PERSPECTIVES

"Standby" EMT and "immune cell trapping" structure as novel mechanisms for limiting neuronal damage after CNS injury

The central nervous system (CNS) contains the two most important organs, the brain and spinal cord, for the orchestration of the mental and physical activities of life. Because of its importance, the human body has evolved barrier systems to protect CNS tissue from the external environment. This barrier is a membrane composed of tightly apposed cells and is selectively permeable to specific molecules by way of membrane transporters. The major barriers in the brain and their corresponding cellular constituents are the blood-brain barrier (BBB) composed of endothelial cells in brain capillaries, the choroid plexus barrier containing ependymal cells, and the meningeal barrier containing arachnoid cells (Lee et al., 2003; Abbott et al., 2006). While previous studies have actively investigated the roles and repair mechanisms of the BBB and choroid plexus barrier under pathological conditions, the meningeal barrier remains an unexplored field. However, recent studies have reported that factors secreted from the meninges are essential for maintaining neuronal integrity under normal conditions, and many cell populations expressing stem cell markers are derived from the meninges after CNS injury (Decimo et al., 2012), suggesting that the meninges could have important roles in maintaining homeostasis and regeneration after CNS injury. Therefore, the meningeal barrier is expected to become a subject of great interest in the field of CNS repair. The meninges are a set of complex membrane structures that cover CNS tissues and are composed of the dura mater, the arachnoid membrane, and the pia mater. The arachnoid membrane consists of arachnoid cells, a type of epithelial cell which forms tight junctions with neighboring cells. Therefore, it functions as a meningeal barrier to separate the CNS from the external environment (Weller, 2005). Meningeal damage is commonly observed with severe CNS injuries induced by falls, vehicle accidents, penetration-like brain trauma, and spinal cord injury. There is significant variability in recovery time for meningeal barrier damage depending on the type of accident and degree of damage. Some patients who show chronic leakage in the meningeal barrier despite medical treatment have a higher possibility of cerebrospinal fluid leakage, meningitis, intracerebral aerocele, and extended secondary damage, which lead to an increased occurrence of permanent disorders and mortality (Leech, 1974). These clinical cases imply that the prompt reconstruction of the impaired meningeal barrier is crucial for reducing additional damage and promoting patient prognosis after CNS injury.

CNS repair after injury commonly proceeds with a well-organized cascade of inflammation, new tissue formation, and remodeling. In the new tissue formation stage, meningeal cells dynamically migrate into the lesion site undergoing epithelial and mesenchymal transition (EMT) where they reconstruct the meningeal barrier between normal tissues and the lesion site as they are stabilized through mesenchymal and epithelial transition (MET). Previous studies have reported that expression levels of transforming growth factor-beta (TGF-β) receptor and the Ephrins receptor, ErbB2 are highly increased in the meningeal cells of the lesion site, implying that TGF-β and ErbB signaling are related to meningeal responses after CNS injury (Bundesen et al., 2003; Komuta et al., 2010).

However, the molecular mechanisms of meningeal EMT/MET during reconstruction remain largely undefined. Since meningeal reconstruction is temporally and spatially coincident with CNS scar formation, angiogenesis, and immune resolution near the lesion site, we have attempted to understand meningeal EMT/MET in terms of interactions between various cell types and the microenvironment near the lesion site. Recently, we reported that two novel protective mechanisms reduce additional neuronal damage during meningeal reconstruction *via* A-kinase anchoring protein 12 (AKAP12) (Cha et al., 2014a, b). AKAP12 was previously known to regulate the movement of mesodermal cells, vessel integrity, and differentiation of the blood neuronal barrier by modulating junction formation during development. Reduced AKAP12 levels in cancer progression induce motility and invasion of cancer cells (Gelman, 2010). Interestingly, these reported functions of AKAP12 are closely related to EMT/MET, which is an essential event for meningeal reconstruction. In practice, our recent studies showed that AKAP12 modulates EMT/MET of meningeal cells by regulating the TGF-β1/non-Smad/SNAI1 pathway in response to the change in microenvironment after CNS injury.

In this perspective review, we introduce these protective mechanisms and discuss their broader implications for the field of CNS repair.

"Standby" EMT mechanism: Cross-talk between TGF-β1, retinoic acid (RA), and oxygen tension during the process of CNS repair immediately induces EMT to repair the meningeal barrier: The high levels of TGF-β1 and RA from the meninges have a crucial role in neuronal differentiation and integrity in the development. Whether there was a cross-talk between the two factors and their function in meningeal homeostasis in the adult stage was, however, unclear. Because the CNS is highly dependent upon oxygen for its function and homeostasis, CNS tissue overcomes hypoxic condition after injury through immediate vessel remodeling near the lesion site. Therefore, oxygen concentration is dynamically changed in the repair process. Although such an alteration in oxygen tension is expected to be involved in meningeal reconstruction, its cross-talk with TGF-β1 and RA is not known.

Our recent study (Cha et al., 2014a) suggested that the cross-talk between TGF-β1, RA from meningeal cells, and the changes in oxygen tension during CNS repair could constitute the "standby" mechanism that enables the in-

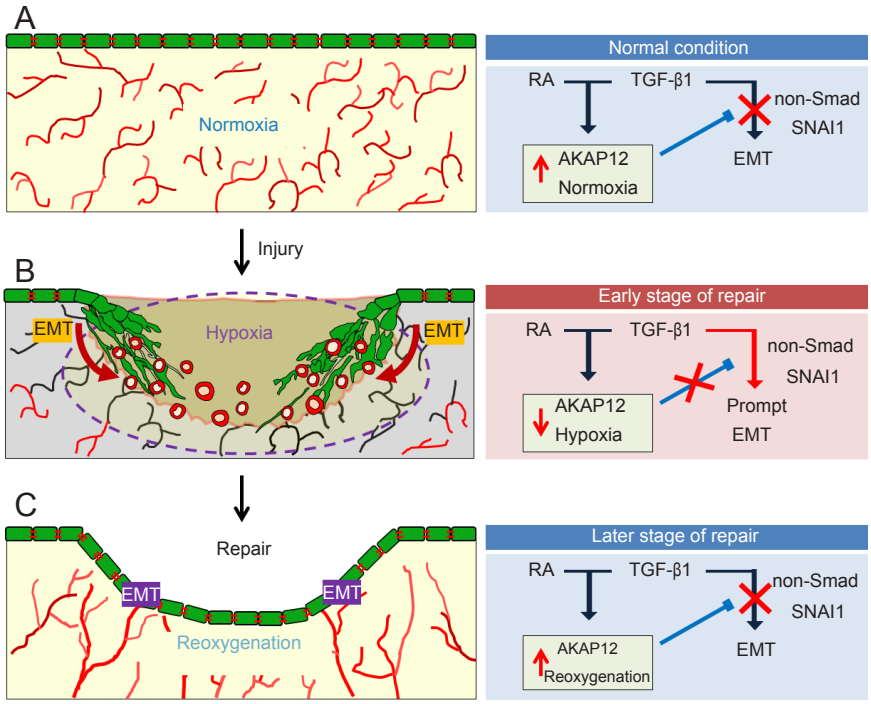
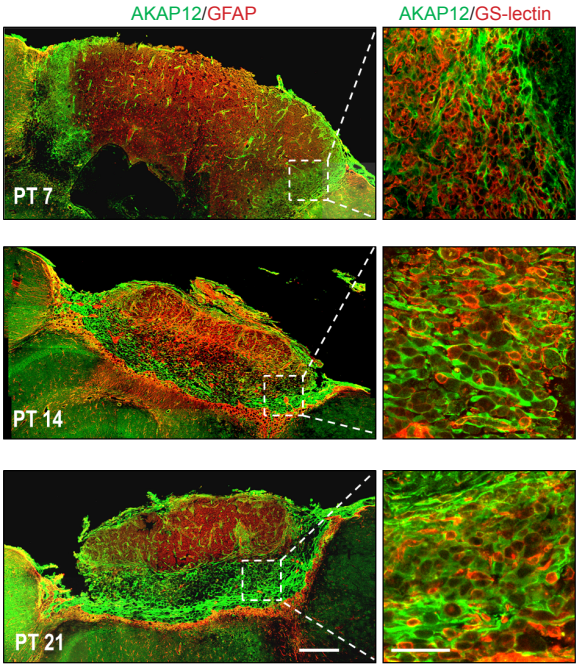
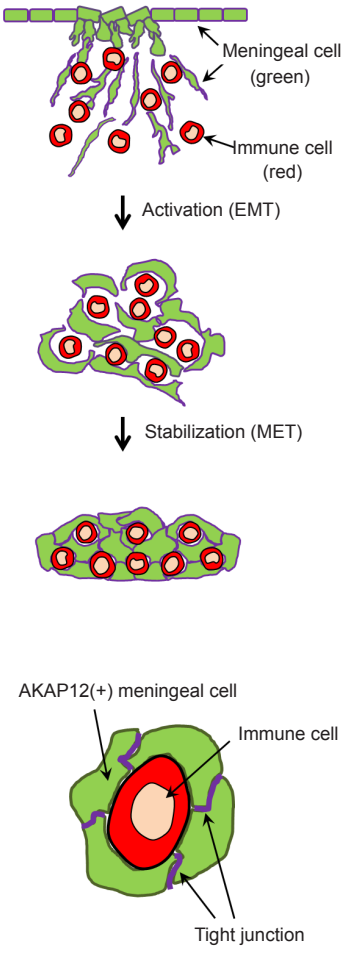


Figure 1 The scheme of "standby" mechanism during meningeal reconstruction.
(A) Transforming growth factor beta l (TGF-β1), retinoic acid (RA), and oxygen induce high levels of AKAP12 in arachnoid cells of normal meninges, which maintains the epithelial properties of the arachnoid cells by mediating the inhibitory effects of RA for TGF-β1. (B) In the early repair stages immediately following CNS injury, hypoxia following vessel damage reduces the level of AKAP12, which then induces epithelial mesenchymal transition (EMT) of arachnoid cells by de-repression the TGF-β1/non-Smad/SNAI1 pathway, resulting in invasion of arachnoid cells transformed to mesenchymal state into the lesion site. (C) In the later repair stages, AKAP12 levels are recovered by oxygen provided by newly formed vessels, which induces re-epithelialization of the invaded arachnoid cells by inhibition of the TGF-β1/non-Smad/SNAI1 pathway. Finally, the meninges are reconstructed around the lesion site.





"Immune cell trapping" structure

- AKAP12(+) meningeal cells surround immune cells, linking to each other.
- AKAP12(+) meningeal cells form a physical barrier by expressing of tight junction proteins.
- TGF-β1 and RA from meningeal cells could have immune suppressing effect.

Figure 2 The scheme of the "immune cell trapping" structure.
Mouse brains were harvested at serial time points after injury induced by photothrombosis (PT). For double labeling with A-kinase anchoring protein 12 (AKAP12), tissue was co-stained with cell-specific markers, such as glial fibrillary acidic protein (GFAP) (astrocyte) and GS-lectin (monocyte and macrophage). Scale bars: 200 μm, 50 μm (magnified images). The number of AKAP12-positive arachnoid cells increases near the lesion site over time after PT injury, and these cells form the "immune cell trapping" structure surrounding the lesion site by linking to each other. GS: Griffonia simplicifolia; TGF-β1: transforming growth factor beta1; RA: retinoic acid; EMT: epithelial mesenchymal transition; MET: mesenchymal and epithelial transition.

duction of immediate EMT for rapid reconstruction of an impaired meningeal barrier after CNS injury. The expression of AKAP12, a candidate effector of this "standby" mechanism, is regulated by cross-talk among TGF-β1, RA, and oxygen tension. TGF-β1, RA, and oxygen induce high level of AKAP12 in arachnoid cells of normal meninges, and AKAP12 maintains the epithelial properties of arachnoid cells by inhibiting the TGF-β1/non-Smad/SNAI1-EMT pathway (**Figure 1A**). Oxygen tension functions as a switch that toggles the "standby" EMT mechanism by regulating the expression of AKAP12. Immediately following CNS injury, hypoxia due to vessel damage reduces AKAP12 levels, resulting in an immediate meningeal EMT by de-repression of the TGF-β1/non-Smad/SNAI1 pathway (**Figure 1B**). In later repair stages, reoxygenation by newly formed vessels restores AKAP12 levels which then induce MET of meningeal cells through inhibition of the TGF-β1/non-Smad/SNAI1 pathway (**Figure 1C**). Consistent with these results, AKAP12 knockout (KO) mice showed a malfunction of the reconstructed meningeal barrier stemming from defects in EMT/MET of meningeal cells during reconstruction, strongly supporting our "standby" EMT hypothesis (Cha et al., 2014b). Collectively, TGF-β1, RA, and oxygen tension coordinately modulate the dynamic changes in AKAP12 expression, which then mediates rapid meningeal reconstruction by regulating EMT/MET of meningeal cells.

In the general wound healing process, the level of TGF-β1 is significantly increased after injury, and TGF-β1 induces EMT of various cell types. While basal levels of TGF-β1 are very low in the normal epithelium of most organs, they are maintained at high levels in the meninges of the CNS even under normal conditions. Because the meninges must maintain their properties as epithelial tissue in order to function properly as a barrier for CNS tissue, it is paradoxical that the meninges have high levels of TGF-β1, a major EMT inducer. Our "standby" hypothesis could explain why the meninges normally secrete TGF-β1, and how the meninges can maintain epithelial properties in spite of high levels of TGF-β1. If the expression of TGF-β1 in the meninges were triggered by signaling cascades activated after injury, similar to other organs, an immediate response would be impossible. Therefore, the meninges may prepare for immediate meningeal EMT in emergencies by keeping levels of TGF-β1 high and, at the same time, maintain epithelial properties by co-expressing high level of RA. Then, TGF-β1 and RA together induce high levels of AKAP12 expression under normoxia. The resultant AKAP12 represses the TGF-β1-induced EMT pathway. Based on this "standby" EMT mechanism, extended studies are warranted to promote meningeal reconstruction by timely regulation of the levels of TGF-β1, RA, and oxygen. These future studies could be helpful in improving patient prognosis after CNS injury.

In the "standby" mechanism, oxygen tension functions as a regenerative microenvironment that regulates the reversibility of meningeal cells during reconstruction of damage to the meningeal barrier. The CNS only occupies 2% of total body mass, but it consumes 20% of the body's oxygen, implying that the CNS has a high dependence on oxygen for its function and homeostasis compared to other organs. Therefore, blood vessel remodeling near a lesion site occurs immediately to overcome hypoxia induced by vessel damage after injury, resulting in dynamic changes of oxygen tension in the CNS repair process. Since oxygen tension is instantly changed dependent upon vessel state, and oxygen is available to affect target molecules directly without engaging a signaling cascade via receptor activation, a change in oxygen tension is expected to be an important microenvironmental factor that could regulate immediate responses for various CNS pathological conditions accompanying vessel damage. Therefore, it will be interesting to investigate the role of oxygen tension in the CNS repair process.

"Immune cell trapping" structure: AKAP12-positive meningeal cells form a physical barrier to restrict inflammation by trapping immune cells in fibrotic scars during meningeal reconstruction: After CNS injury, various cells migrate and form a scar near the lesion site. This CNS scar consists of two distinct layers, the fibrotic scar and glial scar; the fibrotic scar directly surrounds the lesion site and the glial scar forms a boundary between the fibrotic scar and the normal parenchymal tissues. In our focal brain injury model, meningeal reconstruction coincides with fibrotic scaring in the same space (**Figure 2**). After CNS injury, the number of AKAP12-positive arachnoid cells increased over time near the lesion site, and these cells were primarily found in the fibronectin-positive fibrotic scar, showing that the AKAP12-positive arachnoid cell is one cell type contributing to fibrotic scar formation. In the early stages of new tissue formation, arachnoid cells activated through EMT invade into the lesion site under the guide of inflammatory cells. In later stages, becoming stationary through MET, invading arachnoid cells form an interesting "immune cell trapping" structure by linking to each other (Cha et al., 2014b). Based on these serial observations, it is thought that this structure is the middle stage in the reconstruction process of the injured meninges. Since AKAP12-positive arachnoid cells form tight junctions between cells, this structure could physically separate immune cells by trapping. Furthermore, when we applied TGF-β1 and RA under normoxia to macrophage/monocyte cell lines, activation by inflammatory inducers was effectively blocked, implying that TGF-β1 and RA enriched in the fibrotic scar could have immune suppressing effects. Consistent with this finding, AKAP12 KO mice showed more extended infiltration of immune cells into neuronal parenchyma across CNS scars and severe tissue damage with a breakdown of "immune cell trapping" structures. These findings reveal the possibility that the fibrotic scar functions to restrict inflammation after CNS injury, and that the "immune cell trapping" structure formed by AKAP12-positive arachnoid cells could underlie this beneficial property of the fibrotic scar.

Until now, fibrotic scarring has been recognized as an obstacle for CNS repair because fibroblasts within the scar are the main sources of chondroitin sulfate proteoglycans

(CSPGs) and extracellular matrix proteins (ECMs), which disrupt axonal regeneration at the remodeling stage of the CNS repair process (Hellal et al., 2011). Thus, previous studies on fibrotic scarring have focused on blocking scar formation in order to promote axonal regeneration. However, our findings suggest that the fibrotic scar could have beneficial roles in restricting excess inflammation at the new tissue formation stage of the CNS repair process as well (Cha et al., 2014b). Likewise, the fibrotic scar has the double-sided characteristic of being detrimental or beneficial depending on the repair stage. Therefore, further studies are warranted to determine how the protective mechanisms of AKAP12-positive cells intersect with the destructive pathways that inhibit axonal regeneration. Based on such further studies, coordinated approaches are necessary to maintain the protective role of the fibrotic scar and to block destructive pathway activation in a timely manner in order for treatments targeting fibrotic scarring to promote repair and recovery after brain injury.

Despite their importance, neuronal tissues are easily damaged by exposure to external materials and lack regenerative properties following CNS injury. Therefore, it has been thought that the CNS could have a unique, organ-specific repair system to minimize the damage. In this respect, the "standby" EMT mechanism and the "immune cell trapping" structure could be a novel repair system to reduce additional neuronal damage. The microenvironment near the meninges constitutes the "standby" mechanism that enables rapid reconstruction of the impaired meninges after CNS injury. This mechanism could guarantee the neuronal homeostasis by promptly restoring the meningeal barrier function to block the inflow of hazardous substances. During reconstruction of the meningeal barrier, migrating meningeal cells form a structure trapping the immune cells that infiltrated into the lesion site by linking to each other. This "immune cell trapping" structure could reduce secondary inflammatory damage by confining various immune cells to the fibrotic scar, suggesting the beneficial roles of the fibrotic scar newly. These hypotheses are strongly supported by the abnormal reconstruction of impaired meninges observed in AKAP12 KO mice. Compared to WT mice, the reconstructed meningeal barrier structure in the AKAP12 KO mice was loosely assembled and showed loss of tightness between meningeal cells, resulting from reduced expression of tight junction proteins like ZO-1, occludin and E-cadherin. Consequently, AKAP12 KO mice had extensive tissue damage, marked by inflammatory cells and other materials discharged into parenchymal tissues across the compromised barrier. Because junction proteins (epithelial markers) are essential for barrier function by providing tight junctions between cells, such a malfunction of the reconstructed meningeal barrier is well-explained by abnormal EMT/MET of meningeal cells. This suggests that AKAP12 regulates the transition between the epithelial and mesenchymal states of meningeal cells. Collectively, this research comprehensively reveals interactions between various cells in the repair process after CNS injury and crosstalk between related factors from a macroscopic viewpoint. The results of these studies provide not only insights into CNS repair processes that were not previously understood, but also applicable information for more effective treatments that may promote recovery at different stages of the CNS repair process.

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