TROP2 MARKS TRANSIENT GASTRIC FETAL EPITHELium AND ADULT REGENERATING CELLS AFTER EPITHELIAL DAMAGE

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**ABSTRACT**

Mouse fetal intestinal progenitors lining the epithelium prior to villogenesis grow as spheroids when cultured ex vivo and express the transmembrane glycoprotein Trop2 as a marker. In the present study, we report on the characterization of Trop2-expressing cells from fetal pre-glandular stomach, growing as immortal undifferentiated spheroids, and on their relation with gastric development and regeneration. Trop2⁺ve cells generating gastric spheroids differed from adult glandular Lgr5⁺ve stem cells, but appeared highly related to fetal intestinal spheroids. Although they shared a common spheroid signature, intestinal and gastric fetal spheroid-generating cells expressed organ-specific transcription factors and were committed to intestinal and glandular gastric differentiation, respectively. Trop2 expression was transient during glandular stomach development, being lost at the onset of gland formation, whereas it persisted in the squamous forestomach. Undetectable under homeostasis, Trop2 was strongly re-expressed in glands after acute Lgr5 stem cell ablation or following indomethacin-induced injury. These highly proliferative reactive adult Trop2-expressing cells exhibited a transcriptome displaying similarity with that of gastric embryonic Trop2⁺ve cells, suggesting that epithelium regeneration in adult stomach glands involves partial re-expression of a fetal genetic program.

Keywords: Lgr5/ embryonic/ spheroids/ indomethacin/ stomach/Tacstd2
INTRODUCTION

Adult epithelia lining the digestive tract rely on a panel of stem cells to self-renew and maintain tissue homeostasis following injury. In vivo lineage tracing and the development of methods allowing stable culture of “minigut organoids” ex vivo, have been instrumental in identification of the various stem cells in the small intestine (Barker et al., 2007; Sato et al., 2009) as well as in the corpus and antrum regions of the glandular stomach (Hoffmann, 2015). Under homeostatic conditions, actively cycling cells behaving as stem/progenitor cells are essentially located in the isthmus of the corpus glands. Their differentiation generates pit and neck cells secreting mucins, parietal cells generating hydrochloric acid, as well as chief cells and endocrine cells producing zymogens and hormones, respectively (Hoffmann, 2015). Cells expressing TFF2 transcripts, but not the corresponding protein, have been reported to behave as gland progenitors (Quante et al., 2010). Upon epithelial injury, corpus cells with chief characteristics expressing the Tumor necrosis factor receptor 19 (Tnfrsf19/Troy), can de-differentiate and function as reserve stem cells to repopulate the glands (Nam et al., 2010; Stange et al., 2013). In corpus and antral glands, Sox2 traces progenitors and adult stem cells (Arnold et al., 2011). In the antrum, actively cycling stem cells are present in the bottom of the glands and express the Leucine-rich repeat G protein-coupled receptor 5 (Lgr5). They give rise mainly to mucus-secreting and endocrine cells (Barker et al., 2010). Moreover, a pool of rare quiescent Villin-traced cells has been reported to be reactivated upon interferon gamma treatment, leading to repopulation of entire antral gland units, however their molecular signature remains unknown (Qiao et al., 2007).

In addition to its power for identification of adult stem cells from tissues as diverse as intestine, stomach, liver and pancreas (Barker et al., 2010; Huch et al., 2013a; Huch et al., 2013b; Sato et al., 2009), the three-dimensional culture system has recently been used to isolate and characterize epithelial progenitors of the small intestine in the fetus (Fordham et al., 2013; Mustata et al., 2013). In contrast to organoids with their lineage-specific differentiated cell types mimicking adult tissue, these cells grow ex vivo as poorly differentiated immortal hollow spheroids. They keep, however, the potential to convert into adult Lgr5-positive (Lgr5$^{+ve}$) intestinal stem cells both ex vivo and in grafting experiments after epithelial injury in vivo (Fordham et al., 2013; Mustata et al., 2013). These intestinal
progenitors are identified by their high expression levels of the cell surface molecule Trop2, also known as Tumour-associated calcium signal transducer 2 (Tacstd2). Initially discovered as a marker of invasive trophoblasts, Trop2 expression has also been reported in different organs during development and in adult stem cells during homeostasis as well as in regenerative conditions and cancer (McDougall et al., 2015; Shvartsur and Bonavida, 2015).

In the mouse stomach, primary specification of the epithelium occurs before embryonic day E11.5, preceding a secondary phase around E15, which leads to emergence of gastric units in the presumptive glandular region. In the forestomach, a squamous stratified epithelium develops, with characteristics similar to that of esophagus. We show here that Trop2 marks fetal glandular epithelial cells of the stomach, growing as spheroids when cultured ex vivo. In adults, upon injury, Trop2 expression is reactivated in regenerative cells together with part of a fetal-like genetic program.
RESULTS

Gastric Trop2-expressing fetal cells grow as immortal spheroids ex vivo

Fetal progenitors lining the intestinal epithelium before cytodifferentiation were previously identified as Trop2-expressing (Trop2\(^{+ve}\)) cells growing as undifferentiated spheroids when cultured in matrigel in presence of EGF, Noggin and Rspondin 1 (thereafter referred to as ENR culture conditions) (Mustata et al., 2013). In the present study, we explored whether similar cells could be cultured from the fetal stomach. Embryonic E14.5 stomachs were divided into two parts: the “proximal” region and the rest of the stomach referred to as the “distal” zone. Trop2\(^{+ve}\) cells were sorted, seeded in matrigel and cultured in the ENR medium. Trop2\(^{+ve}\) cells from the proximal region generated almost exclusively (98.5%) small round dark elements made of mono or multilayered keratin 14-expressing cells characteristic of squamous epithelium (type 1; Fig. 1A). In contrast, the “distal” samples generated a majority of clear spheroid-like structures, with a morphology reminiscent of fetal intestinal spheroids (49.8%, type 2), together with some organoid-like elements (24.4%, type 3; Fig. 1A) and type 1 squamous contaminants. These spheroids did not express squamous markers, nor the pit mucus marker HGM, which was readily detected in type 3 organoid-like structures, likely corresponding to elements engaged in differentiation (Fig. 1A). Next, spheroid formation capacity of epithelial (Epcam\(^{+ve}\)) cells from the pre-glandular region was studied over time. It was clearly correlated positively both with early developmental stages (E14.5-E15.5) and Trop2 expression (Fig. 1B). Of note, the drop in spheroid formation capacity observed between E15.5 and E17.5 implies some degree of heterogeneity among the Trop2\(^{+ve}\) population over time. Altogether, this indicated that spheroids were generated from poorly differentiated transient Trop2\(^{+ve}\) cells coming from the presumptive glandular region. Spheroids could be efficiently replated under ENR conditions for at least 30 passages (5 months), keeping their hollow spheroid morphology (Fig. 1C). Their growth properties differed from that of adult gastric organoids, the culture of which is optimized in a medium further supplemented with Fgf10, gastrin and Wnt3a (referred to as ENRFGW) (Barker et al., 2010; Stange, 2013). We confirmed that antral glands from adults generate only limited number of elements able to survive under ENR conditions (Fig. 1D).
Gastric spheroid transcriptome was compared to that of antral Lgr5-expressing stem cells by RNA-Seq analysis (Fig. 1E). Spheroids expressed lower levels of markers reportedly associated with adult gastric stem cells or with facultative stem cells (Lgr5, CD44, Tnfrsf19, TFF2, Cckbr) (Hoffmann, 2015). Since some of these markers are Wnt target genes, this suggests lower Wnt signaling activity in fetal spheroids as compared to adult stem cells. Spheroids also displayed lower expression of glandular differentiation markers for mucus neck, chief, or endocrine cells (Muc6, Pgc, Gif, Chga, Chgb, Sst and Gast). In contrast, they showed higher expression levels of reported intestinal progenitors markers (Trop2, Cnx43, Sftpd, Ly6a, Wfde2 or Tubb6) (Mustata et al., 2013), together with chymosin (Cym) described as a marker of immature stomach (Fig. 1E, Fig. S1A) (Chen et al., 2001). This expression profile was stable over at least 20 passages (Fig. S1B). Altogether, these data identified fetal gastric spheroids as self-renewing elements with a phenotype and growing properties clearly distinct from that of adult Lgr5 stem cells.

**Spheroid-generating cells are committed to a gastric glandular fate**

As fetal intestinal spheroids were previously found to express several genes belonging to the gastric differentiation program (Mustata et al., 2013), we compared the transcriptome of fetal gastric and intestinal spheroids. Principal component analysis (PCA) plot of all expressed genes showed that intestinal spheroids clustered with gastric spheroids rather than with intestinal organoids (Fig. 2A). By selecting transcripts 4 fold-upregulated in gastric and intestinal spheroids when compared to intestinal organoids, we defined a list of 692 commonly upregulated genes as the “fetal spheroid signature” (Fig. 2B). GO term analysis of this list revealed a significant correlation with processes related to tissue development, cell migration, adhesion, proliferation and cell differentiation (Fig. 2B, Table S1). Of note, despite the expected divergence between gastric E14.5 Trop2^+^ cells sorted from tissue and cultured spheroid cells (Fig. 2A), both cell types shared expression of embryonic markers, as compared to adult-derived cells (Fig. S2A). This indicated that the global gene expression profile of fetal gastric cells is maintained, at least partly in cultured spheroids.

Despite the high degree of similarity between gastric and intestinal spheroid transcriptomes, key transcription factors associated with patterning of the foregut (Sox2) and midgut (Cdx1) regions, as well as gastric and intestinal differentiation markers (Muc5ac and Muc2, respectively) were expressed...
in a tissue of origin-specific manner (Fig. 2C). Unexpectedly, the intestinal Cdx2 factor was also expressed, though at a lower level, in gastric spheroids (Fig. 2C; Fig. S2B). To investigate the differentiation potential of gastric spheroids ex vivo, spheroids serially passaged in ENR conditions, were plated in the adult-type ENRFGW culture medium. This led to significant upregulation of the adult stem cell marker Lgr5 and cell lineage differentiation markers of the stomach glands at the transcription level (Fig. 2D). Accordingly, morphologically differentiated mucous neck and pit and endocrine (GS-II⁺ve, HGM⁺ve, ChgA⁺ve) cells were observed, similar to those detected in adult-type organoids (Fig. 2E). Although Pgc transcripts were detected, mature chief cells could not be morphologically identified. Besides, shifting spheroids to ENRFGW did not lead to upregulation of the parietal marker ATP4a (Fig. 2D). Concomitantly, expression of the embryonic marker Trop2, detected at the membrane level in spheroids, decreased or disappeared in organoid-like structures emerging from spheroid-derived ENRFGW cultures (Fig. 2E). Of note, some morphologically differentiated cells still co-expressed Trop2, suggesting an ongoing differentiation process in these elements (Fig. S2C). Similar differentiation results were obtained in later passaged spheroids (Fig. S2D). Besides, no evidence for differentiation towards the intestinal or squamous epithelial types was observed in spheroids cultured in ENR medium (Fig. S2E). All over, these experiments indicated that, despite their expression of the intestinal Cdx2 transcription factor, Sox2⁺ve spheroids derived from the fetal stomach are clearly committed to a gastric glandular fate.

**Transient expression of the Trop2 and Cnx43 markers in pre-glandular epithelial cells**

Expression of the spheroid markers Trop2 and Cx43 identified ex vivo was studied during stomach development in the presumptive glandular region by immunofluorescence. At E14.5, Trop2 expression was detected in most epithelial cells (91.8 ± 2.2 %) (Fig. 3A). During gland formation (E15.5 to E18.5), a progressive decrease of Trop2⁺ve/Cnx43⁺ve cells was observed, manifesting mainly in the most basal cells of the developing glandular epithelium (Fig. 3A). This was accompanied, between E14.5 and E17.5, by concomitant increased proportions of proliferating cells displaying Trop2⁻ve/Cnx43⁺ve or Trop2⁻ve/Cnx43⁻ve phenotypes (from 5.4 ± 2.9 to 52.1 ± 1.4 % and from 3.4 ± 1.8 to 37.7 ± 7.0%, respectively). In the postnatal period (from P5 onward), Trop2 as well as Cnx43 were no longer detected in the epithelium, with Cnx43 labeling only present in the mesenchyme (Fig. 3A). This
Transient expression during stomach development was not observed for other epithelial markers such as the Trop2-related marker Epcam1, which was detected throughout the pre- to postnatal periods (E14.5 to P20 stages) (Fig. S3A). Together, these data indicate the existence of a transient population of Trop2+/Cnx43+ve cells in the fetal glandular epithelium during development. In contrast, in the forestomach, strong expression of Trop2 and Cnx43 was maintained in the different layers of the squamous epithelium throughout development and postnatally (Fig. S3B).

Since, the vast majority of epithelial Cnx43+ve cells (95.1 ± 2.6 %) co-expressed Trop2 at E14.5, the fate of Trop2+/Cnx43+ve embryonic cells was followed by lineage tracing using the Cnx43Cre-ER/Rosa26R-YFP mouse strain (Fig. 3B,C). One day following a tamoxifen pulse (1dpp) given at E14.5, YFP+ve cells were detected as sparse small clones in the squamous and presumptive glandular epithelia as well as in the corresponding mesenchymal compartments. Despite similar increase in the size of individual clones in both epithelial regions, at 3 dpp and, more so, at 28 dpp, the proportion of labeled cells contributing to glandular epithelium decreased significantly meanwhile it remained stable in the squamous epithelium (Fig. 3B,C; Fig. S3 B,C). Together with the observed concomitant increase in proliferating Cnx43-ve cells, this suggests that E14.5 Trop2+/Cnx43+ve cells contribute mainly to glandular formation during the fetal stages, with a definite but limited contribution to the postnatal epithelium resulting from dilution of the E14.5-traced cells by later progenitors unrelated to the Cnx43-Cre lineage.

Re-expression of the Trop2 marker in damaged adult stomach

Adult Lgr5+ve stem cells ensure constant renewal of the stomach epithelium under homeostatic conditions (Barker et al., 2010). To investigate the potential re-expression of embryonic markers in the absence of Lgr5+ve cells, we induced their specific ablation (Tian et al., 2011) by injecting Lgr5-DTR(EGFP) mice with diphtheria toxin (DT) (Fig. 4A). As early as 24 hours after the first injection (day2), DT treatment was associated with appearance of apoptotic cells and concomitant loss of Lgr5+ve cells in the bottom of the glands of Lgr5-DTR mice, indicating efficient ablation (Fig. S4A). Significant weight loss was observed in DT-treated heterozygous Lgr5-DTR mice (HE-T), but not in control animals [vehicle-treated heterozygous Lgr5-DTR (HE-NT) or DT-treated wild-type Lgr5-DTR (WT-T)] after the third day of treatment (Fig. S4B). Whereas the spheroid-associated marker Cnx43
was not induced in the epithelium of HE-T mice, strong membrane expression of the Trop2 marker was specifically detected in HE-T glands (Fig. S4C). Trop2⁺ve cells were detected as small clusters at day2 (Fig. 4B). At day3, a stage associated with an overall disorganization of the HE-T glands characterized by presence of many cystic structures, the number of Trop2⁺ve clusters had significantly increased (Fig. 4B,C). At day5, HE-T glands had recovered a normal architecture and contained a large number of Trop2⁺ve clusters, the size of which had further increased in both corpus and antral glands (Fig. 4B-D). Consistent with an ongoing regeneration process, the number of proliferating cells per gland was higher in HE-T as compared to controls, at all time-points investigated, this likely allowing to maintain constant the gland depth in damaged glands (Fig. 4E left panel, Fig. S4D). To test for potential involvement of Trop2⁺ve cells in this process, Ki67/Trop2 co-staining was performed. At day2, a high proliferation rate was observed in Trop2⁺ve cells and it further increased at later times (Fig. 4E right panel), indicating that the majority of Trop2⁺ve cells are actively cycling.

We also explored whether expression of the Trop2 marker may be induced in another type of injury, involving extended general tissue damage. For this purpose, adult mice were injected with indomethacin, an anti-inflammatory drug known to induce gastrointestinal ulcers in humans and in experimental animals (Anthony et al., 2000; Sun et al., 2015) (Fig. 4F). Histological examination of the stomach revealed presence of mucosal/submucosal lesions in all treated mice whereas none was found in vehicle-treated mice (Fig. 4F). A significant induction of Trop2 expression was detected in indomethacin-treated stomach, in both antral and corpus glands, with the number of Trop2⁺ve clusters appearing positively correlated with the extent of injury (Fig. 4F,G; Fig. S4E). Like in the Lgr5-DTR model of injury, a high proportion (32%) of Trop2⁺ve cells were in a proliferative state (Fig. 4H). These data suggest that re-expression of the Trop2 marker may be commonly associated to regeneration processes taking place in the stomach in response to epithelial injury.

**Cells at the origin of the reactive adult Trop2⁺ve cells**

In the absence of tools allowing direct tracing approaches, we attempted to identify the cells at the origin of Trop2⁺ve cells in the two experimental models of injury. In the Lgr5-DTR model of localized specific ablation of the stem cell population, the distribution of reactive Trop2⁺ve cells along the gland was first analyzed over time. Most cells were localized deep in the glands at day2, while they were
found throughout the glands at later stages, indicating that Trop2 is initially expressed by cells close to the stem cell zone (Fig. 5A). Just after damage induction (day2), rare cells were identified in HE-T glands that co-expressed Trop2 and ChgA or the mucus neck-binding GS-II lectin (Fig. 5A), suggesting that Trop2 expression can initially occur in some differentiating or terminally differentiated cells belonging to the endocrine or mucous cell lineages. In the indomethacin-induced injury model, leading to extended damages throughout the epithelium, Trop2 re-expression occurred in differentiated cells from the parietal and mucous cell lineages throughout the gland (Fig. 5B). Altogether, it is suggested that proliferating Trop2-expressing cells may originate from differentiated epithelial cells located close to the damaged area. Additional origin from another kind of yet uncharacterized cell type is not excluded.

**Characterization of reactive adult Trop2\(^{\text{\textbf{+ve}}}\) cells upon Lgr5 stem cell ablation**

We used the Lgr5-DTR injury model to isolate antral reactive adult Trop2\(^{\text{\textbf{+ve}}}\) cells and to perform RNA-Seq analyses (Fig. 6A). PCA on the whole transcriptome indicated that emerging Trop2\(^{\text{\textbf{+ve}}}\) cells exhibited a unique pattern as compared to adult Lgr5\(^{\text{\textbf{+ve}}}\) stem and fetal Trop2\(^{\text{\textbf{+ve}}}\) cells (Fig. 6B). Nevertheless, using the transcriptome of adult Lgr5\(^{\text{\textbf{+ve}}}\) cells as a common reference, 79 % (1922/2436) of the transcripts upregulated in reactive adult Trop2\(^{\text{\textbf{+ve}}}\) were commonly upregulated in fetal Trop2\(^{\text{\textbf{+ve}}}\) cells, indicating partial expression of overlapping genetic programs in regenerating adult and fetal-derived Trop2\(^{\text{\textbf{+ve}}}\) cells (Fig. 6C). Of relevance, 21.4% of the genes (148/692) constituting the “fetal spheroid signature” (defined in Fig. 2B and Table S1) were upregulated both in regenerating adult and fetal Trop2\(^{\text{\textbf{+ve}}}\) cells. GO term analysis of these common genes indicated high correlation with processes related to regulation of development, stem cell differentiation, cell migration and proliferation (Fig. 6C). Of note, reactive adult as well as fetal Trop2\(^{\text{\textbf{+ve}}}\) cells did not show any evidence for transcript enrichment in adult stem cell/progenitor markers as compared to Lgr5\(^{\text{\textbf{+ve}}}\) cells, including the Lgr5 gene itself (Fig. S5A, Table S2). Moreover, RNA-Seq analysis revealed differential expression of the Shh and Ihh ligands as well as Ereg and Areg ligands together with their cognate receptors, in adult and fetal Trop2\(^{\text{\textbf{+ve}}}\) cells as compared to Lgr5\(^{\text{\textbf{+ve}}}\) cells; this suggests potential involvement of these signaling pathways in reactive Trop2\(^{\text{\textbf{+ve}}}\) cells (Table S2). Overall, transcriptome
analysis indicated that adult Trop2$^{+ve}$ cells involved in epithelial regeneration express genes that are part of a fetal developmental program.

Finally, the ex vivo growth properties of regenerating cells were studied by seeding antral glands from Lgr5-DTR HE-T or controls in matrigel in ENR medium (Fig. 6D). At day6, as compared to controls, a majority (77.6 ± 4.6%) of HE-T elements was composed of Trop2$^{+ve}$ cells. At day12, spheroid-like surviving elements showed diameter statistically greater in HE-T versus control glands (Fig. 6D). Upon replating, HE-T samples also exhibited higher survival capacity than controls which, in agreement with Barker et al. (Barker et al., 2010) and our own observation (Fig. 1D), show limited capacity to grow in ENR medium (Fig. 6D). However, after replating, not all adult-derived elements kept the spheroid-like shape, some surviving elements evolved into organoid-like structures with protrusions (Fig. 6D). Despite maintaining Trop2 expression at levels similar to the fetal spheroids, adult-derived replated elements expressed pit and neck mucus differentiation markers (Fig. S5B). Together, these data indicated that, upon injury, emerging Trop2$^{+ve}$ cells show a growth advantage as compared to adult stem cells cultured under ENR conditions. Nonetheless, adult Trop2$^{+ve}$ cells do not exhibit a stable spheroid-like phenotype as the fetal ones, likely due to incomplete fetal re-expression program.
**Discussion**

In the present study, we have shown that Trop2 marks fetal gastric epithelial cells and is re-expressed, together with other fetal markers, in cells contributing to regeneration of the glandular stomach following epithelial injury.

We identified two kinds of Trop2^+ve^ cells in the fetal stomach generating two types of elements when grown ex vivo under the same culture conditions. Trop2^+ve^ cells isolated from the proximal stomach gave rise to circular multilayer organoids of squamous type, similar to those reported in human and mouse adult esophagus (Barbera et al., 2015; Jeong et al., 2015). In contrast, Trop2^+ve^ cells isolated from the pre-glandular epithelium generated hollow spheroids expressing low levels of gastric markers despite showing definite commitment to a gastric fate, as shown when cultured ex vivo. Whereas Trop2 expression persisted in the postnatal and adult periods in the squamous portion of the mouse stomach, Trop2^+ve^ cells were only transiently present in the fetal presumptive glandular stomach. Progressive loss of Trop2 coincided with the onset of gland formation and concomitant cell lineage differentiation, as well as with loss of spheroid-formation capacity ex vivo. A similar observation has been reported for the related Trop2^+ve^ intestinal progenitors isolated from E14.5-E16.5 fetus (Mustata et al., 2013). This supports the notion that the poorly differentiated spheroids grown from fetal stomach and intestine, represent frozen states of embryonic cells displaying commitment to their respective fate. Interestingly, despite the expected distortion of the transcriptome in Trop2^+ve^ cells induced by ex vivo culture, fetal Trop2^+ve^ cells and cultured Trop2^+ve^ spheroids share expression of embryonic markers and similarly express lower levels of Lgr5 as compared to adult glandular stem cells, which fits with the low Wnt tone reportedly associated with gastric development in vivo (Sherwood et al., 2011). In this regard, our immunofluorescence and lineage tracing experiments suggest that the glandular epithelium of the stomach may be generated in two waves. The first one, depending on early Cnx43^+ve^ embryonic epithelial cells, generates mainly the fetal pre-glandular epithelium, but contributes also to a proportion of the postnatal epithelium. A second wave, relying on later highly proliferative Cnx43^-ve^ progenitors, generates the bulk of the postnatal epithelium, leading to progressive dilution of the Cnx43-traced clones. Future studies will be needed to determine the
precise nature of these later progenitors, with Sox2- and Lgr5-expressing cells as potential candidates among others (Arnold et al., 2011; Barker et al., 2010). Recently, it has been reported that mature adult glandular stomach can be obtained from human iPS and mouse ES cells in a process of specification and maturation taking no less than 34 days (McCracken et al., 2014; Noguchi et al., 2015). The present study shows the possibility of converting undifferentiated spheroids obtained from E14.5 fetal stomach into organoids containing the differentiated cell types of the adult glands. The convenience of the isolation and maintenance procedures to culture Trop2\(+\) fetal cells represents an alternative ex vivo system to further dissect the mechanisms involved in primary to secondary transition stages occurring in the glandular stomach.

In adults, the regeneration capacity of a tissue has been correlated to the presence of undifferentiated stems cells or to the plasticity of differentiated cells. In lower vertebrates, differentiated cells can revert to a fetal-like stage with high proliferative capacity to repair the damaged tissue. Thereafter, re-differentiation into the adult cell types allows to restore full tissue functionality (Singh et al., 2015). Lineage tracing approaches in the mouse have revealed that a similar process of de-differentiation can occur during epithelium repair in mammals. Committed secretory cells can acquire a stem-like state upon airway stem cell ablation in lungs or sub-lethal irradiation in the intestine (Tata et al., 2013; van Es et al., 2012). However, the transcriptome of the de-differentiated cells was not investigated. In the present study, we provide evidences that a similar process takes place in the stomach following Lgr5 stem cell ablation or an acute injury secondary to indomethacin administration. Within 24 hours after initiation of the damage, some differentiated cells started to express Trop2 and the majority of reactive Trop2\(+\) cells entered the cell cycle. Contribution of other cell types to the origin of Trop2-expressing cells is presently not formally excluded. Among potential candidates are Tnfrsf19/Troy\(+\) cells, previously described as a corpus reserve stem cell pool in proliferation-depleted glands (Stange et al., 2013). Their involvement seems however unlikely since the kinetics of Troy cell-dependent regeneration was reportedly much slower, taking place after one week only. Besides, contrary to Troy\(+\) cells, reactive Trop2\(+\) cells are prevalent in the antrum and show lower levels of Troy expression as compared to Lgr5 stem cells.
Our RNA-Seq data demonstrated that re-expression of the Trop2 marker in adults is the hallmark for a global change in the expression profile, shifting towards a fetal-like expression pattern, similar to that of fetal Trop2\(^{+ve}\) or spheroid-generating cells. This is characterized by a robust induction of the whole proliferation machinery, coherent with high proportion of Trop2\(^{+ve}\) cells entering the cell cycle and expression of genes associated with tissue morphogenesis and organ development. Comparison of the signaling pathways between reactive adult and fetal Trop2-expressing cells and resident Lgr5 stem cells revealed active involvement of the Hedgehog pathway, well known in lower vertebrates to contribute to tissue regeneration (Singh et al., 2015). Moreover, the Areg/Ereg cascades, reported to participate to epithelial regeneration, may also take part to cell signaling in adult Trop2\(^{+ve}\) cells contrary to Lgr5\(^{+ve}\) stem cells (Liu et al., 2012; Nagai et al., 2014).

Trop2 re-expression has also been reported in other regeneration processes in adult mice: in the liver, upon Diethoxycarbonyl DihydroCollidine (DDC) diet injury and in the prostate, where Trop2\(^{+ve}\) cells can regenerate prostatic tubules in vivo (Goldstein et al., 2008; Okabe et al., 2009). Regarding the fate of the activated Trop2\(^{+ve}\) cells, it has been shown in the DDC model that the clonally expanded regenerating cells have the capacity to differentiate into hepatocytes and cholangiocytes (Okabe et al., 2009). In the present study, the toxicity observed in DT-treated Lgr5-DTR heterozygous mice and the lack of tools allowing long term tracing of Trop2\(^{+ve}\) cells did not permit to fully explore their differentiating capacity. However, the observation that ex vivo cultured adult Lgr5-depleted glands exhibited signs of differentiation into adult epithelial lineages suggests that, in vivo, regenerating Trop2\(^{+ve}\) cells have the capacity to re-differentiate into the adult cell lineages after tissue repair.

Although the function of Trop2 is still not clear, it is overexpressed in many cancers where it plays a role in regulating cell growth and migration (Fornaro et al., 1995; McDougall et al., 2015; Shvartsur and Bonavida, 2015). In the present study, the Trop2-expressing cells emerging along the gastric glands after stem cell ablation are highly proliferative and mainly undifferentiated showing re-acquisition of fetal characteristics. Future studies will be needed to determine whether Trop2 must be considered as a simple marker of embryonic and regenerating cells or plays a functional role in gastric epithelial morphogenesis during development and in adults during regeneration.
**Materials and Methods**

**Mice**

Animal procedures complied with the guidelines of the European Union and protocols were approved by the local ethics committee. Mice strains were: CD1 (Charles Rivers, France); Cnx43-KI-Cre-ER(T) (EMMA); Rosa26R-YFP, Rosa26R-Tomato. *Lgr5-DTR-EGFP* knock-in mice were kindly provided by Genentech (Tian et al., 2011). The day the vaginal plug was observed was considered as embryonic day 0.5 (E0.5).

For lineage tracing experiments, tamoxifen (Sigma-Aldrich) was dissolved in sunflower oil (Sigma-Aldrich)/ethanol mixture (9:1) at 10 mg/ml. Pregnant females were injected intraperitoneally at a dose of 0.1 mg/g of body weight. For specific cell ablation over Lgr5-DTR mice, diphtheria toxin (Sigma-Aldrich) was injected intraperitoneally (50 µg/kg) to 8 weeks-old mice. Control mice were injected with sterile phosphate buffer saline solution. Eight weeks-old CD1 mice were injected subcutaneously with 300 µg/kg of indomethacin (dissolved in DMSO and 5% NaHCO₃ buffer) or with the buffer as control.

**Histology and immunostainings**

Dissected stomachs or ex vivo cultured spheroids and organoids were fixed with 10% formalin solution neutral buffered (Sigma-Aldrich) and sedimented through 30% sucrose solution before OCT embedding. Histological protocols and immunofluorescence/histochemistry experiments were carried out as previously described (Garcia et al., 2009). Primary antibodies are reported in supplementary material. The Alexa Fluor 647 conjugated GS-II lectin was obtained from Molecular Probes (L-32451) and the in situ cell death Tunel detection kit from Roche. Samples were visualized with Zeiss Axioplan 2 or Zeiss Observer Z1 microscope. Quantifications are detailed in supplementary material.
Flow cytometric analysis and cell sorting (FACS)

Embryonic stomach samples or adults Lgr5-DTR antral glands isolated as previously described (Barker et al., 2010), were dissociated with the Stem Pro accutase cell dissociation reagent (Thermo electron) and passed through a 40-μm nylon cell strainer (Greiner).

Fluorochrome-conjugated antibodies or relevant isotype controls were used for staining in PBS-2% BSA-2mM EDTA for 45 min on ice and sorted by using the Facs Aria I (BD Biosciences). For spheroid formation efficiency measurements, sorted cells were cultured ex vivo under ENR conditions. Quantification methods are detailed in supplementary material. For RNA-Seq analysis, Lgr5-DTR-EGFP^{+ve} cells were directly sorted using the FITC channel. Sorted cells were directly collected for RNA-Seq analysis over Qiazol lysis reagent (Qiagen). For adult Trop2^{+ve} and Lgr5-GFP^{+ve} sorting, cells were pooled (2 pools of 2 HE-T samples, 1 pool of 4 HE-NT samples) to extract RNA from total 4,000-8,000 cells. For fetal Trop2^{+ve} cells, a mean of 30,000 cells were sorted in each independent experiment.

Ex vivo culture

Embryos’ stomach and small intestine were dissociated as reported (Mustata et al., 2013) and cultured according to the protocol reported (Sato et al., 2009). Specifically, the culture medium used for growing gastric spheroids (ENR) consisted in a basal medium [Advanced-DMEM/F12 supplemented with 2 mM L-Glutamine, N2 and B27 w/o vit.A (Invitrogen), gentamycin, penicillin-streptomycin cocktail, 10 mM HEPES, and 1 mM N acetyl cysteine] supplemented with growth factors at a final concentration of: 50 ng/ml EGF and 100 ng/ml Noggin (both from Peprotech), and 100 ng/ml CHO-derived R-spondin1 (R&D System). Culture medium was changed each other day and after 5-6 days in culture, spheroids were harvested, mechanically dissociated and replated in fresh Matrigel (BD). Fetal gastric spheroids obtention as well as repeated replatings were done over six independent culture experiments, starting from either individual embryos or pools of embryos.
Adult antral glands from Lgr5-DTR mice were isolated and cultured as reported (Barker et al., 2010). Culture medium used for adult-derived gastric organoids growth (ENRFGW) was basal medium (see above) supplemented with growth factors at a final concentration of: 50 ng/ml EGF and 100 ng/ml Noggin; 200 ng/ml CHO-derived R-spondin1; and 100 ng/ml Fgf10, 100 ng/ml Wnt3a (R&D system), 10 nM Gastrin (Sigma-Aldrich).

Media were supplemented with 10 µM Y-27632 (Sigma-Aldrich) in all initial seeding and replating experiments. Pictures were acquired with a Moticam Pro camera connected to Motic AE31 microscope or with Leica DFC 420C camera using the Leica Application Suite V3.8 software.

Gene expression analysis

qRT-PCR was performed on total RNA as reported (Garcia et al., 2009). Expression levels were normalized to that of the reference genes (RPL13, GAPDH). Gastric and intestinal spheroid samples were obtained from independent pools of E15-16 embryos, while organoid samples were obtained from E16 embryos and adult mice. Each sample was run in duplicate. Primer sequences are reported in supplementary data.

RNA seq and transcriptome analysis

RNA from spheroids/organoids and from sorted cells were extracted with the miRNA isolation kit (Ambion, Life Technologies) and with the miRNeasy Micro Kit (Qiagen), respectively. RNA quality was checked by Bioanalyzer (Agilent). Indexed cDNA libraries were prepared using the TruSeq stranded mRNA sample preparation kit (Illumina) and the Ovation single cell RNAseq system (NuGEN) for RNA extracted from spheroids/organoids and sorted cells, respectively. RNA-Seq and transcriptome analysis methods are further detailed in supplementary material. Transcript profiling: GEO accession number is GSE65395.
**Statistical analysis**

Statistical analyses were performed with Graph Pad Prism 6. All experimental data are expressed as mean±SEM. The significance of differences between groups was determined by the appropriate tests as described in figure legends.

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**Author contributions:**

VFV, ML: study concept and design, acquisition of data, analysis and interpretation of data, statistical analysis, drafting of the ms

SS, GVasile, AL, FL: acquisition of data, statistical analysis

GVassart: study concept and design, critical revision of the ms, obtained funding, study supervision

MIG: study concept and design, acquisition of data, analysis and interpretation of data, drafting of the ms, study supervision

**Conflict of interest:** The authors have nothing to disclose
**Figure 1.** Gastric Trop2-expressing fetal cells grow as immortal spheroids ex vivo. (A) Spheroid-generating cells originate from pre-glandular stomach. **Left:** Sorted Trop2$^{+ve}$ cells from E14.5 proximal and distal stomach regions were cultured in ENR (EGF, Noggin, R-spondin1) conditions for 10 days. Scale bars: 1mm. **Right:** Immunofluorescence for type 1-3 elements showing Krt14 (keratin 14) expression.
HGM (mucins) and Trop2 expression. Arrows indicate stratified cells. Scale bars: 20 μm. The proportion of grown elements subdivided into 3 types is graphed (mean±SEM) (n=2 independent sorting experiments). (B) Spheroid formation efficiency among Epcam⁺ve cells isolated from “distal” stomach depends on embryonic stage and Trop2 expression (n=2 independent sorting experiments). None of the Epcam⁻ve cells generated spheroids. (C) Representative pictures of fetal stomach spheroids from 3 different pools over passages. Scale bars: 200 μm. (D) Representative pictures from initial seeding of adult antral glands under ENR and ENRFGW (FGW for Fgf10, gastrin, Wnt3a) conditions. The same field was followed over time; arrow shows a differentiating organoid element. Scale bars: 200 μm. Bottom, percentage of surviving elements over time (n=4 mice). Two-way ANOVA (p<0.0001 for medium/time effect), Bonferroni (a,b<0.0001). (E) Transcriptome analysis of gastric fetal spheroids (3 independent pools) and sorted adult Lgr5-positive stem cells (4 mice pooled) represented as scatter plot of the log₂ mean. Rpm: reads per million. Genes representing embryonic, adult stem/progenitor or glandular differentiation markers are highlighted.
Figure 2. Spheroid-generating cells are committed to a gastric glandular fate. (A) PCA plot of transcriptome (19,468 genes) from cultured stomach (Sto-Sph) and intestinal (SI-Sph) spheroids, intestinal organoids (SI-Org) and sorted stomach and intestinal Trop2+ve cells (Sto-Trop2+ve and SI-Trop+ve). (B) Venn diagram showing overlap between 4-fold upregulated genes (number of genes in brackets) in stomach and intestinal spheroids (both versus intestinal organoids). Chi-square with
Yates’ correction. GO term analysis for the common “fetal spheroid signature”. (C) **Left**: scatter plot of gastric and intestinal fetal spheroids transcriptomes (3 independent pools), represented as the log2 mean. Rpm: reads per million. Genes representing gastric and intestinal commitment are highlighted; **right**: Sox2 and Cdx2 mRNA expression levels measured by qRT-PCR in stomach spheroids (Sto Sph; n=6), stomach organoids (Sto Org; n=5), small intestine spheroids (SI Sph; n=4) and small intestine organoids (SI Org; n=4). *below the mean value of 0.05. (D) Stomach spheroid cells can differentiate in ENRFGW medium. qRT-PCR of selected gastric differentiation genes in Sto Sph at day6 and day12 (n=4), using Sto Org (n=3) obtained at day12 as positive differentiation controls. Mucin 6 (Muc6), Pepsinogen C (PgC), Chromogranin A (ChgA) and proton pump (ATP4a) are markers of mucus neck, chief, endocrine and parietal cells; respectively. Two-way ANOVA (p=0.0018 for media effect over Sto Sph). (E) Immunofluorescence showing low and high magnification for Trop2, or cell differentiation markers (HGM, GSII and ChgA), over spheroids cultured under ENR or ENRFGW conditions with stomach organoids used as positive controls. Arrows show differentiated cells. Scale bars: 20 µm.
Figure 3. Transient expression of Trop2 and Cnx43 markers in pre-glandular epithelial cells. (A) Left: Immunofluorescence showing expression of Trop2 and Cnx43 during development. Arrows: point to absence of Trop2 expression at the bottom of the glands where positive punctuated staining of Cnx43 is still present. Right: Graphs showing the proportion of apical and basal cells expressing...
Trop2 (upper panel) and the proportion of cells expressing embryonic markers in the most basal cells (lower panel). N=3 embryos per time point. Two-way ANOVA (*p<0.05, ** p<0.01*** p<0.001, **** p<0.0001). (B) Representative pictures for lineage tracing experiments in Cnx43-CreER/Rosa26-YFP mice. Low and high magnification of squamous (Sq) and glandular (Gl) stomach, tamoxifen administrated at E14.5 and analyzed 1, 3 and 28 days post pulse (dpp). Duo: duodenum. Dotted lines evidence epithelial/mesenchymal boundaries. Scale bars: panel A: 20 µm, panel B: 500 and 20 µm (low and high magnification, respectively). (C) Relative proportion of Cnx43-traced clone area per total Gl or Sq epithelial area. Each dot corresponds to a single embryo/mouse. Non-parametric one-way ANOVA (* p<0.05).
Figure 4. Re-expression of the Trop2 marker in damaged adult stomach. (A) Design of the experiment for Lgr5⁺ve cells ablation in Lgr5-DTR mice with diphtheria toxin (DT), EP: endpoint. (B) Immunohistochemistry of antral gland sections showing Trop2 expression after DT treatment of wild-
type (WT-T) or heterozygous Lgr5-DTR (HE-T) mice. Scale bars: 50 µm, insets: 20 µm. (C) Quantification of Trop2
tive clusters per field in HE-T and control (C) animals. Individual C (non-treated heterozygous Lgr5-DTR HE-NT and WT-T) and HE-T mice are represented by green circles and red squares, respectively. Unpaired t-test with Welch’s correction (antrum: *p=0.023, **p=0.002; corpus: *p=0.017, **p=0.007). (D) Quantification of the number of Trop2
tive cells/cluster over time. Mice: n=3 at day2; n=4 at day3 and day5. Two-way ANOVA (**p=0.0008). (E) Left: quantification of total number of Ki67
tive cells per antral gland. Mice: n=3 at any time point for C; n=3, 4 and 7 for HE-T at day2, day3 and day5, respectively. Two-way ANOVA (p<0.0001 for treatment effect), Bonferroni (*p=0.03; **p=0.004). Right: immunofluorescence showing co-expression of Ki67 in Trop2
tive cells in HE-T glands. Inset: percentage of Trop2
tive proliferating cells (n= 3 and 4 at day2 and day5, respectively). Scale bars: 20 µm. (F) Left: experimental scheme for in vivo treatment with indomethacin (Ind), EP=endpoint; right: representative immunohistochemistry showing Trop2 expression in the vicinity of Ind-induced lesions. Scale bars: 200 µm (low), and 100 µm (high magnification). (G) Quantification of Trop2
tive clusters/10 fields in Ind-treated and vehicle-treated control (C) animals. Non-parametric Mann Whitney (antrum and corpus, *p=0.0159). (H) Representative immunofluorescence showing co-expression of Ki67 and Trop2
tive cells in glands of indomethacin-treated mice. Inset: percentage of Trop2
tive proliferating cells (n=2). Scale bars: 20 µm.
Figure 5. Cells at the origin of the reactive adult Trop2$^{+ve}$ cells. (A) Spatial distribution of Trop2$^{+ve}$ cells after Lgr5$^{+ve}$ cell ablation along antral glands (subdivided into top, middle and bottom zones as depicted). Mice: n=3 at day2 and 3, n=4 at day5. **Bottom:** Immunofluorescence showing co-expression of differentiation markers in Trop2$^{+ve}$ cells of HE-T glands at day2. Arrows evidence the double-positive cells. Dotted lines show gland limits and double-positive cells. (B) Immunofluorescence showing co-expression of differentiation markers in Trop2$^{+ve}$ cells from indomethacin-treated mice. Arrows show double-positive cells. Scale bars for all panels: 20 µm.
Figure 6. Characterization of reactive adult Trop2^{+ve} cells following Lgr5 stem cell ablation. (A) Trop2^{+ve} cells isolation by FACS after diphtheria toxin (DT) treatment of Lgr5-DTR mice. Left: experimental scheme. Right: representative FACS plots. Percentages of Lgr5^{+ve} (green circle) or Trop2^{+ve} (red circle) cells per gate are shown as mean±SEM for treated-wild type (WT-T; n=1), non treated-heterozygous (HE-NT; n=4) and treated-heterozygous (HE-T; n=4). (B) PCA plot of the whole transcriptome (19,468 genes) from sorted adult and fetal cells and fetal-derived spheroids. See legends.
of Fig. 2A. (C) **Top:** Venn diagram showing overlap between 4 fold-upregulated genes (number of genes in brackets) in reactive adult and fetal Trop2$^{+ve}$ cells versus Lgr5$^{+ve}$ cells, with the fetal spheroid signature defined in Fig. 2B. **Bottom:** GO term analysis for the genes common to the 3 lists. (D) Ex vivo culture of Lgr5-DTR antral glands after in vivo DT treatment. Control (C) mice are WT-T and HE-NT. Individual C and HE-T mice are represented by green circles and red squares, respectively. **Top:** experimental design (EP=endpoint). **Bottom left:** representative pictures of immunofluorescence showing Trop2-expression at day6 and quantification as percentage of growing elements. Unpaired t-test, p=0.0001. Scale bars: 20 µm. **Bottom center:** representative pictures of growing elements at day12 after initial seeding of antral glands. Arrows point to surviving elements. Quantification of the mean diameter of elements/animal is shown. Unpaired t-test, p=0.0017. Scale bars: 1 mm. **Bottom right:** survival of replated elements at passage 1 day9. Each square corresponds to an individual mouse. Empty and filled squares represent no growth and growth, respectively. Fisher’s exact test. Representative pictures of elements grown from HE-T glands. Scale bars: 100 µm.


