In focus in HCB

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Accepted: 30 July 2021 / Published online: 1 September 2021

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In this September issue of the journal, we will highlight three Original Articles and one Short Communication describing (1) the investigation of DNA methylation changes and reprogramming during human embryogenesis; (2) the spatiotemporal localization of the helix-loop-helix transcription factor atonal homologue 8 during early zebrafish development; (3) protein engineering techniques to create hybrid galectins and characterization of their binding activities; and (4) the introduction of an in vitro model for the senescent blood–brain barrier using cultured murine brain endothelial cells. We hope you enjoy these highlights, as well as the full issue.

Remodeling of DNA methylation in early human preimplantation development

In most mammalian species, including humans, major epigenetic reprogramming takes place during early development (Messerschmidt et al. 2014; Reik et al. 2001). Such epigenetic reprogramming affects DNA methylation, which consists of mainly passive DNA demethylation interwoven with de novo methylation and active DNA demethylation involving oxidation of 5-methylcytosine (5mC) by ten-eleven translocation (TET) enzymes (Amouroux et al. 2016; Gu et al. 2011; Ladstätter and Tachibana 2019). In continuation of previous studies (Arand et al. 2015; Wossidlo et al. 2011), Arand et al. (2021), using quantitative immunofluorescence, have analyzed dynamic changes in 5mC and its derivatives (5-hydroxymethylcytosine, 5hmC; 5-formylcytosine, 5fC; and 5-carboxylcytosine, 5caC) generated by TET enzymes in human zygotes up to blastocysts (Fig. 1).

The abundance of their findings can be summarized as follows. Human zygotes follow human-specific dynamics of DNA demethylation, with variable 5mC and 5hmC appearance and no apparent difference in maternal or paternal pronuclei. In contrast to the mouse, no accumulation of 5fC or 5caC was detected in paternal pronuclei. Similar to mice, in human four-cell embryos up to morula stage, a mosaic pattern for 5mC and 5hmC was observed, suggesting passive dilution of DNA methylation. The majority of the analyzed blastocysts showed a stronger signal of 5mC and 5hmC in the inner cell mass relative to the trophectoderm. Since these blastocysts had significantly fewer cells than the blastocysts with lower signals of 5mC and 5hmC, these two types represent early- and late-stage developing blastocysts. This indicates that in late preimplantation development, global DNA demethylation is still occurring in the pluripotent inner cell mass. By combining noninvasive time-lapse imaging, well-defined cell cycle parameters and immunofluorescence for DNA methylation, normally and abnormally developing human four-cell embryos were analyzed. Interestingly, lower

Fig. 1 Human-specific dynamics of DNA methylation reprogramming in zygotes. Images of immunofluorescence for 5mC and 5hmC, 5fC, or 5caC in human zygotes at late G2 stage. From Arand et al. (2021)

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5mC and 5hmC levels were measured in normal embryos compared to abnormal embryos, whereas the levels of 5caC were very similar between normal and abnormal embryos. The authors concluded that DNA methylation reprogramming is conserved in humans, that human-specific dynamics and extent exist, and that this is essential for successful human embryogenesis.

Spatiotemporal expression of *atoh8* in early zebrafish development

The helix-loop-helix transcription factor atonal homologue 8 is involved in the control of numerous aspects of vertebrate organ development and function (Chen et al. 2011; Massari and Murre 2000; Phillips, 1994; Rawnsley et al. 2013; Yao et al. 2010). Despite the proposed role of *atoh8* for zebrafish development, a detailed in situ mapping of *atoh8* transcripts has not been conducted (Yao et al. 2010). Now, Fragale et al. (2021) have performed a whole-mount in situ hybridization to reveal the expression pattern of *atoh8* (and of *myoD* and *crestin*) during the first 24 h of zebrafish development. They could distinguish two expression phases. The first appearance of *atoh8* started at around 9 hpf in the pre-segmental paraxial mesoderm, and the second phase started around 13 hpf in the developing cerebellum and hindbrain (Fig. 2).

At 16 hpf, the *atoh8* hybridization signal was detectable in three distinct locations along the anterior to posterior axis of the embryo: anterior, in the head, in the posterior portion of the developing eye, in the presumptive hindbrain and cerebellum; in the trunk and tail in segmented and unsegmented paraxial mesoderm, and posterior in the front portion of the tail. In contrast to previous reports, *atoh8* was not detectable in the myotome, which was substantiated by double in situ hybridization for *atoh8* and *myoD*. Furthermore, *atoh8* transcripts were undetectable in neural crest cells. Summarizing their highly detailed findings, the authors proposed a possible

role of *atoh8* in sclerotome, cerebellum, and hindbrain development in zebrafish.

Protein engineering of a human galectin

Lectins are carbohydrate-binding proteins of nonimmune origin. Although especially prevalent in plants and invertebrates, they can be found in many vertebrate species as well (Manning et al. 2017). Given their renowned capability for recognition of very specific carbohydrate structures and sequences, much interest has been generated in using lectins as tools for elucidating the functional significance of cellular carbohydrates (see the Special Issue of *Histochemistry and Cell Biology*—In Focus: the Sugar Code (volume 47, issue 2, February 2017) for detailed reviews of the topic). Moreover, the ability to use chemical and molecular methods to alter or create hybrid lectins as a means to relate the complex structure of the carbohydrate binding domain (CRD) with the overarching bioactivity of the lectin is of great interest. The Gabius research group has been leading such investigations for many years (for instance, Roy et al. 2017). In this current issue, they provide a very detailed and well-presented example of using protein engineering to investigate the intriguing question of why evolutionary mechanisms resulted in a specific lectin CRD, rather than any of the multitudinous variations also possible (Ludwig et al. 2021). As a test lectin, they chose to use galectin-7 (Gal-7), an *N*-acetyllactosamine-specific member of the vertebrate galectin family, whose CRD may be arranged in three types of structures: a non-covalently associated homodimer, a linker-coupled heterodimer, or a CRD combined with a structurally different second part. They used cDNA engineering techniques to produce variants using direct CRD conjugation and insertion of a 33-amino-acid linker of Gal-8 to provide a spacer between the CRDs. They generated stable homodimers with the Gal-7 CRD, as well as heterodimers consisting of the Gal-7 CRD connected to Gal-1 or Gal-3 CRDs. The resulting galectin variants were carefully purified, highly characterized, and then used to assess both their histochemical staining of mouse tissues (Fig. 3) and their impact on cell proliferation in vitro using a human neuroblastoma cell line.

Their very rigorous testing and multiple analyses demonstrated (1) very little difference between the natural non-covalently linked Gal-7 homodimer and artificial covalently-linked variants; and (2) that the binding properties and

Fig. 2 Flat mounts of zebrafish embryo at 22 hpf showing expression of *atoh8* in hindbrain, cerebellum, and eye. From Fragale et al. (2021)

Fig. 3 Illustration of differences in staining patterns of labeled Gal-7 and the heterodimer pair with sequence permutation (Gal-7–Gal-3 and Gal-3–Gal-7) in villi in sections of murine jejunum. From Ludwig et al. (2021)

activity of the heterodimers can be influenced by the order of the lectin domain arrangement as well as the length of the linker. This comprehensive study paves the way for further research into galectin family engineered heterodimers and their potential as biotherapeutic reagents.

Model for the aging blood–brain barrier

The blood–brain barrier (BBB) serves a protective and regulatory function to maintain brain neuronal health. Morphologically, the BBB consists of brain capillary endothelial cells, pericytes, astrocytes, and microglia, together with extracellular matrix and basal lamina working in tandem. During the normal aging process, and as a result of various neurological diseases, the BBB has been found to manifest numerous morphological and physiological alterations (reviewed in Erdo et al. 2017). In an attempt to overcome the

Fig. 4 Alterations in endothelial cells induced by aging and senescence. Treatment with H_2O_2 (100 μM) increases beta-galactosidase ($\beta\text{-gal}$) expression in low passage (LP) cerebellar endothelial cells (cerebEND). From Salvador et al. (2021)

limitations currently imposed by the use of patient samples and ex vivo mouse materials to investigate age-related BBB alterations, Salvador et al. (2021) have now developed an in vitro model based upon cultured murine brain-derived endothelial cells. Using two well-characterized cell lines they had previously derived from murine cerebellar (cerebEND) and cerebral (cEND) microvascular endothelial cells (Helms et al. 2016), the authors induced senescence via treatment with H_2O_2 , and then analyzed the cells by immunofluorescence microscopy, Western blot, transendothelial resistance (TEER), and endothelial permeability. They demonstrated the suitability of their proposed in vitro model by showing that (1) H_2O_2 treatment induced senescence (indicated by increased β -galactosidase-positive cells) in both cerebEND (Fig. 4) and high-passage cEND cells; (2) other markers of cellular senescence, such as p16 and p21, were mostly expressed in high-passaged cells, but not in lower-passaged cultures; (3) GLUT-1 staining, as an indicator of cellular transport function, was decreased and more dispersed in treated cerebEND and cEND cells compared to primary ECs; (4) staining for the tight junctional and adherens proteins VE-cadherin, ZO-1, and claudin-5 showed some variability amongst the different cell treatments and passage, but in general a disruption due to senescence was noted; and (5) cell barrier permeability increased in both cerebEND and cEND cells treated with H_2O_2 , while untreated high-passaged cerebEND and cEND cells showed only a slight increase in permeability compared to low-passaged cells.

Taken in total, their results show that these two immortalized murine brain capillary endothelial cell-derived cell cultures should prove to be a useful and convenient model for the study of the senescent BBB.

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