Notch signalling regulates asymmetric division and inter-conversion between lgr5 and bmi1 expressing intestinal stem cells

Tara Srinivasan1, Elaine Bich Than2, Pengcheng Bu1,3, Kuei-Ling Tung4, Kai-Yuan Chen1, Leonard Augenlicht5, Steven M. Lipkin2 & Xiling Shen1,3,6

Rapidly cycling LGR5+ intestinal stem cells (ISCs) located at the base of crypts are the primary driver of regeneration. Additionally, BMI1 expression is correlated with a slow cycling pool of ISCs located at +4 position. While previous reports have shown interconversion between these two populations following tissue injury, we provide evidence that NOTCH signaling regulates the balance between these two populations and promotes asymmetric division as a mechanism for interconversion in the mouse intestine. In both in vitro and in vivo models, NOTCH suppression reduces the ratio of BMI1+/LGR5+ ISCs while NOTCH stimulation increases this ratio. Furthermore, NOTCH signaling can activate asymmetric division after intestinal inflammation. Overall, these data provide insights into ISC plasticity, demonstrating a direct interconversion mechanism between slow- and fast-cycling ISCs.

In the murine intestine1, fast-cycling LGR5-expressing (Leucine-rich repeat-containing G protein-coupled receptor 5-expressing) crypt base columnar (CBC) stem cells are the cells primarily responsible for maintaining homeostasis by replacing cells as they mature and are sloughed into the lumen. LGR5+ CBCs can self-renew, or produce transit-amplifying (TA) daughter cells that rapidly divide and terminally differentiate into distinct lineages that populate the intestinal epithelium1,2. There are also additional stem or progenitor cell populations3, which have been associated with markers including BMI1, HOPX, TERT and LRIG-14–8. Single-molecule transcript analyses suggest that the presence of LGR5 and BMI1 mRNAs is more prevalent than that indicated by antibody staining and that they potentially overlap in a subset of cells, raising the possibility that post-translational mechanisms may amplify the difference in protein levels and these two populations may be more plastic than previously thought9,10.

Remarkably, it has been shown that Lgr5+ stem cells can produce +4 cells as daughters4, and +4 ISCs can reciprocally produce Lgr5+ CBC daughter cells as a compensatory mechanism following experimental ablation of Lgr5-expressing cells4. The interconversion between faster proliferating Lgr5+ vs. more quiescent Bmi1+ ISC populations demonstrates the fluidity of crypt cell type hierarchy, which can help maintain homeostasis and adapt to different types of intestinal micro-environmental conditions. Our newly gained knowledge about ISC plasticity provokes the question as to which mechanism regulates the choice of each identity.

In mouse intestinal crypts, Notch signalling is known to be an important pathway associated with stem cell self-renewal11–14. Accordingly, the proliferative zone of intestinal crypts contains essential Notch pathway components, such as receptors NOTCH1 and NOTCH2, ligands DLL-1, DLL-4, and JAG-1, and downstream effector
genes Hairy and Enhancer of Split 1 (Hes1) and Hes514–16. Here we demonstrate that NOTCH signaling is a key mechanism that regulates the balance between highly proliferative and relatively quiescent stem cells, and activates asymmetric division when the tissue is under stress. Maintaining both fast- and slow-cycling stem cells may provide a survival strategy for maintaining homeostasis within intestinal tissue.

Results

**NOTCH signaling balances BMI1+ and LGR5+ populations in intestinal organoids.** Single mouse LGR5-EGFP+ intestinal stem cells (ISCs) were isolated (Supplementary Fig. 1A) using FACS17 and propagated as organoids to quantify the relative ratio of BMI1+ and LGR5+ ISC under conditions in which NOTCH signaling was modulated17. When NOTCH signaling was inhibited with the γ-secretase inhibitor DAPT for 48 hours and visualized by co-IF, the ratio of BMI1+/LGR5+ cells decreased vs. DMSO-treated controls (p = 0.001; Student t-test) (Fig. 1a,b). Western analysis for NICD confirmed inhibition of NOTCH activity due to DAPT treatment (Supplementary Fig. 1B).

POFUT-1 (Protein O-fucosyltransferase 1) is an enzyme responsible for the addition of fucose by O-linkage on EGF domains of NOTCH receptors and is required for functional NOTCH signaling 18–20. To confirm the results from chemical inhibition of NOTCH signaling, ISCs derived from mice expressing a LGR5-EGFP-creER/POFUT-1flox/flox genotype were treated in vitro with 4-hydroxy-Tamoxifen for approximately 48 hours to inactivate the POFUT-1 gene, and cells were then imaged by IF (Fig. 1a). Similar to DAPT treatment, the BMI1+/LGR5+ cell ratio decreased vs. DMSO-treated controls (p = 0.001; Student t-test) (Fig. 1b). Western analysis showed, as expected, that POFUT-1 and NICD were not detectable in this model of NOTCH suppression (Supplementary Fig. 1C).

A complementary experiment then examined the effect of stimulation of the NOTCH pathway via soluble JAG-1 embedded in Matrigel, the substrate on which the organoids were propagated4. JAG-1 stimulation of NOTCH in ISCs generated from LGR5-EGFP mice significantly increased the ratio of BMI1+/LGR5+ vs. DMSO-treated controls (p = 0.001; Student t-test) (Fig. 1a,b). As expected, JAG-1 treatment also increased NICD levels (Supplementary Fig. 1D).

The effects on NOTCH modulation on intestinal stem cell populations in vitro were then further validated based on ASCL2 expression, an alternative marker for LGR5+ ISCs12,21. Consistent with our earlier findings, DAPT treatment and POFUT-1 deletion decreased the ratio of BMI1+/ASCL2+ ISCs while JAG-1 stimulation increased the ratio of BMI1+/ASCL2+ compared to DMSO-treated controls (p = 0.001; Student t-test) (Supplementary Fig. 1E,F). Taken together, these findings show that NOTCH signaling increases the ratio of BMI1+/LGR5+ (ASCL2+) ISCs, whereas NOTCH inhibition reduces this ratio in intestinal organoids.

**NOTCH signaling balances BMI1+ and LGR5+ populations in vivo.** To confirm the organoid studies, intestinal sections from LGR5-EGFP mice treated with DMSO were analyzed by IF for LGR5 (detected by GFP antibody) and BMI1 expression. BMI1+ cells largely localized to nuclei in the +4 position and LGR5+...
NOTCH signaling was also inhibited using LGR5-EGFP-creER/POFUT-1 flox/flox mice that were administered Tamoxifen (Supplementary Fig. 3A,B). BMI1 on ASCL2 expression (Supplementary Fig. 2B,C), suggesting that NOTCH suppression decreases the ratio of BMI1+ cells (n = 5 mice/treatment) for conditions in (a). Data represents mean ± s.d of 5 mice/condition with n = 500 crypts/mouse measured (***p = 0.001, one-way ANOVA). Bottom: Western Blot for POFUT-1 and NICD expression. Actin was used as a control.

Figure 2. NOTCH balances LGR5+/BMI1+ ISC populations in vivo. (a) Treatments were administered by i.p injections: DMSO (on LGR5-EGFP mice); DAPT (on LGR5-EGFP mice every 12 hours for 3 days); Tamoxifen (on LGR5-EGFP-creER/POFUT-1 flox/flox mice every 24 hours for 5 consecutive days); or Tamoxifen (on LGR5-EGFP-CreERT2/Rosa26-YFP-NICD mice every 24 hours for 8 consecutive days). Shown are representative intestinal crypts from the duodenum: Anti-GFP antibody (green) detects LGR5 (green); BMI1 (red) and DAPI (blue). Scale bar: 200 μm (H&E), 20 μm (IF). (b) Top: Quantification of BMI1+ and LGR5+ cells (n = 5 mice/treatment) for conditions in (a). (b) Treatments were administered by i.p injections: DMSO (on LGR5-EGFP mice); DAPT (on LGR5-EGFP mice every 12 hours for 3 days); or Tamoxifen (on LGR5-EGFP-creER/POFUT-1 flox/flox mice every 24 hours for 5 consecutive days); or Tamoxifen (on LGR5-EGFP-CreERT2/Rosa26-YFP-NICD mice every 24 hours for 8 consecutive days). Shown are representative intestinal crypts from the duodenum: Anti-GFP antibody (green) detects LGR5 (green); BMI1 (red) and DAPI (blue). Scale bar: 200 μm (H&E), 20 μm (IF). (b) Top: Quantification of BMI1+ and LGR5+ cells (n = 5 mice/treatment) for conditions in (a). Data represents mean ± s.d of 5 mice/condition with n = 500 crypts/mouse measured (***p = 0.001, one-way ANOVA). Bottom: Western Blot for POFUT-1 and NICD expression. Actin was used as a control.

Asymmetric BMI1+/LGR5+ division of ISC organoid cells. To assess a potential role for NOTCH signaling in regulating LGR5+ and BMI1+ (HOPX+) normal stem cell populations, we examined in vitro organoid cultures of single ISCs derived from mice carrying an EGFP knock-in driven by the LGR5 promoter (LGR5-EGFP). Murine crypts were isolated, dissociated into single cells, embedded in Matrigel overlaid with growth media, and observed 16 hours post-plating to visualize the mitotic outcome of single stem cells by IF. Using α-TUBULIN staining, we observed single ISCs producing BMI1+/LGR5+, LGR5+/LGR5+, and BMI1+/BMI1+ daughter pairs in the final stages of cell division (Fig. 3a). To confirm antibody specificity, ISCs were treated with a microtubule-depolymerizing agent (Colchicine) for 4 hours following the pair cell assay, which showed an absence of α-TUBULIN expression in Ki67+ dividing pairs (Supplementary Fig. 4A). Next, we tested additional microtubule markers, including β-TUBULIN (Supplementary Fig. 4B) and γ-TUBULIN.
(Supplementary Fig. 4C) in pair cell assays, which consistently showed the generation of BMI1+/LGR5+ asymmetric ISC daughters. We also found asymmetric distribution of LGR5 and the cell polarity marker PARD3A in ISC daughter pairs prior to completion of cell division using mitotic spindle labeling, indicating intrinsic asymmetric division (Supplementary Fig. 4D).

Single ISCs were analyzed further using the pair cell assay for conditions that modulate NOTCH signaling (Fig. 3b). RT-PCR analysis of Hes1 and Hes5 confirmed NOTCH decreased and increased signaling upon treatment with DAPT or JAG-1, respectively (Supplementary Fig. 4E). The frequency of BMI1+/LGR5+ cell pairs was reduced upon NOTCH inhibition and increased upon NOTCH stimulation relative to the DMSO-treated control (p = 0.002; one-way ANOVA). Quantification based on ASCL2 expression was consistent with these findings, indicating that DAPT decreased the percentage of asymmetric BMI1+/ASCL2+ division while JAG-1 elevated this frequency compared to DMSO-treated controls (Supplementary Fig. 5A, B). To understand whether this process can be influenced by stress to the system, we treated organoids with TNF-α, a pro-inflammatory cytokine linked to chronic colitis and carcinogenesis, and increased apoptosis of organoid cells. TNF-α was administered at a low dosage of 10ng/ml to LGR5-EGFP ISCs over 72 hours. We found that TNF-α up-regulated NICD by Western blot analysis, as well as expression of Hes1 and Hes5 by RT-PCR (Fig. 3c). TNF-α treated ISCs showed a marked increase to 4.3% BMI1+/LGR5+ divisions (p = 0.003, one-way ANOVA) (Fig. 3d). When DAPT was added to the culture medium during the last 48 hours of TNF-α treatment, NICD, Hes1 and Hes5 levels all decreased (Fig. 3c) and notably, BMI1+/LGR5+ asymmetric division was reduced to 0.1% (p = 0.002, one-way ANOVA).
ANOV A) (Fig. 3d). Quantification based on ASCL2 expression was consistent with these results, indicating that TNF-α increased the percentage of asymmetric BMI1+/ASCL2+ division while TNF-α + DAPT decreased this frequency compared to DMSO-treated controls (Supplementary Fig. 5C). FACS analysis was then used to quantify the BMI1+ vs. LGR5+ ISC population balance in TNF-α+ and TNF-α+DAPT treatment groups (Fig. 3e). Consistent with our earlier findings, the BMI1+/LGR5+ double positive population containing BMI1+/LGR5+ pairs increased with TNF-α treatment and decreased with TNF-α + DAPT treatment. The ratio of BMI1+/LGR5+ ISCs increased with TNF-α treatment and decreased with TNF-α + DAPT treatment (p = 0.01; Student t-test). These data suggest that normal ISCs are capable of NOTCH-dependent asymmetric BMI1+/LGR5+ division, which can be triggered by stress.

Asymmetric BMI1+/LGR5+ division in vivo. LGR5-EGFP intestinal tissue was then used to study ISC division using α-TUBULIN and Ki67 expression. We detected only LGR5+ vs. BMI1+ symmetric division (Supplementary Fig. 6A). This finding shows that asymmetric BMI1+/LGR5+ division under homeostatic conditions in vivo is rare, unlike in organoids, where stem cells are promoted to proliferate by growth factors such as WNT, R-SPONDIN, and NOGGIN. To examine the effect of stress on ISC division,
LGR5-EGFP mice were treated with 3% dextran sulfate (DSS) in the drinking water for 5 days followed by a 5-day recovery period with plain water. DSS has been shown to promote small intestinal inflammation in addition to chronic colonic inflammation that increases intestinal cell apoptosis. Since BMI1+ ISCs are not present in the colon, the effects of DSS on BMI1+/LGR5+ asymmetric division were evaluated in the small intestine. Consistent with TNF-α treatment in organoids, we detected asymmetric BMI1+/LGR5+ daughters in α-TUBULIN+/γ-TUBULIN+/Ki67+/ dividing pairs (Fig. 4a). DSS treatment increased BMI1+/LGR5+ asymmetric division frequency to 3.9% (p = 0.002; one-way ANOVA) (Fig. 4b). When DSS-treated mice were injected with DAPT during the last 3 days of the plain water diet, the number of asymmetric BMI1+/LGR5+ cell pairs reduced to 0.2% (p = 0.004; one-way ANOVA) (Fig. 4b). Quantification based on ASCL2 expression was consistent with these results, indicating that DSS increased the percentage of asymmetric BMI1+/ASC2+ division while DSS + DAPT decreased this frequency compared to the control (Supplementary Fig. 6b,c). DSS treatment increased NOTCH signaling levels, while the addition of DAPT reduced NOTCH signaling levels, in terms of NICD, Hes1, and Hes5 expression (Fig. 4c). DSS and DSS + DAPT intestinal tissues were then analyzed by FACS to quantify BMI1+ vs. LGR5+ population balance (Fig. 4d). Again, the double-positive population containing BMI1+/LGR5+ pairs increased with DSS treatment and decreased with DSS + DAPT treatment. The ratio of BMI1+/LGR5+ ISCs increased with DSS treatment and decreased with DSS + DAPT treatment (p = 0.01; Student t-test). Therefore, these data suggest that stress can trigger asymmetric BMI1+/LGR5+ division in the intestine, potentially increasing conversion between BMI1+ and LGR5+ cells.

Discussion

We show that BMI1+/LGR5+ divisions, regulated by NOTCH signaling levels, exist in mouse intestinal organoids and in the intestinal mucosa. In mouse intestine, LGR5+ CBCs are fast-cycling and proliferate largely through symmetric division, while BMI1+/HOPX+ cells are mostly quiescent. However, single-molecule RNA FISH suggests that the mRNA levels of these markers do not as clearly distinguish the fast and slow cycling populations, raising the possibility of plasticity and interconversion among these populations. Targeted ablation of LGR5+ ISCs in transgenic mice with diphtheria toxin revealed that intestinal crypt homeostasis could be rescued by rare, normally quiescent ISCs. Remarkably, the two populations can be replenished when each is depleted. Potential plasticity between LGR5+ ISCs and other quiescent cell types in response to tissue injury has also been suggested.

Our data suggest potential roles for the NOTCH pathway to regulate the balance between fast- and slow-cycling populations, and asymmetric BMI1+/LGR5+ division can potentially be activated to aid direct interconversion when the balance is disrupted and needs to be restored. The frequency of such BMI1+/LGR5+ division does not need to be high, given that the normally quiescent ISCs are relatively rare and long-lasting. However, the low frequency of quiescent cells may still serve an important role as reserve stem cells, establishing an important link to repopulation and maintenance of homeostasis.

Methods

Animal Experiments. LGR5-EGFP (also known as Lgr5-EGFP-IRES-creERT2) mice originally purchased from the Jackson Laboratory and LGR5-EGFP-creER/POFUT-1floxflox mice on a mixed 129/C57BL/6 background were provided by Dr. Augenlicht’s research group. For in vivo studies, DAPT was administered every 12 hours for 3 days by ip injection in LGR5-EGFP mice, and Tamoxifen (Sigma) was administered by daily i.p. injections for 5 consecutive days in POFUT-1floxflox mice. For DSS treatment, LGR5-EGFP mice were administered 3% Dextran Sodium Sulfate (DSS) (MP Biomedicals) in the drinking water for 5 days, followed by plain water for 5 days. During the last three days of the plain water diet, mice were injected i.p. with DAPT according to the regimen described earlier. The entire length of the small intestine was harvested for RT-PCR and Western blotting analyses or snap frozen in O.C.T. cryo-sectioned, and stained by IF. Additionally, harvested single intestinal cells were subjected to FACS analysis using a Beckman Coulter flow cytometer. FlowJo software was used to analyze data and to gate populations according to 7-AAD viability, and forward and side scattering. Cutoff thresholds were provided by using unstained cells as a negative control. All experiments were performed in accordance with the ethical and care guidelines established by the Research Animal Resource Center of Weill Cornell Medical College followed the protocol (2009-0029). Additionally, all experimental protocols were approved by the Research Animal Resource Center of Weill Cornell Medical College.

Murine ISC Analysis. LGR5-EGFP+ ISCs from LGR5-EGFP and LGR5-EGFP-creER/POFUT-1floxflox mice were isolated using FACS analysis and cultured as organoids as previously described. In vitro studies, LGR5-EGFP organoids were seeded on chamber slides and treated with one of the following: 10μM DAPT (EMD Millipore) added to the media for 48 hours, or embedded in Matrigel containing 1μM JAG-1 (AnaSpec) for 48 hours followed by IF. LGR5-EGFP-creER/POFUT-1floxflox ISCs were treated with 500nM 4-hydroxytamoxifen (Sigma) added to the media for 48 hours to induce Cre recombinase followed by IF. Single ISCs were embedded in Matrigel overlaid with growth medium and incubated at 37°C for 16 hours before IF in pair-cell assays.

Pair-Cell Assay. Pair-cell analysis was performed as described. For pair-cell assays involving single mouse intestinal stem cells (ISCs), cells were embedded in Matrigel overlaid with growth medium and incubated at 37°C and 5% CO2 for 16 hours before fixation and IF. Specifically, single LGR5-EGFP ISCs were treated with one of the following: DMSO, 10μM DAPT (EMD Millipore) for 48 hours, or 1μM JAG-1 (AnaSpec) for 48 hours. ISCs were then fixed and stained for BMI1, LGR5 and TUBULIN expression to observe dividing pairs. In order to determine TUBULIN antibody specificity following a 16 hour pair cell assay, single ISCs were treated with 10μM colchicine (Santa Cruz) for 4 hours prior to fixation. In a separate pair cell assay, single
LGR5-EGFP ISCs were treated with 10 ng/ml TNF-α (R&D) dissolved in culture medium for 72 hours. TNF-α-treated ISCs were simultaneously treated with DMSO or DAPT during the last 48 hours as described above. Subsequently, ISCs were subjected to FACS analysis for BMI1 and LGR5 expression.

**Statistical Analysis.** The data displayed are represented as mean ± s.d. Statistical comparisons between two groups was made using Student t-test or one-way ANOVA for multiple groups. P < 0.05 was used to establish statistical significance.

**References**
1. Barker, N. Adult intestinal stem cells: critical drivers of epithelial homeostasis and regeneration. Nature reviews. Molecular cell biology 15, 19–33 (2014).
2. Snippert, H. J. et al. Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. Cell 143, 134–144 (2010).
3. Yan, K. S. et al. The intestinal stem cell markers Bmi1 and Lgr5 identify two functionally distinct populations. Proceedings of the National Academy of Sciences of the United States of America 109, 466–471 (2012).
4. Takeda, N. et al. Interconversion between intestinal stem cell populations in distinct niches. Science 334, 1420–1424 (2011).
5. Montgomery, R. K. et al. Mouse telomerase reverse transcriptase (hnTert) expression marks slowly cycling intestinal stem cells. Proceedings of the National Academy of Sciences of the United States of America 108, 179–184 (2011).
6. Powell, A. E. et al. The pan-ErbB negative regulator Lrig1 is an intestinal stem cell marker that functions as a tumor suppressor. Cell 149, 146–158 (2012).
7. Sangiorgi, E. & Capecchi, M. R. Bmi1 is expressed in vivo in intestinal stem cells. Nature genetics 40, 915–920 (2008).
8. Tian, H. et al. A reserve stem cell population in mouse intestine renders Lgr5-positive cells dispensable. Nature 478, 255–259 (2011).
9. Munoz, J. et al. The Lgr5 intestinal stem cell signature: robust expression of proposed quiescent ‘+’4’ cell markers. The EMBO journal (2012).
10. Fre, S. et al. Notch signals control the fate of immature progenitor cells in the intestine. Nature 435, 964–968 (2005).
11. VanDussen, K. L. et al. Notch signaling modulates proliferation and differentiation of intestinal crypt base columnar stem cells. Development 139, 488–497 (2012).
12. van Es, J. H. et al. Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. Nature 435, 959–963 (2005).
13. Sato, T. et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature 459, 262–265 (2009).
14. Guilmot, S. et al. Intestinal deletion of Pofut1 in the mouse inactivates notch signaling and causes enterocolitis. Gastroenterology 135, 849–860, e841–846 (2008).
15. Shi, S. & Stanley, P. Protein O-fucosyltransferase 1 is an essential component of Notch signaling pathways. Proceedings of the National Academy of Sciences of the United States of America 100, 5234–5239 (2003).
16. Stahl, M. et al. Roles of Pofut1 and O-fucose in mammalian Notch signaling. The Journal of biological chemistry 283, 13638–13651 (2008).
17. van der Flier, L. G. et al. LRH-1-mediated glucocorticoid synthesis in enterocytes protects against inflammatory bowel disease. Proceedings of the National Academy of Sciences of the United States of America 104, 13098–13103 (2007).
18. Oh, S. Y., Cho, K. A., Kang, J. L., Kim, K. H. & Woo, S. Y. Comparison of experimental mouse models of inflammatory bowel disease. International journal of medicinal chemistry 33, 333–340 (2014).
19. Lopez-Garcia, C., Klein, A. M., Simons, B. D. & Winton, D. J. Intestinal stem cell replacement follows a pattern of neutral drift. Science 330, 822–825 (2010).
20. Roth, S. et al. Paneth cells in intestinal homeostasis and tissue injury. PLoS one 7, e38965 (2012).
21. Sikandar, S. S. et al. NOTCH signaling is required for formation and self-renewal of tumor-initiating cells and for repression of secretory cell differentiation in colon cancer. Cancer research 70, 1469–1478 (2010).
22. Bulte, R. S. et al. Mammalian Par3 regulates progenitor cell asymmetric division via notch signaling in the developing neocortex. Neuron 63, 189–202 (2009).
23. Yui, N. et al. Basolateral targeting and microtubule-dependent transcytosis of the aquaporin-2 water channel. American journal of physiology. Cell physiology 304, C38–48 (2013).

**Acknowledgements**
This work was supported by NIH R01GM95990, NIH R01GM114254, NSF 1350659 career award, NSF 1137269, and NYSSTEM C029543.

**Author Contributions**
T.S., S.M.L. and X.S. conceived the concept, experimental design, and co-contributed to the manuscript. T.S. performed the experiments with the assistance of E.B.T. P.B., K.-I.T. and K.-Y.C. created LGR5-EGFP-CreERT2 × Rosa26-YFP-NICD mouse strain. L.A. contributed to the manuscript and provided LGR5-EGFP-CreER/POFUT-1<sup>fl</sup>/fl<sup>mouse</sup> mice.
Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Srinivasan, T. et al. Notch signalling regulates asymmetric division and interconversion between lgr5 and bmi1 expressing intestinal stem cells. Sci. Rep. 6, 26069; doi: 10.1038/srep26069 (2016).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/