Cancer Stem Cells of the Digestive System

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INTRODUCTION

Cancers of the digestive system are as a group the largest cause of cancer-related mortality in Japan. Surgery is the mainstay of curative treatment for most tumours of the digestive system. Taking colorectal cancer as an example, it is associated with more favourable prognosis among cancers of the digestive system, but the overall 5-year survival of 55% highlights the need for novel strategies in the treatment of cancers of the digestive system as a whole. One of the major breakthroughs in the recent decades is the growing evidence supporting the existence of cancer stem cells, including cancers of the digestive system, providing promise of a realistic target for effective oncological treatment in the future.

An important notion of the cancer stem cell theory is that similar to any proliferative tissues in adults, the growth of tumours is sustained solely by small numbers of stem cells that are responsible for self-renewal. Following this logic, tumours like other normal adult organs are thought to predominantly consist of rapidly proliferating cells and post-mitotic differentiated cells that are not capable of self-renewal. In other words, the eradication of cancer stem cells should be necessary and at the same time sufficient in order to halt the progression of cancer.

An important observation of cancer stem cells is that they are more resistant to radiation and chemotherapeutic agents when compared with other cancer cells. Cancer stem cells surviving the existing cancer therapies may explain the frequent recurrences of cancers at a later date, even when there

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has been good response to radiation or chemotherapy at first. Supporting the argument that cancer stem cells are responsible for cancer recurrence are observations that colorectal cancers with high expression of stem cell markers are associated with increased rates of recurrence (7,8) as well as poorer prognosis (9). Therefore, if the emphasis of cancer treatment is against the self-renewable cells, this may lead to successful cure of cancer (6,10,11).

In this review, we have summarized our current understanding of the cancer stem cell biology of the digestive system and how this knowledge could be used in the development of novel strategies for treating cancers in the future.

IDENTIFICATION AND THE ORIGIN OF CANCER STEM CELLS OF THE DIGESTIVE SYSTEM

STEM CELLS OF THE GASTROINTESTINAL TRACT

The existing knowledge of the normal gastrointestinal stem cells have helped a great deal in the research of cancer stem cells of the digestive system, given many shared characteristics between the two (12). The stem cells of the gastrointestinal tract are ideal for conducting research because they are abundant and highly proliferative, making their identification possible. Also, stem cells are organized into highly structured units called crypts, which are similar if not identical in architecture and function to one another, aiding their studies enormously (13–15). It is therefore of no surprise that much of our understanding of the solid organ stem cell biology has come from the gastrointestinal tract.

Stem cells reside at or close to the crypt base dividing around once a day, resulting in the production of transiently amplifying (TA) cells that migrate towards the intestinal lumen. TA cells divide rapidly around five times and move out of the crypt to line the luminal surface, having fully differentiated into either enterocytes, goblet cells or entero-endocrine cells. In mice, the cellular proliferation in the crypt is very active, resulting in the luminal epithelium being completely replaced around every 5 days (Fig. 1) (13,14,16).

Through studying the normal gastrointestinal stem cells, the signature cell surface markers of stem cells have been discovered, such as CD133 and Lgr5. CD133 is a membrane bound glycoprotein whose function is yet to be elucidated, but has proven to be a reliable marker for stem cells of different organs (17,18). On the other hand, Lgr5 is a functional marker of Wnt signalling, faithfully representing the active Wnt signalling in the intestinal stem cell population (19). Stem cells residing at the crypt base express Lgr5 as well as CD133 (17), and are thought to be maintained by signals released from the closely associated Paneth cells including the Wnt protein and epidermal growth factors. There is another distinct population of stem cells residing around four cell levels up from the base of the crypt, which express Bmi1 and are relatively more quiescent than the stem cells at the crypt base (20). There is ongoing debate about which of these different populations of

Figure 1. The organization of the intestinal crypt. Intestinal Lgr5+ stem cells are located at the base of the crypt and BMI1+ stem cells around four cells up from the crypt base. Lgr5+ cells are closely associated with Paneth cells that provide signals to support the stem cell ‘niche’. The proliferation of these stem cells feed cells into the transiently amplifying compartment, which in turn produces daughter cells that are differentiated and repopulate the intestinal luminal surface.
cells are true stem cells (20), although it is possible that both are stem cells with each having a different function from one another. Regardless of this contentious issue, markers of healthy adult stem cells have proven to be crucial in identifying cancer stem cells of the digestive system.

**Identification of Cancer Stem Cells of the Digestive System**

In 2007, Ricci-Vitiani et al. and O’Brien et al. both independently identified a population of cancer initiating cells from human colorectal cancer. They used flow cytometry to isolate the cancer initiating cells, using CD133 as the marker of these cells. The tumour-initiating capability of CD133+ cells was demonstrated by their ability to form viable xenografts in non-obese diabetic/severe combined immunodeficiency mice (Fig. 2). These became the first experiments suggesting the existence of cancer stem cells in cancers of the digestive system.

Similar experiments identified Lgr5 to also be a marker of colorectal cancer initiating cells. The functional significance of Lgr5 for stemness was also demonstrated, whereby overexpression of Lgr5 lead to the enhancement of the tumourigenic potential of colon cancer cells (21).

Both Lgr5 and CD133 are markers of normal and cancer stem cells, meaning for example that drugs targeting these would also affect the normal stem cells. Therefore, the recent discovery of Dclk1, a marker specific for colorectal cancer stem cells marks a significant step forward in cancer stem cell research. Accordingly, selective ablation of Dclk1-positive cells resulted in the regression of intestinal polyps in mice without damage to the normal intestinal tissue (22), reinforcing at the same time the importance of cancer stem cells in intestinal cancer.

**Origin of Cancer Stem Cells of the Digestive System**

One of the fundamental questions regarding cancer stem cells is where they arise from, as such knowledge may allow prevention of cancer stem cells being generated in the first instance. There is conflicting data on the origin of cancer stem cells of the digestive system, although these are not necessarily mutually exclusive (Fig. 3).

Gastrointestinal stem cells may be inherently susceptible to transform into cancer stem cells, due to their extended lifespan as well as their rapid turnover making them prone to acquiring genetic mutations leading to carcinogenesis (23). In an elegant study, selective and conditional knock-out of the Adenomatous polyposis coli (APC) gene in the Lgr5+ stem cells of the murine intestines demonstrated the production of intestinal tumours. Moreover, the APC deletion in non-stem cells did not progress to tumours demonstrating that carcinogenesis only originated from stem cells (24). A similar study has been conducted with similar results when CD133 was used as the marker of intestinal stem cells instead of Lgr5, providing further support that cancer stem cells originate from normal stem cells of the gastrointestinal tract (17).

The second potential source of the cancer stem cells of the digestive system is from the differentiated intestinal cells. In an analysis of human colorectal adenomas, dysplastic cells with gene expression signatures of cancer were located towards the luminal end of the crypts, while the cells at the bottom of the same crypts appeared to be normal (25). The authors of this study state the possibility of having missed an incriminating cell at the bottom of the crypts due to the way the histological samples were sectioned, leading to misinterpretation of the results. However, there is another study that supports differentiated intestinal cells being the potential source of cancer stem cells.
cells, showing that activation of the Wnt pathway can induce de-differentiation of non-stem cells in the intestines and allow them to acquire tumour-initiating capacity (26). These two studies have used different approaches to demonstrate the differentiated cells being the potential source of cancer stem cells, adding credibility to this concept.

The third potential source of the cancer stem cells of the digestive system is from the bone marrow. Lineage tracing of mice transplanted with lac-Z expressing bone marrow stem cells showed these cells to repopulate the stomach in the presence of Helicobacter pylori induced inflammation and that these cells progressed through the sequence of metaplasia and dysplasia before transforming to intraepithelial cancer cells (27).

The fourth potential source of cancer stem cells may be from cancer cells without stem cell-like properties, which has been demonstrated in breast cancer, but not in malignancy of the digestive system (28).

In conclusion, the source of cancer stem cells of the digestive system in humans is not yet clear but may arise from multiple sources.

**CHARACTERISTICS OF CANCER STEM CELLS**

**ROLE OF METABOLISM IN CANCER STEM CELLS**

Warburg (29) first proposed that metabolism may drive carcinogenesis, reasoning that cancer cells had markedly different metabolism from normal cells and that multiple carcinogens affected metabolism. Since then, diet (30), inactivity (31) and obesity (32) have been established to be independent risk factors for colorectal cancer, providing strong evidence behind the role of metabolism in carcinogenesis. Much research conducted since have shown the importance of metabolism in carcinogenesis, including for the generation of tumour-initiating cells (33).

The rationale for cancer adopting a different metabolic profile to normal tissue may be to support the high rate of proliferation and protection from oxidative stress (34). The unique features of cancer metabolism described by Warburg et al. (35) include the preference for glycolysis mediated energy production and lactic acid fermentation (Fig. 4). While this is a much less efficient way of metabolizing glucose than through oxidative phosphorylation in the mitochondria employed by normal cells, the products of glycolysis provide the neoplastic cells with necessary molecules to fuel the high rate of proliferation characteristic of cancer cells (36,37). The preference of cancer cells for the pentose phosphate pathway leads to the synthesis of the reduced form of glutathione, providing these cells with protection from reactive oxidative stress, thereby conferring them with resistance against chemotherapy and radiotherapy (38).

The evidence linking metabolism with cancer stem cells of the digestive system have begun to emerge. The metabolic enzyme glycine decarboxylase (GLDC) is overexpressed in colorectal cancer and is necessary to maintain the tumorigenic potential of the colorectal cancer cells. In non-small cell lung cancer, GLDC has been found to play a critical role in the induction as well as maintenance of tumour-initiating cells, these being demonstrated by the transformation of non-tumour-initiating cells to tumour-initiating cells through overexpression of GLDC (39).

These studies indicate that metabolism is a critical component of cancer stem cell biology, and therefore is promising as a target for cancer therapy in the future.
Signalling and Epigenetics in the Regulation of Cancer Stem Cells

Wnt signalling is necessary for both normal and cancer stem cell homeostasis in the gastrointestinal organs (40,41). Alteration of the Wnt signalling is important in the transition of normal colonic mucosa to adenocarcinoma (42,43). The transcriptional regulator β-catenin is normally held in check by the multi-protein complex containing the tumour suppressor APC. Wnt ligands activate Frizzled receptors, which in turn disrupts the stability of the APC multi-protein complex. In the absence of stable APC complex, β-catenin translocates into the nucleus, binding to the TCF/LEF family of transcription factors and activating the Wnt target genes (Fig. 5). APC mutation is an early event toward colorectal tumourigenesis and accounts for ≈80% of cases, and so one may expect all the cells harbour β-catenin activity. However, β-catenin activity was only seen in a small subpopulation of cells and that these cells display the properties of cancer stem cells. β-Catenin activity and cancer stem cell characteristics are maintained by hepatocyte growth factors released by neighbouring myofibroblasts in the cancer niche, and these myofibroblasts were also capable of transforming the differentiated cancer cells into cancer stem cells. This demonstrates not only the importance of Wnt signalling in colon cancer stem cells but also the importance of interaction of cancer stem cells with its microenvironment (44).

Other signalling pathways important in cancer stem cells of the digestive system include transforming growth factor β, Notch and Hedgehog signalling (43).

Epigenetics refers to the control and alteration of gene expression through mechanisms other than through changes in

Figure 4. The difference in metabolism between normal cells and cancer cells. Normal cells metabolize glucose efficiently by oxidative phosphorylation in the mitochondria. Warburg observed and described that most cancer cells have a preference for energy production by glycolysis and lactic acid formation, even in normoxia.

Figure 5. Wnt signalling. (A) In the absence of WNT ligand, the APC multi-protein complex prevents the translocation of β-catenin into the nucleus. (B) (1) In the presence of WNT ligand, (2) Frizzled receptor is activated, which disrupts the stability of the APC multi-protein complex, (3) allowing nuclear translocation of β-catenin and (4) allowing it to bind to TCF family of transcription factors to activate Wnt target gene expression.
the DNA coding sequence, and is important for the function of normal cells as well as in malignancies. MicroRNAs (miRNAs), DNA methylation and chromatin remodelling are important machinery for epigenetic regulation (45).

miRNAs are small single-stranded non-coding RNAs that are powerful endogenous regulators of transcription and controller of cell fate, including cancer. New evidence suggests that miRNAs preserve stemness in cancer stem cells. Microarray analysis of colorectal cancer cells revealed 19 miRNAs that have altered levels when comparing CD133\(^+\) cells and CD133\(^-\) cells, which have previously been shown to regulate tumour suppressors and epithelial–mesenchymal transition (EMT), suggesting a role in the maintenance of the cancer stem cells (46). In another study, miR34a was shown to be an inhibitor of stemness in colorectal cancer cells, instead promoting differentiation, through inhibition of Notch signalling (47).

Histones regulate gene expression through remodelling of chromatin complexes. In turn, Bmi1 is a component of the polycomb repressive complex 2 that regulate the expression of many genes through histone methylation, resulting in a chromatin that is transcriptionally silent. A recent study has shown that the abrogation of Bmi-1 with a small molecule inhibitor, PTC-209, can irreversibly diminish the self-renewing capabilities and tumorigenicity of colorectal cancer cells (10). Encouragingly, the dose of PTC-209 used in the experiments was not sufficient to disrupt the function of the normal intestinal stem cells (10).

DNA methylation induces transcriptional silencing of genes and plays a role in many cancers (48). However, there is no direct evidence linking DNA methylation to cancer stem cells of the digestive system.

Metastasis and Epithelial Mesenchymal Transition

Metastasis is responsible for much of cancer-related morbidity and mortality. Metastasis requires cancer cells to accomplish several events in succession, such as tissue invasion, vascular invasion, transportation in the blood stream, extravasation and seeding in their target organ. Epithelial–mesenchymal transition refers to the process of transformation of cells from the epithelial to the mesenchymal phenotype, enabling cells to become depolarized and migrate across the basal lamina, which is a critical initial process of metastasis allowing cells to enter invade tissue and enter the blood stream (Fig. 6).

Cancer stem cells have also been shown to play a critical role in the process of metastasis, as demonstrated by the observation that the proportion of CD133\(^+\) CXCR4\(^+\) tumour-initiating cells is raised in colorectal liver metastasis in comparison with the primary tumour. CD133\(^+\) CXCR4\(^+\) cells not only displayed the genetic signatures of EMT, but also demonstrated increased capability for metastasis in vitro and in vivo. These findings translated to clinical evidence of poorer prognosis of colorectal cancer patients with a high proportion of CD133\(^+\) CXCR4\(^+\) cells in their primary lesions. On the other hand, CD133\(^-\) cells were unable to form tumours or metastasis in vivo regardless of the CXCR4\(^+\) status, suggesting here at least that the capability of metastasis may be limited to cancer stem cells (49).

Many molecular mechanisms have been described that link the process of EMT with stemness of cancer cells, at transcriptional, translational and post-translational levels. Snail is a zinc finger transcription factor that is widely known for its ability to induce EMT, and it was found in colon cancer cells that Snail also induced the cancer stem cell phenotype, as

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**Figure 6.** Epithelial mesenchymal transition (EMT). EMT involves epithelial cells losing their polarity, loss of tight and adherens junctions and gaining a mesenchymal phenotype allowing migration across the basal lamina.
confirmed by *in vitro* and *in vivo* methods (50). The depletion of the tumour suppressor FBXW7 also induces EMT and cancer stem cell phenotypes in colorectal cancer cells, but this effect was countered by the mTOR inhibitor rapamycin (51). Igf2 mRNA binding protein 1 (IMP1) is a messenger RNA binding protein, shown to be important in increasing the number of colorectal tumour cells entering the circulation as well as inducing the cancer stem cell phenotype. IMP1 is also associated with poor prognosis in colorectal cancer patients (52). The pro-inflammatory cytokine IL-1β is associated with many cancers and was shown to promote stemness and EMT in colorectal cancer cells (53).

The above is an intriguing finding that the biological process of EMT also appears to generate cells with cancer stem cell characteristics, which endows the cells with capability not only of invading blood vessels but to seed and repopulate at distal organs (54,55). Perhaps this strong mechanistic relation between stemness and EMT could be due to the important physiological role of EMT in embryogenesis as well as in adults.

**DORMANCY**

Long-term quiescence or dormancy is a key feature of normal stem cells, given the avoidance of active proliferation is thought to shield stem cells from accumulating mutations, differentiation and losing their capacity to proliferate (56–58). Dormant stem cells can be isolated by their DNA label retaining traits, with opposite characteristics being seen in their daughter cells undergoing rapid cell division (59–61). Evidence suggests cancer stem cells may also display dormancy, which would explain the occurrence of recurrence at a much later date. Moreover, the ineffectiveness of the current chemotherapy and radiotherapy may be because the non-proliferating cells are naturally more resistant to chemo and radiotherapy, which often target proliferative mechanisms of cells (62).

Dormant cells can be by their very nature difficult to visualize and study. However, in liver and pancreatic adenocarcinoma, the quiescent nature of the cancer stem cells has been demonstrated by their dye-retaining properties (63,64). In the pancreatic cancer cell line, the dye-retaining cells not only had the basic requirements of stem cells in terms of appropriate cell surface markers, soft agar colony forming ability, and ability to grow as xenotransplants, but also had increased invasive potential, had signatures of EMT and associated with chemoresistance. Of interest was the fact that while dye-retaining cells could repopulate the heterogeneous cancer tissue, non-quiescent cells were also able to transform into quiescent cells. Also, there was only partial overlap between the dye-retaining cells and the CD24+/CD44+ and CD133+ cells, which are common markers for pancreatic cancer stem cells, raising the questions about which markers are more specific and whether there are heterogeneous groups of cancer stem cells with different degrees of quiescence (63). The latter point is a likely explanation given heterogeneity within the tumour-initiating cell population in terms of their tumour-initiating potential has been reported previously (65). Specific targeting of the quiescent cancer stem cells in liver cancer can lead to decreased tumourigenicity, suggesting the importance of targeting dormant cells for effective treatment of cancer (64).

**THERAPY RESISTANCE OF CANCER STEM CELLS**

Normal stem cells are endowed with mechanisms to protect themselves against a wide range of insult during the human life-span and allow for life-long regeneration of normal adult tissue (66,67). These protective mechanisms have been studied in colorectal and pancreatic cancer cells and include the expression of ABC drug pumps (68,69), aldehyde dehydrogenase that can metabolize certain chemical toxins (59,60), anti-apoptotic/pro-survival profile (70,71) and enhanced DNA damage response (72,73). These mechanisms by default also confer resistance against chemotherapy and radiotherapy, and may, for example, explain the resistance of CD133+ colorectal cancer stem cells to a wide range of chemotherapeutic drugs such as paclitaxel, temozolomide, etoposide and carboplatin when compared with CD133− cells (67).

**PERSPECTIVES**

One of the limitations of a significant number of the studies of cancer stem cells is that these cells have been studied outside of their native environment, for example, as *in vitro* assays or as xenotransplant models. Therefore, the true nature of cancer stem cells remains largely unknown.

A new approach is required to investigate the biology of cancer stem cells. Intravitral microscopy is an emerging technique allowing cellular and biomolecular processes to be studied longitudinally in real-time *in vivo* (74). As the depth of vision is limited to ~1 mm, it has hitherto been developed to study specimens or cellular processes occurring at the surface of the animals (75). While the abdominal cavity has been difficult for direct visualisation, creation of an abdominal window has allowed cellular processes to be observed in the abdominal organs in live mice (76).

Many of the markers of cancer stem cells reported to this date are non-selective and will be problematic as targets for cancer therapy in the future. The identification of the specific markers for cancer stem cell will provide a good target for drug therapy. Cancer stem cell selection of cell surface markers is enhanced by using a combination of cell membrane markers, which could be viewed as a vantage for specific targeting of these cancer stem cells. Novel biochemical and nanomedical technologies could be employed to deliver cytotoxic drugs as inactive components that are only activated when they co-localize to the cancer stem cells expressing a particular combination of surface glycoproteins.

Targeting cancer stem cells appears to be a promising strategy for the treatment of cancer (10,77,78). The key to any novel therapies against cancer stem cells is specificity, in
order to avoid, for example, detrimental effects on the normal stem cells (79). The current understanding of the unique characteristics of cancer stem cells in terms of metabolism, EMT, dormancy and signalling, therefore, provide direction for further research as promising targets that would be specific against cancer stem cells.

CONCLUSION

There is growing evidence for the existence of a subset of cancer cells of the digestive system that have their own unique epigenetic, metabolic and phenotypical features endowing them with tumour-initiating capabilities. The abrogation of the tumourigenic potential of the colorectal cancer cells by selectively targeting the cancer stem cells (10,22) adds further weight to the importance of these cells, and provide evidence that targeting of cancer stem cells is a promising method for the treatment of cancer.

Future work required in the field includes the search for reliable and specific markers of stem cells across the digestive system. This will allow better characterization of cancer stem cells. Emphasis of research also needs to be directed to study cancer stem cells in their native environment in order to study their true biology in the future.

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Conflict of interest statement

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