Extended Abstracts for the 38th International Symposium of the Princess Takamatsu Cancer Research Fund: Current Challenges in the Understanding and Management of Colon Cancer

Nakahara Memorial Lecture: Basic and Applied Issues in Colon Cancer Studied in the Min Mouse and Pirc Rat Kindreds

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We are using two different murine models for colon cancer, the Min mouse and the Pirc rat, to address four issues in colon cancer:

- the somatic genetics and epigenetics in tumorigenesis;
- modifier loci that influence these pathways of colon cancer formation;
- the development of molecular markers and the distinction between these markers and factors that are causative in pathogenesis; and
- cellular interactions in tumor formation – the issue of polyclonality

Our overall shared goal in this Symposium is the understanding and management of human colon cancer. Thus I shall focus on ways in which the use of these experimental models may help us to move forward. We investigators in the field need to work together to identify key technical and conceptual issues to be addressed, and to seek together ways in which the particular avenue of murine models that we are developing might synergize with other avenues of inquiry being pursued, worldwide. Cancer research crossing national and disciplinary borders mirrors the career of Waro Nakahara, in whose honor I am giving this Lecture, and the spirit of the Princess Takamatsu International Symposia.

The Min mouse, discovered by its phenotype after germline mutagenesis, develops tumors throughout the intestinal tract, with most in the small intestine. Unlike the human, there are rather few tumors found in the colon. In searching for a more precise model of human colon cancer, we have investigated whether a rat carrying a mutation in its *Apc* gene develops intestinal cancer which is more restricted to the colon. Again, we mutagenized the entire genome with the point mutagen ENU and created a library of first-generation offspring. The nonsense mutation in animal #1360 of this library was detected by cloning the large exon 15 of *Apc* into a yeast universal gap repair plasmid that expresses the AD2 function; the cloned fragment carried a nonsense mutation that disrupted the AD2 function.

Rats carrying this nonsense allele present a strong colonic predisposition, in contrast to the known mouse models for familial colon cancer (Table 1); the strain is therefore dubbed "Pirc" for Polyposis in the rat colon. Interestingly, these rats show enhanced susceptibility in males compared to females. This difference between males and females has not been prominent in any of the mouse models for colon cancer but has been observed in human colon cancer (Table 2).

We summarize below our analysis of the four issues of this talk.

Table 1. Colonic cancer predisposition and male *versus* female incidence in Pirc rats

Age, months	Sex	N of rats	Tumor count, mean ± SD	
			Colonic	Small intestinal*
3	Male	5	2 ± 1	7 ± 9
	Female	5	3 ± 2	0 ± 0
4-6	Male	10	8 ± 3	14 ± 5
	Female	11	5 ± 3	2 ± 2
7-13	Male	17	14 ± 8	22 ± 9
	Female	6	7 ± 5	4 ± 5

* Only tumors over 0.5mm in diameter were counted.

Amos-Landgraf and Kwong et al., PNAS 104:4036-4041 (2007)

Table 2. Male *versus* female incidence in humans

Age, years	Sex	Incidence of colonic adenoma or cancer found on colonoscopy
40-49	Men	12.1% (319/2646)
	Women	8.0% (355/4460)
50-66	Men	20.3% (3124/15366)
	Women	11.8% (3272/27676)

Regula et al., NEJM 355:1863-1872 (2007)

Somatic genetics and epigenetics in tumorigenesis

In adenomagenesis, the wild-type *Apc* allele of the *Min*/+ heterozygote loses function by one of at least three different processes: homologous somatic recombination[1,2]; intragenic mutation[3]; and apparent epigenetic silencing[2,4]. The Pirc rat confirms that conservative somatic recombination can be sufficient for adenomagenesis, with no apparent pre-exisiting genomic instability[5].

The apparent epigenetic pathway for loss of wildtype Apc function is deserving of detailed molecular analysis. Beyond the hypermethylation of CpG islands, mammalian genes can also be silenced by the acquisition of stable polycomb complexes, an ancient regulatory system found extensively in Drosophila. We are therefore exploring primary assays for silencing at the cDNA level. When the gene of interest is heterozygous for single nucleotide (SNP) differences, it is feasible to assess whether the silencing is monoallelic or biallelic (Figure 1).

As we begin to document the different molecular pathways to colon cancer in these murine models, we hope to gain a perspective on the possibility that, in the human, different pathogenetic routes represent colonic tumors with distinct progenitors and distinct biological potential[6].

Figure 1. Epigenetic processes

- Stochastic events
  - Monoallelic silencing?
    - For example, X-inactivation in mammalian females
    - See Darryl Shibata in this symposium
- Programmed events
  - Biallelic silencing?
    - For example, developmental epigenetics
- CIMP tumors
  - See Rick Boland in this symposium
- Assays for silencing

Modifier loci that influence these pathways of colon cancer formation

We are familiar with the tumor suppressor genes, called gatekeepers by Vogelstein and Kinzler. When they lose function, they open up a particular neoplastic pathway. By contrast, when proto-oncogenes are activated by mutation or translocation, they create active oncogenes, also determining a particular neoplastic pathway. But for each pathway, there is a large set of modifying loci whose allelic status influences the efficiency of passage through that particular pathway, making it either more or less efficient in tumor incidence, or qualitatively affecting progression. It seems that the most comprehensive assays for modifying genes are carried out in the intact organism: some modifiers act non-autonomously. For example, hormones that effect breast cancer phenotypes are made by tissues outside of the tumor tissue itself. So, one can detect them best by working in the intact organism, using model systems. This is precisely the

setting that Dr. Nakahara studied when he began his career in cancer research at the Rockefeller Institute.

Worldwide access to the Min strain on the fixed genetic sensitive background of C57BL/6J from The Jackson Laboratory has allowed many laboratories to discover loci at which allelic variation affects the phenotype of Min (Table 3). Each one of these modifying loci gives a clue for further diagnostic or therapeutic tools for colon cancer.

We mapped the *Mom1* modifier to mouse chromosome 4. Lying within this region was the secretory phospholipase, expressed in active form in the resistant alleles of *Mom1* and mutated in sensitive alleles. A transgene for the active form of this phospholipase conferred a measure of resistance on the sensitive C57BL/6-Min strain.

Another modifier, discovered by Rudi Jaenisch and Peter Laird, is the maintenance DNA methylase *Dnmt1*, for which a loss-of-function mutation in heterozygous form reduces tumor multiplicity in Min mice. Both the *Mom1* resistance and the *Dnmt1*$^{-}$ deficiency affect tumor multiplicity only quantitatively. But, when put together, these two modifiers are synergistic (Table 4). Thus, an important strategy is to discover single modifiers on well-controlled conditions and then investigate what pairs of modifying alleles will synergize. Progress toward this goal may discover new routes of combination therapeutics.

In the laboratory rat, major investments in the development and genotyping of hundreds of inbred and recombinant-inbred strains will enable powerful studies of polymorphic quantitative and qualitative modifiers of the Pirc phenotype. Many of these investments have been made by investigators in Japan.

Table 3. Genetic modifiers of *Apc*^{Min}

Modifier effect	Gene
Karyotypic stability	*BubR1, Cdx2, Terc*
DNA mutation rate	*Pms2, Mlh1, Msh2, Msh3/Msh6, Fen1, Myh*
Recombination rates	*Rb9, Recq14, Blm*
Differentiation	*EphB2, EphB3*
DNA methylation	*Mbd2, Mbd4, Dnmt1*
Stromal regulation	*Fox11, TSP1*
Cell growth and regulation	*C-Jun, Cyclin D1, Egfr, p21, p27, p53, Igf2, Pla2g2a, Atm*
Pleiotropic	*Matrilysin, BAH, E-cadherin, PPAR-δ, Netrin-1, Smad4*

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In: Nathke and McCartney, eds. *Apc Proteins*. In press.

Table 4. *Mom1*^{R} synergizes with *Dnmt1*$^{N/+}$ to reduce intestinal adenomas

	Tumor count, mean ± SD (N of mice)	
	Dnmt1 $^{+/+}$	*Dnmt1* $^{N/+}$
Mom1$^{S/S}$	88 ± 30 (35)	41 ± 15 (23)
Mom1$^{R/S}$	44 ± 21 (29)	13 ± 9 (22)
Mom1$^{R/R}$	16 ± 7 (6)	2 ± 2 (16)

R.T. Cormier and W.F. Dove
Cancer Res. 60: 3965-3979 (2000)

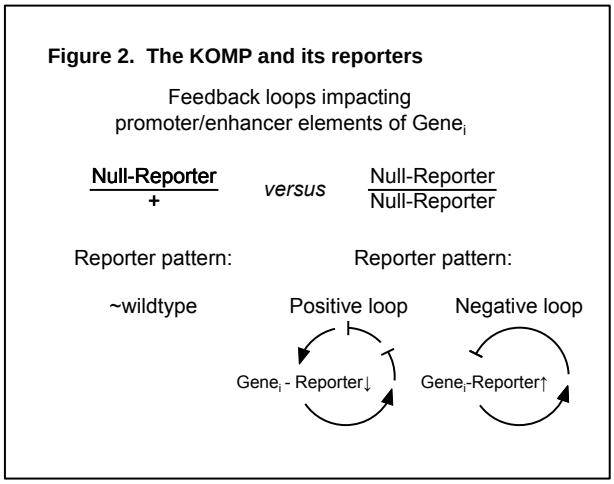
The development of molecular markers and the distinction between these markers and factors that are causative in pathogenesis

Murine models are contributing to the discovery of molecular markers for colon cancer. The genetics of the mouse models then can lead to determining which of these markers has a causative role in oncogenesis. By cDNA subtraction, we have found that the secreted glycoprotein clusterin is strongly expressed in Min intestinal adenomas. Clusterin expression has been analyzed at cellular resolution by both immunohistochemistry and *in situ* hybridization. This molecule is diagnostic of the early human adenoma, both tubular and villous, and is much less strongly expressed in normal tissue, in the more benign hyperplastic polyp, and in the more advanced adenocarcinoma[7].

The knockout mouse project, recently implemented by the Human Genome Research Institute, can strongly impact this realm of investigation[8]. It permits further discovery of

markers, and provides homozygous mutant tissue to serve as rigorous negative controls in which to test cross-reaction. Crucially, a knockout allele also allows one to find out whether a marker has a causative function in the pathogenesis of the colonic tumor. For example, are clusterin-null tissues negative for immunohistochemistry and *in situ* hybridization with the available reagents for clusterin? Is there a detectable effect on the Min phenotype by nullizygosity for clusterin?

The mouse knockout project has been designed in a way that is important for the discussions in this Symposium: to discover the logical structure of the set of genes expressed in tumors. Which genes are essential elements in feedback groups, either creating homeostasis in normal self-renewal by negative feedback control, or establishing fixed nodes in tumor development by the action of positive loops? These issues can be addressed with the lacZ or GFP reporters linked to the null targeted allele. When the null allele is heterozygous, the reporter indicates where in biological space and time that gene is expressed, in normal tissue and in tumors. Consider, then, the situation when the null allele is made homozygous, either constitutionally or conditionally. If the gene is an essential element of a positive loop in the space and time of interest, the gene will no longer be expressed in the null homozygote. If, by contrast, it is a necessary element of a negative loop, that gene will become constitutively expressed in that space and time of normal or neoplastic development (Figure 2).



Cellular interactions in tumor formation – the issue of polyclonality at least in early stages of tumor formation

We have studied the polyclonality of Min adenomas, using aggregation chimeras made by joining together early embryos that differ in respect to the lineage marker Rosa26 that expresses lacZ in all derivatives of its somatic lineage, whether they are embryonic, differentiated or neoplastic. The fine-grained chimeras in these experiments have very small patch sizes, allowing us to study interactions that operate over very short distances. In contrast to a model of random collision, we have found that, even at very low tumor multiplicities, a high fraction of mixed tumors is observed[9].

Here is our current view: homeostatic normal self-renewal of crypts is clonal in the adult mouse. When homeostasis is lost, the conversion to dysplasia is enhanced by clonal cooperation. The factors mediating this cooperation may provide an important target for chemoprevention[10]. Adenoma growth and progression can be accompanied by clonal purification, either by productive cooperation within a clone, or by mutation followed by selection of the dominant clone within the adenoma, or simply by neutral drift within the polyclonal adenoma. Lineage marking at high resolution is needed to determine whether further clonal cooperation operates in the more advanced stages of colon cancer, including metastasis.

We intend to bring forward our studies with these well-controlled animal models toward an understanding and management of human colon cancer. One very important way to manage human colon cancer is to develop methods for early detection. We have only begun to explore ways in which to use the Min and Pirc models to utilize candidate secreted molecules such as clusterin for the detection of early neoplasms. To detect chemotherapeutic and chemopreventive agents by the use of these models involves an appreciation for the non-linear dynamics in cancer

(Figure 3). The normal self-renewal process is robust and requires multiple events to be disrupted - clonal cooperation as in polyclonality, or age-dependent epigenetic changes, and certainly two hits to the Apc gene. Are these two genetic hits; can they be two epigenetic hits? On the other side, the neoplastic state is also a robust state that is addressed best by combination of genetic modifying factors, *Mom1* and *Dnmt1*. Similarly, Dr. Vogelstein and his colleagues, working with EGF inhibitor EKB569 from Ayerst, showed that the NSAID Sulindac and this EGF inhibitor each have a small effect on the Min phenotype, but together have a very pronounced effect. Overall, the discovery of single factors each with a small effect, followed by wise judgment of effective combinations, is enhanced by having experimental models, such as the Min mouse and the Pirc rat, in which genetic and environmental variation can be minimized.

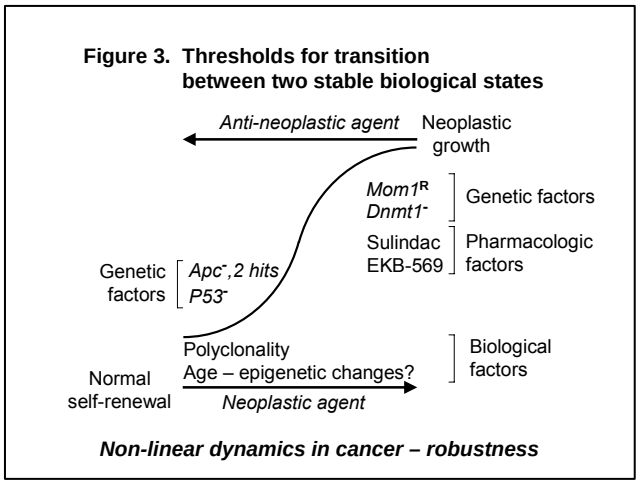


Figure 3. Thresholds for transition between two stable biological states

Non-linear dynamics in cancer – robustness

Thus, we need to develop ways to predict what synergies are involved in tumorigenesis and what synergies will be effective for therapeutics. But also, by using these models, we need to learn from the natural history of individually annotated tumors. Current longitudinal studies by our clinical colleagues at Wisconsin have followed colonic adenomas in human patients by virtual colonoscopy[11]. A significant subset of polyps regresses spontaneously. An adenoma that regresses is, from the point of view of the tumor, a failed neoplasm. What are some possible causes for this? Perhaps that neoplasm has too much genomic instability, or perhaps it has too much epigenetic silencing, or perhaps it is too immunogenic and is attacked by the host (as Dr. Nakahara would have liked to see!). There may be other explanations that we haven't yet considered. By following tumors longitudinally, taking biopsies at the beginning of the experiment, and finally observing the fate of the tumor one can begin to learn what are the natural causes of success and failure for early tumors. Longitudinal studies in murine models for colon cancer are made facile by the Storrs Coloview Endoscope that Marcus Neurath has helped to develop[12].

These are the ways in which we have used the Pirc rat and the Min mouse to develop the issues of somatic genetics and epigenetics, modifier loci, molecular markers versus causative factors, and cellular interactions of polyclonality. We don't believe that either of these models is a complete recapitulation of the landscape of human colon cancer. We simply use them as models in which well-controlled experiments can be done to test explicit hypotheses. At the end of the day, we need to know whether a hypothesis that holds true in both the Min mouse and the Pirc rat also holds true for corresponding stages in human colon cancer. To paraphrase the 1950s Presidential candidate Adlai Stevenson, [An animal model, like smoking,] is all right if you don't inhale!

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