scar formation following injury [39].

In experimental systems and TBI models, cellular co-expression of PDGFRβ and GFAP in reactive cells has been shown in some [13, 19, 21, 39] but not all studies [34, 37]. In a recent lineage-tracing experiment, *Tbx*18-expressing pericytes did not co-localise with GFAP-astrocytes following injury [22]. In the present study, there were rare, overlapping PDGFRβ$^+$/GFAP$^+$ processes located close to the ICR lesions. A more striking qualitative observation in this study, however, was the 'zonality' or relative compartmentalisation in the ICR injuries, with reactive PDGFRβ$^+$ pericytes and nestin$^+$ cells forming the inner border of the organising scar with a surrounding rim of predominantly GFAP$^+$ reactive glia. Such 'layering' of organising scars has been previously noted in experimental models [34, 38] as well as in human strokes where PDGFRβ$^+$ proliferating cells were demarcated from the surrounding astrocytic gliosis [35]. The findings of the present study therefore support two components to brain repair: the formation of a central, chronic, contracted glial scar, dependent on early proliferation and then decline of PDGFRβ$^+$ and nestin$^+$ cell types and an outer predominantly GFAP$^+$ glial scar.

PDGFRβ$^+$ CNS parenchymal cells

There is accumulating evidence that PDGFRβ is also expressed in resting brain parenchymal cells other than pericytes. We previously reported, in the white matter of adult epilepsy cases with FCD, small multipolar cells, away from capillaries, which co-expressed PDGFRβ and PDGFRα as well as NG2 and likely represented subsets of NG2 progenitor glia [40]. An increase in ramified PDGFRβ$^+$ parenchymal cells was observed in human temporal lobe epilepsy (TLE) that co-localised with NG2 but not Iba1 [41] and their redistribution shown following experimental status, suggesting they are reactive populations [42]. Kyyriainen *et al.*, recently described PDGFRβ$^+$ cells with small soma and ramified processes in normal mice brain, shown to be either PDGFRα$^+$ or GFAP$^+$ and dynamic changes in their distribution was also shown following both experimental status and trauma. [21]. In the present study, small PDGFRβ$^+$ parenchymal cells were more evident away from the injury site in normal white matter and their focal co-expression with nestin and, to lesser extent GFAP, was shown but not SMA, supporting their distinction from pericytes. We cannot exclude that pre-existing parenchymal PDGFRβ$^+$ glia also contribute to reactive, proliferating populations at injury sites.

Oligodendrocyte lineage progenitors (OPCs) are in close apposition with pericytes in the perivascular space, with evidence for mutual interactions, promoting proliferation [43]. Surgical TBI resections have shown an increase in OPC number at injury site [44] and transient proliferation of OPC noted between 7 to 21 dpi in a diffuse TBI model [45]. In our study significant proliferation was confirmed, with Olig2/MCM2$^+$ cells peaking at 13 dpi then falling to control levels in chronic lesions. The morphology of Olig2/MCM2$^+$ remained ‘oligo-like’ with no definite evidence of glial/pericyte maturation; however, no double labelling of Olig2 with nestin or PDGFRβ was performed in the present study. We have also recently identified nestin$^+$ radial glial cell types in adult temporal lobe with regenerative

capacity, glial maturation *in vitro* and focal co-localisation with PDGFRβ [8]. Heterogeneous populations of regenerative parenchymal cells could therefore potentially contribute to astroglial scar formation following injury. Further work is needed to define their cellular interactions and signalling pathways that are pivotal to optimal brain repair.

Connexin43 and functional glial markers

Expression of astroglial markers Cx43, Aq4 and GS in reactive cell types is further evidence of their differentiation, and also reflects functional alterations over the time course of brain repair, of potential relevance to epileptogenesis and other post-traumatic sequela [23]. Cx43 is the main gap junction protein in astrocytes [24] and Cx43 hemi-channels, mediating 'gliotransmission' and release of bioactive molecules from astrocytes [25, 26]. Both connexins and Aq4 are implicated in spreading depolarisation following injury [27, 28]. Connexins are also critical to cell-cell interactions and establishing homeostatic cell communication following injury [46], driving cell reactivity [3] and promoting neuronal recovery [47] ; for example, the extent of gliosis is larger in Cx43 KO mice [48] .

We noted spatio-temporal alterations in expression, with initial reduction at the injury site for Cx43 and Aq4 labelling compared to control regions but increased labelling of Cx43 with dpi and its cellular co-expression with GFAP (see summary Supplemental Figure 3). In experimental TBI models, an initial decrease in Cx43 was also reported followed by an increase from 6 to 15 dpi [48]. We noted only occasional co-localisation of Cx43 with perivascular PDGFRβ+ pericytes but exaggerated endothelial labelling; loss of Cx43 in pericytes has been linked to functional decline and vascular instability [49] and increased endothelial expression with defective blood brain barrier [50]. Dynamic changes in Cx43 expression in the neurovascular unit in brain repair warrant further investigation regarding physiological versus detrimental role.

Limitations

All of the patients had refractory focal epilepsy and it is likely that seizures influence the baseline glial populations, including densities of nestin [8], PDGFRβ+ glial cells [41, 42] and Cx43 expression on astrocytes [24] in zone 3 used as the control region as in previous studies. Nevertheless, we favoured this as optimal control tissue over post-mortem tissue with agonal changes and delayed and longer fixation times which could affect staining.

In summary, in the ICR brain injury model we have identified that (i) PDGFRβ$^+$ and Olig2$^+$ cells contribute to the proliferative fraction with evidence of pericyte migration away from the vessels into the organising scar, (ii) the formation of a glial scar is composed of two zones, the central zone enriched in nestin$^+$ pericytes and an outer zone of reactive GFAP astrocytes, (iii) PDGFRβ also identifies populations of small neuroglial cells and (iv) glial differential markers, as Cx43, show altered distribution in reactive cells and the neurovascular unit during repair which could have functional implications.

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STATEMENT OF AUTHOR CONTRIBUTION

JL, CR and APJ were involved in the section preparation and analysis. MT was involved in data collection and study design. JL and MT conducted all the data analysis and interpretation. JL, CR and MT were involved in manuscript preparation. SS conceived the initial injury study design and reviewed the manuscript.

Figures and Legends

Figure 1: Intracranial recording (ICR) injury at low power.

A. A subacute lesion (13 days post injury) involving the cortical surface which represents one of the larger foci of injury in the series. H&E section highlighting the main regions used for qualitative evaluation and delineation of the Zones for study. The Necrotic core (Zone 0) contains mainly non-viable material and macrophages. Zone 1 is the rim of viable material surrounding the core and contains reactive cells and vessels and Zone 2 surrounds Zone 1. **B.** The same section showing the superimposed measured Zone 1 with a width of 350 microns which was tiled for image analysis. **C**. PDGFRβ chromogenic stained section (purple) with MCM2 labelling of nuclei (brown, visible in inset) in the same case to highlight the labelling in Zone 1 of vessels and in **D.** with immunofluorescent labelling for PDGFRβ with nestin highlighting capillaries and reactive glial cells in Zone 1. **E.** GFAP with PDGFRβ shows the relative compartmentalisation of labelling at low power with the majority of GFAP positivity external to the PDGFRβ. **F.** Cx43 with PDGFR β at low power and shown in the inset at higher magnification, with a close relationship between PDGFRB pericytes surrounding Cx43-positive endothelium. Higher magnifications for C are shown in Figure 4, D in Figure 2, E in Figure 3 and F in Figure 6. Bar is 700 microns.

Figure 2: Pericytes and nestin-expressing reactive glial cell types in intracranial recording (ICR) injuries of different stages

A-C: Nestin/PDGFRβ in acute injuries in zone 1. A. At three days post injury (dpi) nestin labelling was relatively confined to vessel endothelium and PDGFRβ in pericytes, forming an incomplete layer around the endothelium. **B-C**. At 8 to 9 dpi, increased nestin expression in perivascular, reactive and bipolar stromal cells (arrows) with increased PDGFRβ cells around small vessels (chevrons), some with focal nestin co-expression (arrowhead; asterisks indicate capillary lumen); In **C** the nestin and PDGFRβ are shown as single channels. **D, E. Nestin/PDGFRβ in subacute injuries in zone 1. D.** At this stage nestin expression is noted in elongated processes and reactive, bipolar and multipolar cells between capillaries and co-expression with PDGFRβ is observed (arrowhead; split channel images shown in supplemental figure 1). **E**. 'Lace-like' proliferations of PDGFRβ$^+$ cells lifting away from the vasculature (asterisk) is noted (chevrons) (split channels shown in supplemental figure 1). **F. Nestin/PDGFRβ in intermediate age injuries in zone 1.** By this stage more prominent increase in the number and networks of PDGFRβ$^+$ cells in zone one is noted, not associated with vessels (asterisk) and with some focal nestin co-expression (split channels shown in supplemental Figure 2); these cells declined in number with age and, in **G.** shown at 52 dpi, PDGFRβ expression is more limited to vasculature (asterisk). **H. and I. Nestin/PDGFRβ in chronic injuries in zone 1** at 209 and 301 dpi respectively, show a residual increase in

nestin+ fibrous processes, demarcating the scar site whereas PDGFRβ expression is mainly perivascular. **J. Nestin/PDGFRβ in acute injuries in zone 3** in acute (8 dpi) and intermediate phases (**K**. 28dpi and **L**. 52 dpi) show multipolar cells that are nestin^{+} (arrow), PDGFRβ^{+} (chevron) or show double-labelling (arrowhead) (asterisks indicate capillary) (**J** to **L** : shown at higher magnification in supplemental Figure 1C'). **M to P**. **SMA/PDGFRβ** double labelling in zone 1 confirms co-localisation in pericytic cells surrounding capillaries (arrowheads) but not in small multipolar PDGBRβ^{+} cells (arrows). In all figures, asterisks denote capillary vascular channels, arrowheads double labelled cells and arrows and chevrons single labelled cells as indicated. Scale bar shown in P is equivalent to 500 microns in A-G, 1000 and 20 microns in J to P.

Figure 3 **Pericytes and GFAP-expressing reactive glial cell types in intracranial recording (ICR) injuries of different stages**

A. Scatter graphs of labelling index of nestin, PDGFRβ and GFAP plotted against dpi (expressed as log10); only nestin showed a significant regression with the age of the lesion. **GFAP/PDGFRβ in zone 1**: B (acute), **D** (subacute), **C** (intermediate) and **E** (chronic) stages. The necrotic core of the penetrating injury is highlighted with yellow asterisk. Increased numbers of GFAP^{+} fibres and PDGFRβ expressing cells are intermingled but form distinct populations at low magnification. Shown at higher magnification in **F** (subacute) and **G** (chronic, same case as E) shows approximation with intermingled and overlapping networks of GFAP and PDGFRβ processes in zone 1 with apparent co-localisation in single processes (arrowheads Figure G) although orthogonal views were not available. Scale bar shown in G equivalent to 100 microns in B to E and 50 microns in F and G.

Figure 4.

A. MCM2/PDGFRβ at 11 days post injury with the lace like networks of cells, some of which show double labelling (arrows); **B.** Double labelling for MCM2 with PDGFRβ at 13dpi confirms double labelled cells in perivascular location (arrowheads) in addition to MCM2/PDGFRβ^{-} cells (arrow). **C**. **MCM2/Olig2** at 9dpi and **D**. at 13dpi showing double labelled cell (arrowhead). **E**. Scatter graph of MCM2/Olig2 double-labelled cell density (/mm^2) with dpi showing a significant declined in Zone 1 ($p=0.028$). **F.** Confocal images showing nestin-expressing cells grown from surgical temporal cortex of patients with focal epilepsy underwent cell division and incorporated EdU A, 24 hours after mechanical scratch injury. **G.** Quantification showed that more dividing cells were noted further away from the scratch. Scale bar in C is 60 microns in A-D; scale bar in F is 100um, and scale bar in (blue channel) is 20um.

Figure 5. Functional astrocytic markers Cx43, Aq4 and GS in ICR injuries

A-F. Connexin 43 (Cx43): A. Low power of an ICR lesion involving the superficial cortex with a defined loss of labelling demarcating injury (asterisk) from adjacent parenchyma. **B**. Within the injury, labelling of endothelium as well as bipolar cells (arrowhead) is noted at 30 dpi. **C**. The injury margin at 70dpi shows increased labelling in reactive cells and processes and **D.** Plump reactive cells are noted. **E**. In chronic lesions (264 dpi) intense labelling of cells and processes at the scar site is noted. **F.** Control region (Zone 3) with delicate, diffuse labelling of astrocytic processes. **G.** Scatter graphs of Cx43 labelling index with days post injury (shown as log) with a significant increase shown for the lesion core ($p=0.03$). **H-J Aquaporin 4 (Aq4)**: **H.** ICR injury at low magnification with loss of labelling in lesion core (asterisk). **I.** In the margins at 8 dpi intense labelling of processes is noted**. J.** Control region with extensive labelling of glial processes. **K-M Glutamine synthetase (GS)**: **K.** Reactive appearing GS positive cells in margin of injury at 13 dpi and **L.** intensely positive cells at 417 dpi. **M.** In control region (Zone 3) labelling of astrocytic cells was shown. Scale bar equivalent to 500 microns in A and H and 50 microns in other figures.

Figure 6. Cx43 labelling in glial cell types in intracranial recording lesions (ICR).

Staining patterns in different stages of repair all in Zone 1 (margin of the ICR lesion): acute 8 days post injury (dpi**) (A-C),** subacute 11dpi **(D-F),** intermediate 30 dpi **(G-I)** and chronic 417 dpi **(J-L**). **Nestin/Cx43: A**. In acute lesions, peripheral Cx43 labelling was noted around large reactive nestin-expressing cells (arrowhead) with focal co-localisation in processes (arrow) (Split channel shown in Supplemental Figure 3F). **D**. In subacute lesions more prominent focal co-localisation in endothelium and perivascular cells was noted; this was still evident in elongated process in intermediate-age ICR lesions (arrowhead) although some large Cx43-positive cells were negative for nestin (chevron) **G. J.** In chronic lesions co-expression was noted in a proportion (but not all) nestin cells at the scar site. **GFAP/Cx43**: **B.** In acute lesions, marginal expression of Cx43 was noted around large reactive $GFAP^+$ cells (arrow) as well as co-localisation in some processes (arrowhead) (Split channel shown in Supplemental Figure 3E). **E.** In subacute and **H**. intermediate lesions, co-labelling of processes was noted (arrowheads) although many large Cx43-positive reactive appearing cells (chevron) were negative for GFAP. **K.** In chronic lesions, a more general overlap between the meshwork of glia fibres in the scar and Cx34 was observed. **PDGFRβ/Cx43: C.** In acute lesions there were many Cx43 processes that were not PDGFRβ-positive. **F.** In subacute lesions some co-localisation was noted in pericyte like cells around capillaries (arrowhead; shown in split channels in Supplemental figure 3D). **I.** In intermediate lesions, lack of co-labelling is appreciated between large reactive $Cx43^+$ cells (chevron) cells and foot processes around capillary pericytes and in **L.** this was also appreciated in chronic lesions. **Control Zone 3: M** Extensive labelling is noted with Cx43 in the cortex however a proportion of GFAP astrocytic domains are devoid of Cx43 labelling. **N.** Patchy or non-confluent labelling with Cx43 is noted and separate from the PDGFRβ in capillaries; (inset) occasional multipolar cell shows co-localisation. **O.** Linear regression analysis for the

relationship between co-localisation of the three markers (GFAP, Nestin, or PDGFRβ) and Cx43 with dpi (expressed as Log_{10}); there was a statistically significant increase noted for GFAP and nestin with chronicity of injury. Scale bar in M is equivalent to 20 microns in A-J, 100 microns in K-N.

Supplemental Figure 1. Immunofluorescence images shown as split channels in green and purple.

A. **PDGFRβ/NESTIN** in subacute injury lesion in zone 1 (**from Figure 2D**) and **B (from Figure 2E);** arrow is single labelled endothelial cell with nestin and arrowheads indicate double labelled perivascular cell. **C**. **PDGFRβ/NESTIN** in intermediate injury in zone 1 showing overlap of labelling in reactive cells at injury margin **(from figure 2F). C' From figures 2J to L with cellular detail at higher magnification. D**. **PDGFRβ/Cx43** in subacute lesion **(from Figure 6F)** showing Cx43 labelling in endothelium and focal co-labelling in pericyte cells (arrowheads). **E. Cx43/GFAP** in acute injury in marginal Zone 1 showing co-expression in some processes and peripheral labelling of Cx43 of some reactive large glial cells (arrowhead) **(from Figure 6B). F Cx43/Nestin** in acute injury in marginal Zone 1 showing co-expression in some processes and peripheral labelling of Cx43 of some reactive large glial cells (arrowhead) **(from Figure 6A).**

Supplemental Figure 2. Nestin, PDGFRβ and GFAP labelling with injury age

A. Scatter graphs of labelling index of nestin, PDGFRβ and GFAP plotted against dpi (expressed as log10) for zone 3 with no significant regressions.

B. Graphical representation of the relative area of co-localisation between GFAP or nestin with PDGFRβ at different stages of injury; both showing significant differences for all cases between Zone 1 and 3 but only nestin showing significant difference at the subacute stage.

Supplemental Figure 3. Summary schematic illustrating the expression of different proliferative cell types around the lesion at various intervals post injury. This is based on the data in current and previous study [5]. The height of the bar refers to level of expression or numbers of dividing cell types. Immediately after injury, the number of dividing Iba1+ microglia and nestin+ expressing cells are upregulated around the lesion, reaching maximal numbers around two weeks post lesion. At two weeks, an increased number of dividing PDGFRβ+ and Olig2+ expressing cells were also observed around the lesion. After a month post lesion, the number of Iba+ microglia, and Olig2+ oligodendrocytes decreased dramatically, reaching similar number of cells found in Zone 3 (normal level). In contrast, many dividing GFAP+ astroglia were observed only after one month after injury. A higher number of MCM2+/GFAP+ cells were still found around lesion after four months post lesion, together with few nestin+ and PDGFRβ+ cells. The level of co-expression between PDGFRβ+ and nestin (red bars within nestin+) were maximally observed a week after post and gradually decrease with time. In contrast, the PDGFRβ+ and GFAP was maximally observed two months after lesion. The expression of CX43 was significantly reduced from normal level, immediately following injury, but increased after a month, coinciding with the

upregulation of GFAP. Some PDGFRβ+and nestin+ expressing cells in Zone 1 were found to express CX43 one month after lesion (Bar colours - Blue, Iba1; purple, nestin; yellow, GFAP; red, PDGFRbeta; brown, olig2 and grey, Cx43).

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