Received: 30 May 2023 | Accepted: 10 June 2023

DOI: 10.1111/cas.15891

REVIEW ARTICLE

Cancer Science WILEY

Genetic and nongenetic mechanisms for colorectal cancer evolution

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Funding information
Japan Agency for Medical Research and Development, Grant/Award Number: 22ck0106541h0003 and 22gm4010012h0002; Japan Society for the Promotion of Science, Grant/Award Number: 22H00454, 22H02899, 22KF0154 and 23K06631

Abstract

The stepwise accumulation of key driver mutations is responsible for the development and malignant progression of colorectal cancer in primary sites. Genetic mouse model studies have revealed combinations of driver gene mutations that induce phenotypic changes in tumors toward malignancy. However, cancer evolution is regulated by not only genetic alterations but also nongenetic mechanisms. For example, certain populations of metastatic cancer cells show a loss of malignant characteristics even after the accumulation of driver mutations, and such cells are eliminated in a negative selection manner. Furthermore, a polyclonal metastasis model has recently been proposed, in which cell clusters consisting of genetically heterogeneous cells break off from the primary site, disseminate to distant organs, and develop into heterogenous metastatic tumors. Such nongenetic mechanisms for malignant progression have been elucidated using genetically engineered mouse models as well as organoid transplantation experiments. In this review article, we discuss the role of genetic alterations in the malignant progression of primary intestinal tumors and nongenetic mechanisms for negative selection and polyclonal metastasis, which we learned from model studies.

KEYWORDS
cancer evolution, mouse model, multistep tumorigenesis, organoid, polyclonal metastasis

1 | INTRODUCTION

Colorectal cancer is a leading cause of cancer-related mortality and morbidity worldwide.[1] The major cause of CRC-related death is metastasis and relapse, as it often spreads to the liver and lung even after surgical resection and neoadjuvant treatment. It has been established that CRC progresses through the stepwise accumulation of genetic alterations, a known concept of multistep tumorigenesis.

High-throughput sequencing technologies not only allow the identification of genomic and transcriptomic alterations associated with CRC development but also reveal considerable ITH.[2,3] Heterogeneity leads to a complex interplay between clonal cell populations, giving rise to cancer evolution such as metastasis, relapse,

Abbreviations: α-SMA, α smooth muscle actin; $Apc^{\Delta 716}$, *Apc* truncation mutation at codon 716; CAF, cancer-associated fibroblast; CIMP, CpG island methylator phenotype; CRC, colorectal cancer; FIGF, c-FOS-induced growth factor; GAP, GTPase-activating protein; GOF, gain-of-function; iCAF, inflammatory CAF; ITH, intratumor heterogeneity; $Kras^{G12D}$, *Kras* missense mutation at codon 12 (Gly to Asp); MDSC, myeloid-derived suppressor cell; MSI, microsatellite instability; myCAF, myofibroblastic CAF; pEMT, partial epithelial–mesenchymal transition; TME, tumor microenvironment; Treg, regulatory T-cell; $Trp53^{R270H}$, *Trp53* missense mutation at codon 270 (Arg to His).

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and drug resistance.[4] However, it has been reported that there are no fundamentally different genetic alterations between primary and metastatic CRC, suggesting that nongenetic mechanisms promote metastasis development.[5,6]

To better understand the mechanisms underlying CRC development and metastasis, we generated mouse models carrying major driver mutations in various combinations in intestinal epithelial cells and established metastasis models via the transplantation of organoids derived from mouse model tumors. These model studies helped to clarify the in vivo mechanisms underlying the malignant progression of primary tumors, negative selection in the tumor cell population, and polyclonal metastasis with heterogeneous cell clusters, as discussed below.

2 | GENETIC ALTERNATION PROGRAMS IN PRIMARY CRC

2.1 | APC and Wnt activation for initiation of CRC development

Genetic alterations in the Wnt, MAPK/ERK, PI3K, p53 and TGF-β pathways are primary mediators of CRC development.[7] Wnt signaling is crucial in maintaining stemness in normal intestinal stem cells and is commonly dysregulated in most CRC cells.[8] Almost 75% of CRC cases have *APC* mutations that cause constitutive Wnt signaling activation, leading to adenomatous polyp development in the intestine (Figure 1). The causal link of *Apc* mutation to intestinal polyposis was genetically confirmed in $Apc^{\Delta 716}$ mice (A) that carried an *Apc* truncation mutation at codon 716.[9] Interestingly, an *Apc* mutation is not sufficient for polyp development, but the induction of COX-2 expression in stromal cells and subsequent activation of PGE_2 signaling are required for tumorigenesis by generating an inflammatory microenvironment, thus the COX-2/PGE_2 pathway can be a possible prevention target against CRC (Figure 1).[10,11]

Constitutive Wnt signaling activation is also induced by *RNF43* mutations. Loss-of-function mutations in *RNF43* lead to a failure of Wnt receptor degradation by R-spondin through the proteasome pathway, which increases sensitivity to Wnt ligand stimulation. A CRC patient-derived organoid analysis indicated that *RNF43* mutations are found in MSI-positive and CIMP-positive CRC, suggesting a different genetic mechanism for the malignant progression from *APC* mutation-initiated CRC.[12] Furthermore, that study also showed that a Wnt ligand inhibitor effectively suppressed patient-derived xenograft (PDX) tumor development using *RNF43* mutant CRC-derived organoids, suggesting a possible therapeutic strategy of Wnt ligand inhibition for MSI-type CRC.

2.2 | Malignant progression by p53 gain-of-function mutations

In addition to APC dysregulation, a comprehensive genome analysis showed a high mutation incidence of *TP53* (60%) and *KRAS* (42%), which cooperates to drive malignant progression with *APC* mutations.[2,13] p53 plays a tumor suppressor role through the regulation

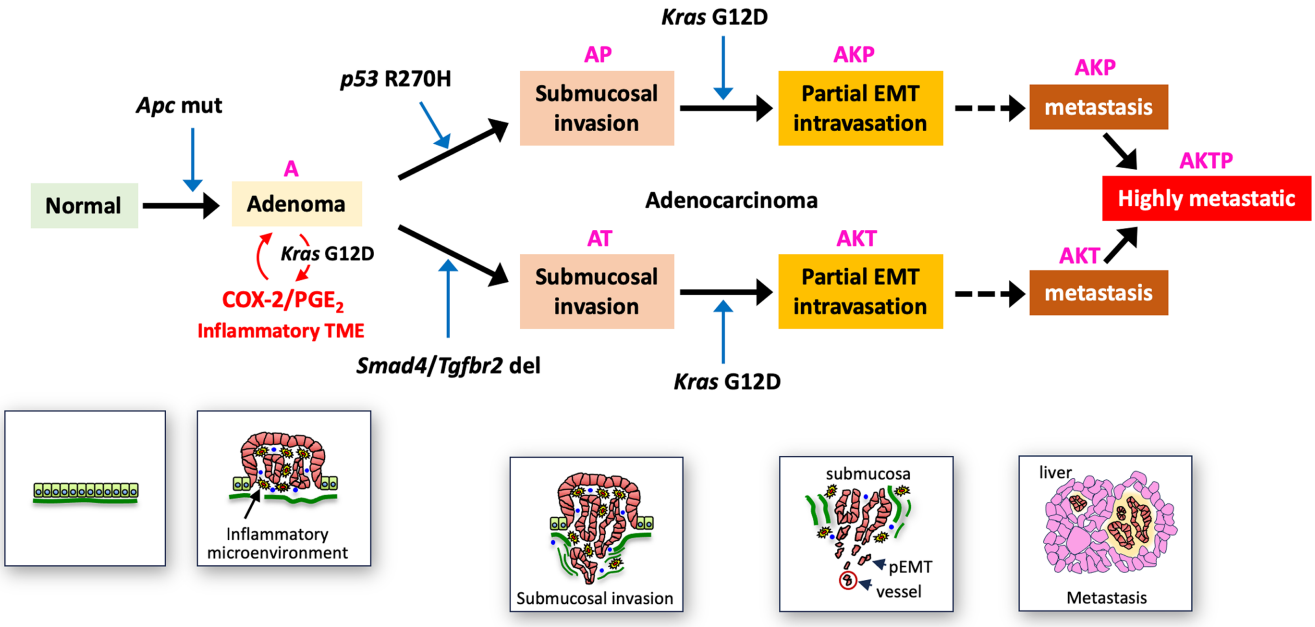

FIGURE 1 Genetic alterations and malignant progression of intestinal tumors initiated by the *Apc* mutation. The *Kras* G12D mutation in adenoma cells causes increased polyp formation, possibly through inflammatory tumor microenvironment (TME) generation by the COX-2/PGE_2 pathway activation (red arrows). A gain-of-function *p53* mutation or blockade of the TGF-β pathway causes submucosal invasion, and an additional *Kras* G12D mutation leads to advanced malignant phenotypes such as partial EMT (pEMT) and intravasation. The metastatic ability was examined using organoid transplantation experiments. Genotypes, A, $Apc^{\Delta 716}$; K, $Kras^{G12D}$; T, $Tgfbr2^{-/-}$; and P, $Trp53^{R270H}$ are indicated by magenta. Corresponding histology image drawings are provided in the lower part of the figure.

of multiple biological processes, including cell cycle arrest, DNA damage repair, apoptosis, and the immune response.[14] Most *TP53* mutations in CRC are missense mutations that not only impair the p53 function but also play a novel oncogenic role in p53 by GOF mechanisms, such as increased stemness, invasion, and metastasis.

To study the role of the GOF mutant p53 R270H (corresponding to human R273H) in intestinal tumorigenesis, we generated $Apc^{\Delta 716}$ $Trp53^{R270H}$ compound mice (AP) and found that mutant p53 R270H induced submucosal invasion of intestinal tumors (Figures 1 and 2).[15] Consistently, tumor-derived organoids with the $Trp53^{R270H}$ mutation showed the acquisition of invasiveness, which is associated with the complex glandular structures of organoids.[15] A transcriptome analysis revealed that GOF mutant p53 causes activation of Wnt/β-catenin signaling and inflammatory pathways, possibly through increased promoter accessibility, which may contribute to the acquisition of invasiveness.

2.3 | Malignant progression by the combination of *Kras* and *Trp53* GOF mutations

Oncogenic *KRAS* mutations confer constitutive activation of MAPK/ERK and PI3K signals, leading to increased cell proliferation, survival, migration, and invasion, which is associated with a poor prognosis in CRC patients.[16] However, we did not find an advanced malignant phenotype in $Apc^{\Delta 716}$ $Kras^{G12D}$ compound mouse tumors.[17] Rather, the *Kras* mutation significantly increased the number of intestinal adenomatous polyps. Kras activation may induce COX-2 expression, which accelerates the generation of an inflammatory microenvironment that supports the survival and proliferation of tumor cells (Figure 1).[10,17] Notably, the combination of $Apc^{\Delta 716}$ $Kras^{G12D}$ $Trp53^{R270H}$ triple mutations (AKP) induced pEMT and intravasation at submucosal invasion lesions, which was not found in $Apc^{\Delta 716}$ $Trp53^{R270H}$ double-mutant mice.[17] Consistently, another genetic model also indicated that the oncogenic *Kras* mutation is dispensable for polyp growth but important for invasive and metastatic growth.[18]

Notably, organoids derived from $Apc^{\Delta 716}$ $Kras^{G12D}$ $Trp53^{R270H}$ mouse intestinal tumor (AKP) developed liver metastasis tumors when transplanted to the spleen, while organoids with any two of the mutations did not (Figure 1). Accordingly, these results indicated that mutations in *APC*, *KRAS*, and *TP53* are the minimum combination required for malignant progression and metastasis of CRC.

2.4 | Malignant progression by *Kras* mutation and TGF-β pathway inhibition

Alterations in the downstream components of the TGF-β pathway are involved in CRC development. TGF-β signaling induces differentiation and growth suppression of epithelial cells, thus functioning as a tumor suppressor. Consistently, mutations in *SMAD4*, encoding an essential downstream effector of the TGF-β pathway, and the TGF-β type II receptor gene *TGFBR2* are frequently detected in nonhypermutated or hypermutated CRC cases and are associated with the promotion of metastatic trait development.[2,19] Notably, suppression of TGF-β signaling in the colonic mucosa drives tumor progression through not only epithelial transformation but also tumor–stromal interactions.[20] For example, an inflammatory and fibrotic microenvironment with protease expression is generated in $Apc^{\Delta 716}$ and $Tgfbr2^{-/-}$ or $Smad4^{-/-}$ mouse intestinal tumors (AT), which promotes submucosal invasion phenotypes (Figure 1).[21,22] Furthermore, intestinal tumors in $Apc^{\Delta 716}$ $Kras^{G12D}$ $Tgfbr2^{-/-}$ compound mice (AKT) showed pEMT and intravasation, and spleen transplantation of the tumor-derived organoids induced liver metastasis with increased incidence and multiplicity (Figure 1).[17] Accordingly, these results indicate that mutations in *APC*, *KRAS*, and *TGFBR2*, or *SMAD4* are another minimum core of metastasis of CRC.

Notably, TGF-β signaling can also act as a tumor promoter, as it is highly expressed during the late stages of CRC[23], thus TGF-β is considered a double-edged sword in cancer development. We will not discuss the oncogenic effect of TGF-β in the present paper.

3 | CANCER EVOLUTION SHAPES METASTATIC ABILITY

3.1 | Combination of *Trp53* GOF mutation and LOH for metastasis

Consistent with the results of mouse model studies, the combination of *KRAS/NRAS*, *TP53* and *SMAD4* mutations is associated with recurrence and worse survival compared with any two mutations after surgical resection of liver metastasis.[24] In particular, the *TP53* mutation is quantitatively enriched in liver metastasis of CRC.[25] The GOF mutant p53 plays a role in epigenetic regulation through interaction with the chromatin remodeling complex or histone modification, which may contribute to the acquisition of malignant properties by increasing promoter accessibility.[26]

To further study the mechanism of GOF mutant p53 in metastasis, we established intestinal tumor-derived organoids from $Apc^{\Delta 716}$ $Kras^{G12D}$ $Tgfbr2^{-/-}$ $Trp53^{+/R270H}$ quadruple-mutant mice (AKTP), termed $AKTP^{+/M}$ cells (note that the *Trp53* genotype is heterozygous +/R270H).[17] As expected, multiple liver metastatic foci developed when $AKTP^{+/M}$ organoids were transplanted into the spleen. Importantly, however, in approximately 50% of liver metastasis lesions, tumor cells showed loss of the wild-type *Trp53* gene via LOH to become $AKTP^{R270H/LOH}$ (hereafter, $AKTP^{M/LOH}$).[27] In contrast, wild-type *Trp53* was retained in the splenic transplanted primary tumors. These results indicated that loss of wild-type *Trp53* by LOH is important for the promotion of metastasis development (Figure 2).

Ourselves and others have identified that wild-type p53 interferes with the nuclear stabilization of mutant p53 protein in heterozygous cells, thus p53 LOH is an important prerequisite for the stable oncogenic function of mutant p53.[27,28] Indeed, $AKTP^{M/LOH}$ cells showed an increased survival rate and clonal expansion of the single

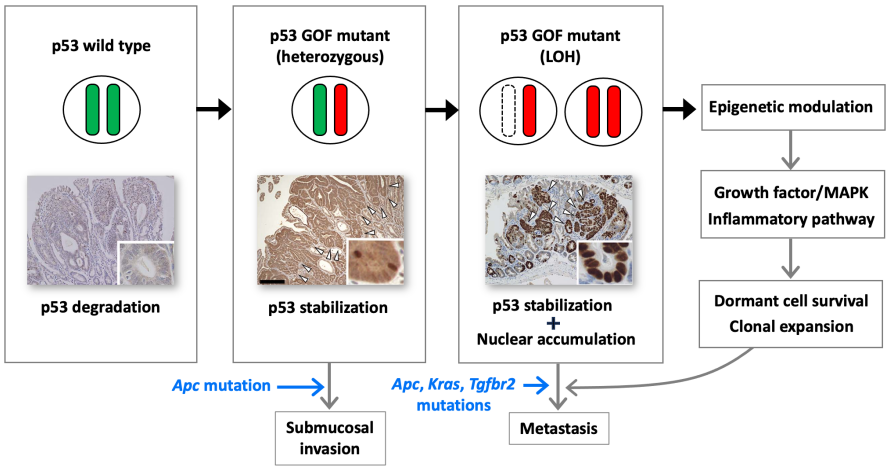

FIGURE 2 Schematic drawing of the *p53* genotypes and associated phenotypes in intestinal tumors. Representative photographs of immunohistochemistry for p53 in the intestinal polyps are provided. Green, wild-type *Trp53* allele; red, GOF mutant *Trp53* allele. In *p53* wild-type tumors (left), p53 protein is continuously degraded. In *p53* GOF heterozygous tumors (center), p53 is stabilized, and the submucosal invasion of tumor cells is induced. In *p53* GOF and LOH tumor cells (right), mutant p53 clearly accumulates in the nuclei, which causes increased oncogenic functions. Growth factor/MAPK and inflammatory pathways are activated, resulting in the promotion of metastasis in combination with other driver mutations. (Modified from Nakayama et al., Nat Commun 2020; 11: 1–4 (Ref. 27); and Nakayama et al., Oncogene 2017; 36: 5885–5896 (Ref. 15)).

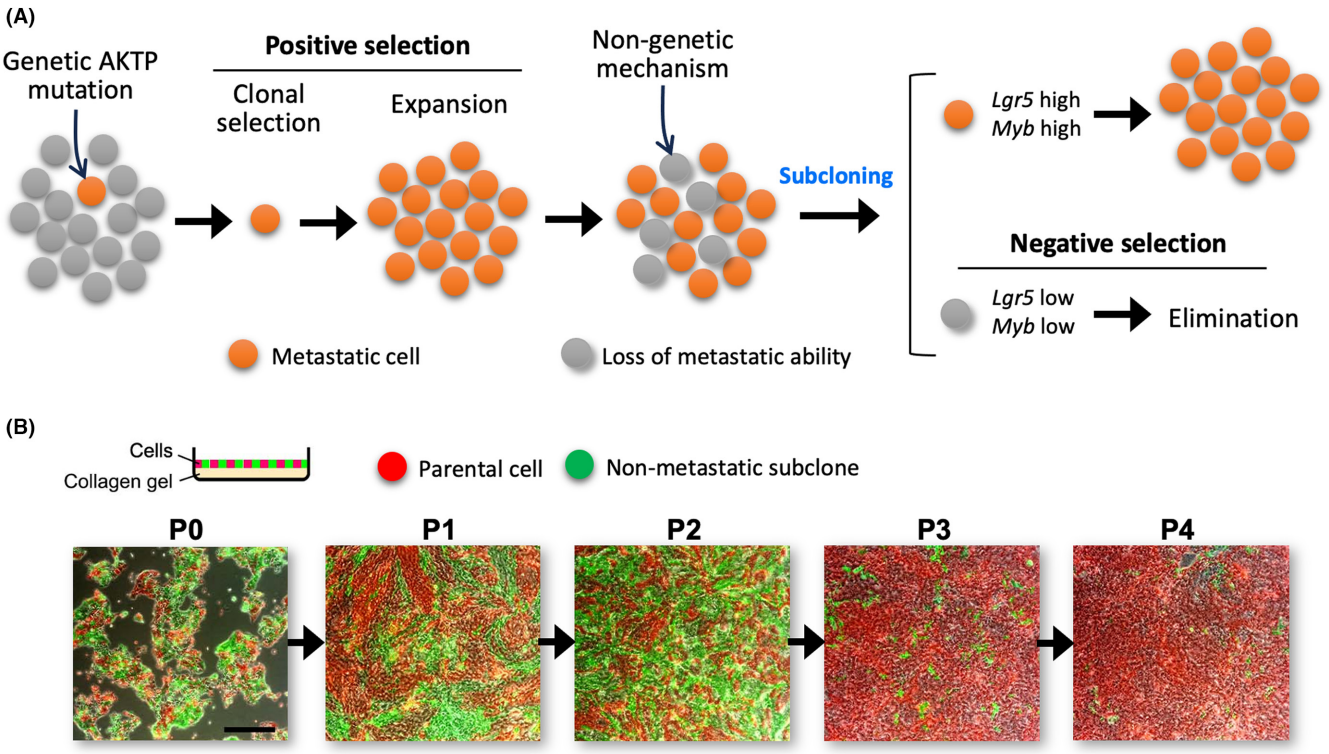

FIGURE 3 Cancer evolution by positive and negative selection. (A) Schematic drawing of positive and negative selection. When tumor cells acquire a growth advantage by accumulating genetic alterations, as in AKTP cells (orange), tumor cells are selected and expanded in a positive-selection manner. If the metastatic ability is lost in cancer cells (gray) with decreased stemness, such cells are eliminated from the population by negative selection. (B) Representative photographs of negative selection of the nonmetastatic subclone during co-culture. When metastatic parental cells (tdTomato) and nonmetastatic subcloned cells (Venus) are co-cultured, nonmetastatic cells are gradually eliminated from the population. (Modified from Morita et al., Cancer Sci 2023; 114: 1437–1450 (Ref. 32)).

dissociated cells compared to AKTP$^{+/M}$ and AKTP$^{-/-}$ cells, which are important properties for dormant disseminated cells in distant organs (Figure 2).[27] Taken together, these model studies indicated that a heterozygous GOF p53 mutation induces submucosal invasion in the primary site, and additional LOH of p53 contributes to metastasis development by stabilizing GOF mutant p53 (Figure 2).

3.2 | Combination of *Kras* and *Trp53* mutations for metastasis

A gene expression analysis of AKTP organoid cells indicated that the *Trp53* GOF/LOH mutation induces the upregulation of growth factor/MAPK and inflammatory pathway signatures, which may cause the acquisition of high metastatic ability with *Apc*, *Kras* and *Tgfbr2* mutations (Figure 2).[27] The results also suggested that the GOF mutant p53 enhanced the oncogenic response by *KRAS* through activation of the MAPK pathway. A similar synergism between mutant p53 and KRAS has also been reported in pancreatic ductal adenocarcinoma, where mutant p53 dysregulates alternative splicing of GAPs, which promotes maximal activation of KRAS.[29] It has therefore been suggested that correction of mis-splicing events in GAPs is a potential strategy for treating tumors with co-existing *KRAS* and *TP53* mutations.

3.3 | Cancer evolutions that do not involve multistep tumorigenesis

The evolutionary tempo of primary CRC toward malignancy is promoted by the accumulation of driver mutations. At the same time, during malignant progression processes, genetic alterations generate ITH with varying capacities to adapt to the TME. Sequencing of multiple regions of CRC tissues has shown that major driver mutations accumulate at the early stage of CRC development, and subsequent ITH develops by a neutral evolution mechanism.[3,30]

Furthermore, the Big Bang model of CRC development proposes that tumors grow exponentially in the early-growth phase, generating a high degree of ITH and subclone mixing in CRC.[31] It has been suggested that ITH is involved in tumor progression and metastasis, differing markedly from the multistep tumorigenesis model. However, the details regarding the exact mechanism of ITH underlying malignant progression and metastasis remain unclear.

3.4 | Positive selection vs. negative selection

Cancer evolution is promoted by positive selections based on acquired malignant phenotypes obtained by genetic alterations. Using highly metastatic AKTP$^{M/LOH}$ organoids, we recently demonstrated the potential involvement of negative selection in the maintenance of malignant phenotypes of the tumor cell population. Surprisingly, approximately 30% of AKTP$^{M/LOH}$ tumor-derived subclones showed a loss of metastatic ability, despite carrying the same set of driver mutations as parental cells with downstream activation of the β-catenin, p53, and ERK1/2 pathways (Figure 3A).[32] Highly metastatic subclones generate a metastatic niche in the liver with Stat3 activation, while nonmetastatic subclones do not. Stat3 is essential for the survival and expansion of normal intestinal stem cells and thus plays an important role in micrometastasis development.[33] Furthermore, RNA sequencing results indicated that stemness-related genes, including *Lgr5* and *Myb*, in metastatic AKTP$^{M/LOH}$ cells were downregulated in nonmetastatic subclones (Figure 3A).

A recent study showed that depletion of *Lgr5*-expressing cells after transplantation of intestinal tumor-derived organoids halted liver metastasis development in mice, indicating that increased stemness is important for metastatic ability.[34] Notably, the subclones that lost metastatic ability were gradually eliminated from the parental cell population when co-cultured on collagen gel (Figure 3B). Therefore, the proportion of nonmetastatic subclones may continuously rise and fall in the cancer cell population.

Taken together, these findings suggest that cancer evolution and maintenance of malignant characteristics of tumor cells may be regulated by both positive and negative selection. Therefore, understanding the nongenetic mechanism underlying the loss of metastatic ability in cancer cells may contribute to the development of novel therapeutic strategies against metastatic spreading.

4 | INTERACTIONS AMONG SUBCLONES PROMOTE METASTASIS

4.1 | Concept of polyclonal metastasis

Genome analyses of matched primary and metastatic tumors suggest that metastatic seeding involves the cooperation of heterogeneous cells and, consistently, polyclonal seeding in metastatic lesions has been reported in CRC.[35–37] Such polyclonal cell clusters may explain how genetic heterogeneity is transferred from the primary tumor to the metastatic site. The heterogeneous cancer cells within tumors with different growth and metastasis abilities were recognized in the 1970s.[38,39] Therefore, an increasing number of mouse model studies have examined the interaction of clonal populations within tumors and their possible role in metastatic spread.[40]

A multicolor lineage tracing approach using a cancer cell line demonstrated that polyclonal cell clusters derived from primary sites disseminate to distant organs to form polyclonal metastases.[41] Furthermore, it has been shown that tumor cell clusters generate metastatic foci in the lung with more than 100-fold incidence relative to single cells in mouse experiments. These data indicated that cell cluster formation is advantageous for metastasis formation.

In addition, a mouse xenograft model study showed the effect of polyclonal cell clusters with different expression profiles. Interestingly, the incorporation of interleukin 11 and FIGF-overexpressing cells as a minor population in the cluster, including nonmetastatic cells, caused the development of aggressive cancers and polyclonal metastasis.[42] The findings of that xenograft study suggested an important interaction of subclones toward cancer malignancy progression.

4.2 | Polyclonal metastasis of genetically heterogeneous subclones

Most studies on polyclonal metastasis have used differentially labeled cancer cells with the same or similar genetic backgrounds. To clarify the polyclonal metastasis mechanism with genetic and

phenotypic variations, we performed spleen transplantation experiments by co-injection of nonmetastatic (*Apc*$^{\Delta 716}$ *Trp53*^{R270H} double-mutant organoid, AP cells) and metastatic intestinal tumor-derived organoid (quadruple-mutant organoid, AKTP cells).[15,17] Importantly, nonmetastatic AP cells were able to develop metastatic tumors in the liver when co-transplanted with metastatic AKTP cells (Figure 4A–C).[43] We found that AP cells did not contribute to metastatic foci when transplanted independently from AKTP cells via different injection routes, suggesting that co-dissemination with metastatic cells is required for polyclonal metastasis. Although nonmetastatic cells can metastasize by polyclonal mechanisms, the proliferation of less malignant cells is supposed to be dependent on more malignant cells in the metastatic lesions. Importantly, however, nonmetastatic AP cells are able to survive and continue proliferation to form large tumors if AKTP cells are experimentally depleted from polyclonal metastatic lesions.[43] Therefore, targeting mutant KRAS may be insufficient to treat metastatic tumors if the tumor foci contain both *KRAS* mutation-positive and mutation-negative cells. Accordingly, understanding the genetic heterogeneity of metastatic foci is important when considering an effective therapeutic strategy.

5 | TUMOR MICROENVIRONMENT REGULATES POLYCLONAL METASTASIS

5.1 | Cancer-associated fibroblasts in the TME

The TME is a multicellular system that includes immune cells, endothelial cells, and fibroblasts.[44] CAFs are one of the most abundant stromal cells in the TME, and increased numbers of CAFs are

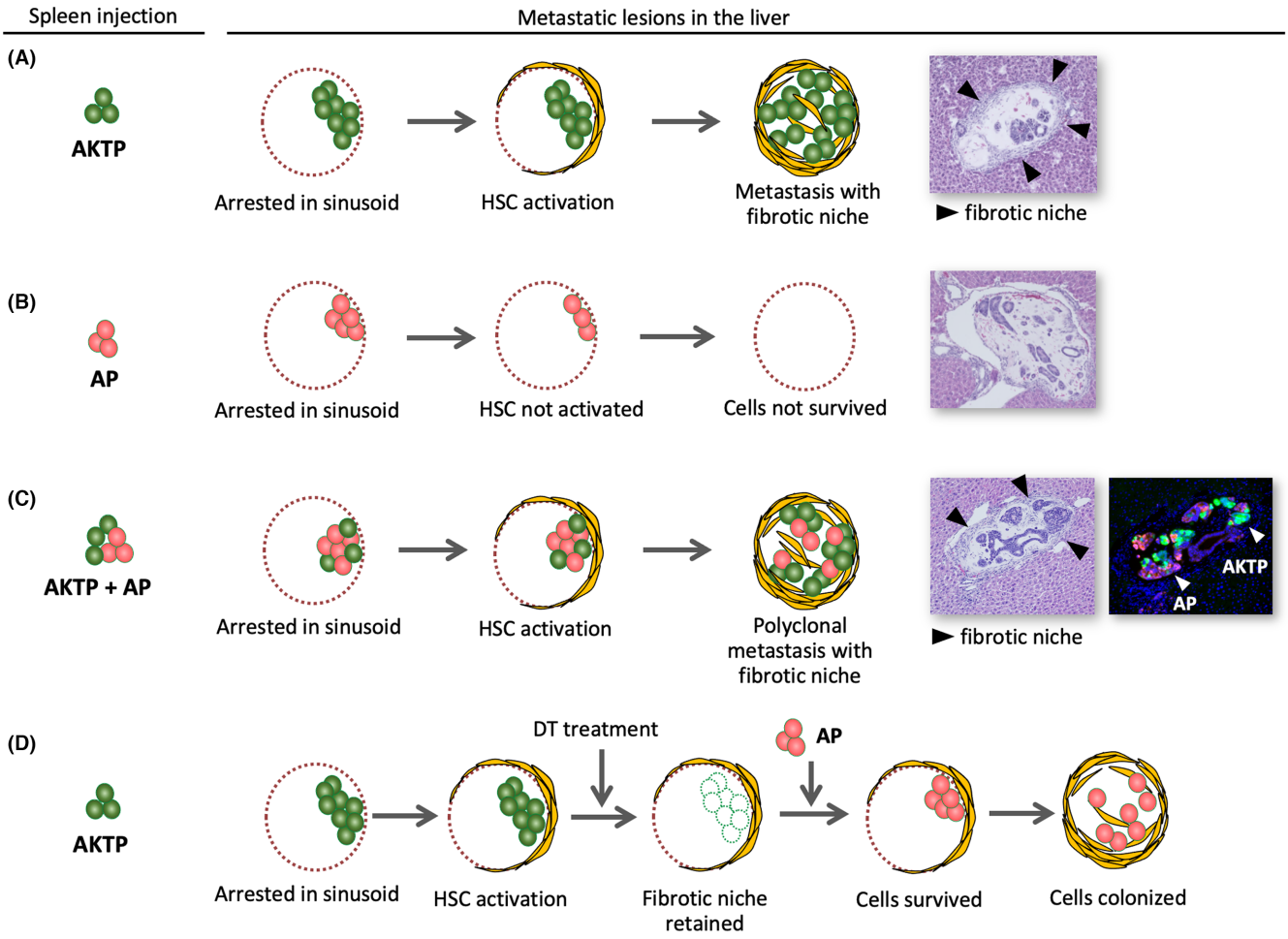

FIGURE 4 Schematic drawing of polyclonal metastasis in the liver of spleen transplantation mouse models. (A) When metastatic AKTP cells are transplanted and arrested in the sinusoid, hepatic stellate cells (HSCs) are activated to form a fibrotic niche (arrowheads in the photograph), and metastatic tumors develop. (B) When nonmetastatic AP cells are transplanted and arrested in the sinusoid, a fibrotic niche is not generated and tumor cells cannot survive. (C) When metastatic AKTP and nonmetastatic AP cells are co-transplanted and co-disseminated in the sinusoid, both cells survive with a fibrotic niche, and polyclonal metastasis develops. An immunofluorescence histology image of polyclonal metastasis is provided. (D) When metastatic AKTP cells are experimentally depleted by the diphtheria toxin (DT) system, the fibrotic niche is retained in the liver. Importantly, AP cells transplanted into DT-treated mice survived and colonized the liver. (Modified from Kok et al., Nat Commun 2021; 12: 863 (Ref. 43)).

often associated with an unfavorable prognosis.[45,46] A recent analysis of CRC tumors defined CAF subtypes, including myCAFs and iCAFs.[47,48] MyCAFs are characterized by matrix-producing contractile and highly proliferative phenotypes, and they comprise ECM networks that serve as physical barriers to protect cancer cells from immune cells and drugs.[49] myCAFs also promote tumorigenesis via direct or indirect interaction with cancer cells. In contrast, iCAFs show a secretory phenotype and are mostly related to immune evasion of tumor cells via chemoattraction of immunosuppressive cells, such as MDSCs and Tregs.[50]

5.2 | Fibrotic niche generation for polyclonal metastasis

Using mouse intestinal tumor-derived organoids, we found that fibroblastic cells proliferated surrounding AKTP cells that were arrested in liver sinusoids, and a fibrotic microenvironment with collagen fiber deposition was generated in metastatic lesions (Figure 4A,C).[43] Such α-SMA-positive CAFs are possible myCAFs and originate from hepatic stellate cells (HSCs), which were not observed if nonmetastatic AP cells were arrested in the liver sinusoid (Figure 4B). We further demonstrated that nonmetastatic AP cells can survive and colonize the liver if the fibrotic niche is retained after the depletion of AKTP cells from metastatic lesions by diphtheria toxin treatment (Figure 4D). Accordingly, malignant cell-induced fibrotic niche generation by CAFs is an important mechanism underlying polyclonal metastasis with nonmetastatic cells.

TGF-β is a cytokine responsible for HSC activation and liver fibrosis. As expected, metastatic AKTP cells did not induce fibrotic niche generation in the livers of *Tgfbr2* knockout mice, and metastasis development was also suppressed significantly.[43] It has also been shown that increased stromal TGF-β signaling enhances the survival of CRC cells and metastatic colonization, suggesting that inhibition of TGF-β signaling can suppress metastatic progression.[51] It has also been shown that targeting TGF-β signaling suppresses metastatic tumor development by activating antitumor immunity.[52]

6 | CONCLUSIONS

Comprehensive genome analyses revealed the major driver mutations in CRC and their heterogeneous characteristics. Mouse model studies revealed the relationships between genetic alterations and output tumor phenotypes during malignant progression in primary tumors. Furthermore, organoid transplantation experiments showed the interaction of tumor cells and host responses to generate a fibrotic TME by CAFs, which promoted polyclonal metastasis. With respect to the great heterogeneity in CRC and its polyclonal interactions with the TME during metastatic progression, there is an urgent need to understand the precise genetic and nongenetic cancer evolution mechanisms in order to provide precision medicine for metastatic CRC.

AUTHOR CONTRIBUTIONS

Investigation, S.Y.K., M.N., A.M., and H.O. Writing – Review and Editing, S.Y.K. and M.O. Funding acquisition, S.Y.K., M.N., H.O., and M.O.

ACKNOWLEDGMENTS

We thank Manami Watanabe, Ayako Tsuda, and Yoshie Jomen for their technical assistance in our mouse model and organoid studies.

FUNDING INFORMATION

This work was supported by Grants-in-Aid for JSPS fellows (22KF0154), Scientific Research (A) (22H00454), (B) (22H02899) and (C) (23K06631) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; and AMED (22ck0106541h0003; 22gm4010012h0002) from the Japan Agency for Medical Research and Development, Japan.

CONFLICT OF INTEREST STATEMENT

The corresponding author, Masanobu Oshima, is an Editorial Board Member of *Cancer Science*. All other authors have no conflicts of interest.

ETHICS STATEMENT

Approval of the research protocol by an Institutional Reviewer Board: N/A

Informed Consent: N/A

Registry and the Registration No. of the study/trial: N/A

Animal Studies: All animal experiments were carried out according to the protocol approved by the Committee on Animal Experimentation of Kanazawa University, Japan.

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How to cite this article: Kok SY, Nakayama M, Morita A, Oshima H, Oshima M. Genetic and nongenetic mechanisms for colorectal cancer evolution. *Cancer Sci*. 2023;114:3478-3486. doi:10.1111/cas.15891