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Identification of Stem Cells in the Epithelium of the Stomach Corpus and Antrum of Mice

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BACKGROUND & AIMS: Little is known about the mechanisms of gastric carcinogenesis, partly because it has been a challenge to identify characterize gastric stem cells. Runx genes regulate development and their products are transcription factors associated with cancer development. A Runx1 enhancer element, eR1, is a marker of hematopoietic stem cells. We studied expression from eR1 in the stomach and the roles of gastric stem cells in gastric carcinogenesis in transgenic mice. **METHODS:** We used in situ hybridization and immunofluorescence analyses to study expression of Runx1 in gastric tissues from C57BL/6 (control) mice. We then created mice that expressed enhanced green fluorescent protein (EGFP) or CreERT2 under the control of eR1 (eR1-CreERT2;Rosa-Lox-Stop-Lox [LSL]-tdTomato, eR1-CreERT2;Rosa-LSL-EYFP mice). Gastric tissues were collected and lineage-tracing experiments were performed. Gastric organoids were cultured from eR1-CreERT2(5-2);Rosa-LSL-tdTomato mice and immunofluorescence analyses were performed. We investigated the effects of expressing oncogenic mutations in stem cells under control of eR1 using eR1-CreERT2;LSL-KrasG12D/+ mice; gastric tissues were collected and analyzed by histology and immunofluorescence. **RESULTS:** Most proliferation occurred in the isthmus; 86% of proliferating cells were RUNX1-positive and 76% were MUC5AC-positive. In eR1-EGFP mice, EGFP signals were detected mainly in the upper part of the gastric unit, and 83% of EGFP-positive cells were located in the isthmus/pit region. We found that eR1 marked undifferentiated stem cells in the isthmus and a smaller number of terminally differentiated chief cells at the base. eR1 also marked cells in the pyloric gland in the antrum. Lineage-tracing experiments demonstrated that stem cells in the isthmus and antrum continuously gave rise to mature cells to maintain the gastric unit. eR1-positive cells in the isthmus and pyloric gland generated organoid cultures in vitro. In eR1-CreERT2;LSL-Kras G12D/+ mice, MUC5AC-positive cells rapidly differentiated from stem cells in the isthmus, resulting in distinct metaplastic lesions similar to that observed in human gastric atrophy. **CONCLUSIONS:** Using lineage-tracing experiments in mice, we found that a Runx1 enhancer element, eR1, promotes its expression in the isthmus stem cells of stomach corpus as well as pyloric gland in the antrum. We were able to use eR1 to express oncogenic mutations in gastric stem cells, proving a new model for studies of gastric carcinogenesis.

Keywords: Stomach Cancer; Mouse Model; Cancer Stem Cell; Oncogene.

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Understanding the cells of origin in cancer is important for the development of effective strategies to detect, prevent, and treat cancer. Tissue stem cells are generally studied as candidate cells from which cancer originates. Adult stem cells with a minimal differentiation phenotype[1] and fully differentiated cells that have acquired the capacity to replicate and differentiate have been described.[2–4] Furthermore, recent developments in stem cell research revealed multiple types of stem cells identified by different markers in the same tissues.[5–7]

Gastric cancer is the second most frequent cause of cancer death in the world.[8] However, until recently, molecular understanding of gastric carcinogenesis has been hampered by the paucity of studies concerning stem cells in the stomach epithelium. Anatomically, the stomach is roughly divided into the distal part (antrum) and the main body (corpus).[9] In the antrum, the upper three-quarters of the pyloric gland contain Muc5ac-producing cells, while the

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Abbreviations used in this paper: EGFP, enhanced green fluorescent protein; EYFP, enhanced yellow fluorescent protein; LSL, Lox-Stop-Lox; MAPK, mitogen-activated protein kinases; TG, transgenic.

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bottom portion contains rapidly growing stem/progenitor cells. By contrast, the gastric unit in the corpus contains Muc5ac-producing surface epithelial cells (pit cells). These cells are rapidly turned over and their half-life is about 3 days. Below this is a small region called the isthmus, where corpus stem cells are presumed to be present based on radiolabeling and morphologic studies using electron microscopy. Below the isthmus there is a neck region where GS-II$^+$ mucous neck cells and acid-producing parietal cells are located. Pepsinogen C–producing zymogenic cells, called chief cells, are present at the base.[10] The half-life of parietal cells and chief cells is several months (see diagram in Supplementary Figure 1*A*).[9,11]

Runx genes are developmental regulators and their products function as transcription factors and are often involved in cancer. There are 3 Runx genes in mammals, namely, Runx1, Runx2, and Runx3.[12] Runx1 is a key regulator of hematopoiesis, is required for the generation of hematopoietic stem cells from endothelial cells,[13] and is also critical for the maintenance of hematopoietic stem cells.[14,15] Anomaly of RUNX1 is widely involved in leukemia.[16] The noncoding region between the P1 and P2 promoters of Runx1 is considered to be composed of multiple cis-regulatory elements in a mosaic fashion. One such element (approximately 270 bp) was identified as an enhancer of Runx1 expression in hematopoietic stem cells and termed the *+24 mouse conserved noncoding element* (referred to as Runx1 enhancer element [eR1] in this study).[17,18] More recently, Runx1 was also demonstrated to have stem cell functions in skin.[19] The many recent reports regarding Runx1 expression in epithelial cells of several organs[20–23] prompted us to study Runx1 expression and eR1 activity in various tissues, and we found that eR1 marked tissue stem cells of the stomach corpus and antrum.

Materials and Methods

Detailed Materials and Methods are described in Supplementary Materials.

Mice and Treatment

Transgenic mice expressing eR1-enhanced green fluorescent protein (EGFP) were described previously.[17] Transgenic mice expressing eR1 and tamoxifen-inducible CreERT2 were generated using the eR1 sequence, the mouse heat-shock protein 68 basal promoter sequence, and the CreERT2 transgene following a strategy similar to that described previously.[17] The generation and details of other mice are described in the Supplementary Material. All animals were handled in strict accordance with good animal practice as defined by the Institution of Animal Care and Use Committee. All animal work was approved by the Institution of Animal Care and Use Committee and the Office of Safety, Health, and Environment at the National University of Singapore.

To induce gene expression, 3- and 6-week-old, eR1-CreERT2;Rosa-Lox-Stop-Lox (LSL)-tdTomato (Rosa-tdTomato), eR1-CreERT2;Rosa-LSL-enhanced yellow fluorescent protein (EYFP) (Rosa-EYFP), eR1-CreERT2:LSL-K-ras$^{G12D/+}$, and eR1-CreERT2:Rosa-tdTomato:LSL-K-ras$^{G12D/+}$ mice were injected with 2, 4, or 6 mg tamoxifen. Mice were analyzed 1 day, 1 week, 3 months, 6 months, and 1 year after treatment. LSL-K-ras^{G12D} will be refer to as K-ras^{G12D} in this article.

Results

Runx1 Expression in Mouse Stomach Epithelial Cells

In situ hybridization with a Runx1 anti-sense probe detected Runx1 messenger RNA expression in the upper and lower parts of the corpus gastric unit (Figure 1*A*). Immunofluorescence staining using an anti-Runx1 antibody showed that Runx1 was expressed in E-cadherin$^+$ epithelial cells in the upper part of the mouse stomach corpus (Figure 1*B*), and was more infrequently expressed at the base (Figure 1*C*, the histologic locations of various parts of the stomach are illustrated in Supplementary Figure 1*A*). CD45$^+$ immune cells expressed higher levels of Runx1 than epithelial cells (Figure 1*B*, *arrows*). The expression level of Runx1 protein relative to that of Runx1 messenger RNA is much lower in the lower part of the epithelium. This may be due to the difference in translation rate of Runx1 protein in the lower part of the epithelium. The differential expression of mRNA and protein was also described for Trefoil factor 2.[24]

The majority of Ki67 labeling was located in the isthmus, and 86% co-localized with Runx1$^+$ cells (Supplementary Figure 1*B* and *G*). Subfractions of Muc5ac$^+$ cells (Figure 1*D*) and GS-II$^+$ neck cells (Figure 1*E*) were Runx1$^+$. In total, 76% of Ki67$^+$ cells expressed Muc5ac (Supplementary Figure 1*C* and *G*), 14% of Ki67$^+$ cells were GS-II$^+$ (Supplementary Figure 1*D* and *G*), and 3.5% of Ki67$^+$ cells expressed HK-ATPase (Supplementary Figure 1*E* and *G*). Some Ki67$^+$ cells at the base were also positive for pepsinogen C, but these constituted only 0.58% of the total Ki67$^+$ cell population (Supplementary Figure 1*F* and *G*).

In addition to the corpus, Runx1 was also expressed in epithelial cells at and near the bottom of the pyloric gland (Supplementary Figure 1*H*). Well-characterized stem cell marker Lgr5 was expressed at the bottom (Supplementary Figure 1*I*).[23]

Identification of eR1-Enhanced Green Fluorescent Protein$^+$ Cells in the Corpus

A pilot investigation suggested that eR1 activity is also detected in stomach corpus epithelial cells. We examined EGFP$^+$ cells in the corpus epithelium of transgenic mice harboring eR1-EGFP (see experimental design in Figure 2*A*). EGFP signals were mainly detected in the upper part of the gastric unit (Figure 2*B*), and EGFP$^+$ cells also expressed E-cadherin (Figure 2*C* and *D*). EGFP$^+$ cells were found in 43% of gastric units and negative ones in 57% (Supplementary Figure 2*A*). In accordance with a previous report, eR1-EGFP signals were not detected in CD45$^+$ differentiated blood cells (Supplementary Figure 2*B*), and some signals were observed in the stroma and muscle layer of the corpus (Figure 2*B*).[17] In total, 83%, 7%, and 10% of EGFP$^+$ cells were located in the isthmus/pit region, neck, and base, respectively (Supplementary Figure 2*C*).