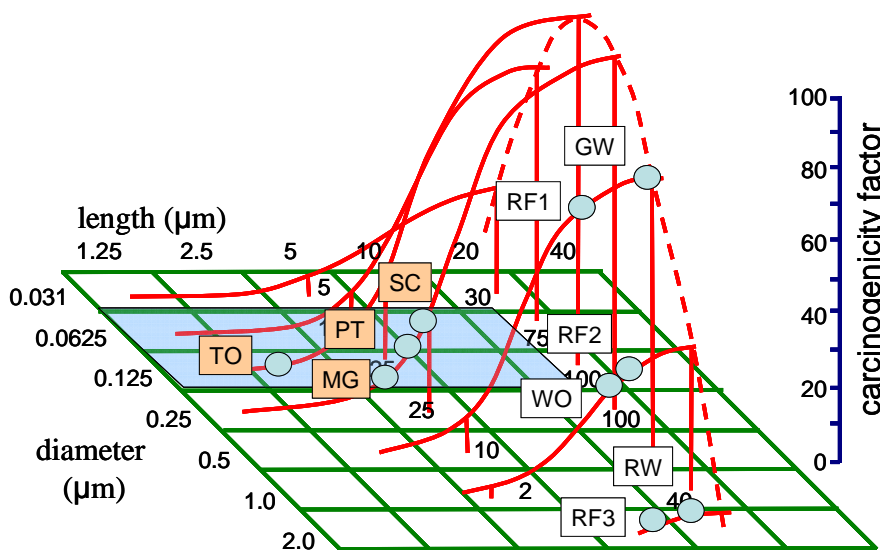


Reply of Dr. Kanno and Dr. Hirose to Comments/Critique by Prof. Ken Donaldson during his Interview with ICON, as posted at the ICON Website  
 (http://icon.rice.edu/resources.cfm?doc\_id=12344)

Thank you for giving us an opportunity to comment on the work of Dr. Ken Donaldson's recent paper, as well as to reply to his critiques of our paper in his Interview as posted on the ICON website.

First of all, Dr. Donaldson's paper supports our findings that, although the observation period was only 7 days, MWCNT (of the same origin) elicits biological responses similar to asbestos in abdominal mesothelial surfaces of C57BL/6 mice (the same strain background to our p53+/- mice), as well as showing *in vitro* that "frustrated phagocytosis" is a common phenomenon to rod shaped particles sharing a certain range of specifications. The paper also confirms that the observation/hypothesis of F. Pott (Staub-Reinh. Luft, 38:486-490, 1978) applies to MWCNT, although it would have been best if MWCNT of one make, for example the Mitsui MWCNT, was fractionated into different lengths and compared, rather than comparing fibers of different makes. (One issue that we cannot understand is that the iron content of the Mitsui MWCNT is reported as ND (non-detectable) by Poland, et.al; our data showed 3,500ppm of iron content.)

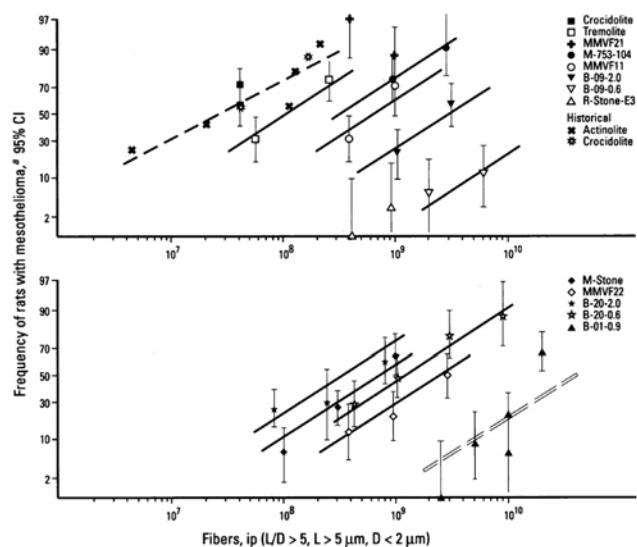


Suspected tumorigenicity of JFM standards fibers on the hypothetical curve by Pott(1978) From: Adachi et al., (2001)(Each spot indicates the geometric mean of length and width. PT:Potassium titanate whisker; SC:silicon carbide whisker; TO:titanium oxide whisker; MG:Micro glass fiber)

(From Adachi et al., Ind Health. 2001 Apr;39(2):168-74.)

Our study is based on the hypothesis that rigid and biopersistent fibrous particles of a size and shape similar to asbestos, or more precisely those fulfilling the dimensions postulated by Pott in the 1970s, share the property of mesotheliomagenic potential. For the dose selection,

we referred to the paper of M. Roller, F. Pott et al. (EHP 105(S5), 1253-1256, 1997). As shown in Figure 1 of that paper, the “weakest” mesotheliomagenic fibers showed potency at around  $10^{10}$  fibers i.p. (longer than 5 micrometers). To identify the mesotheliomagenic potency of the MWCNT as a first step in a hazard identification, we adopted this value as a top dose. In other words, if results are negative at this dosage, it is highly possible that MWCNT will be negative for mesotheliomagenesis. We consider that this approach is also supported by the clinical/epidemiological and mechanistic understanding that there would be no threshold for the fiber mesotheliomagenesis.



**Figure 1.** Probit analysis of the relationships between injected number of fibers and frequency of rats with peritoneal mesothelioma in ip injection studies. Data from three series of experiments (1990–1992) were combined for analysis and presented in two separate panels for clarity. For comparison, results from asbestos dusts depicted previously (6) have been added. The corresponding broken line has been independently fitted to the combined historical data of actinolite and crocidolite. \*Historical data: mesothelioma/sarcoma. Abbreviations: L, length; D, diameter.

In this regard, our paper assesses the micrometer-sized particles within the bulk Mitsui MWCNT, which is a mixture of a wide range of particles from micrometer down to nanometer dimensions. Our study does not assess any of the biological responses caused by the nanometer particles in this material.

We would like to reply to the critiques posed by Dr. Donaldson in the ICON interview.

1. The delivery of the dose. In toxicology the dose is everything. The dose drives the response. In our study, the effective dose is long nanotubes. Their dose was big clumps of nanotubes, 100 microns in diameter, far bigger than any cell. They would never have reached the part of the lung where macrophage phagocytosis, a key factor in determining fiber pathogenicity, would have occurred. Nor are such big lumps likely to reach the sensitive mesothelium.

### Reply:

The phrase “In toxicology the dose is everything” probably originated from the statement made famous by Paracelsus, a 15<sup>th</sup> century physician and alchemist: “All things are poison and not without poison; only the dose makes a thing not a poison”. This statement is often

referred to as the basic concept of toxicology. Originally, it provided more or less a legacy for a pharmacologist to seek “hormesis”, or the low dose beneficial effects of various toxins. Mechanism-based toxicology, however, clearly shows that “Paracelsus is not enough”. A good example is asbestos mesotheliomagenesis. Frustrated phagocytosis theory, as well as experimental and epidemiological data, indicates that there is no threshold - there is no non-toxic (nor beneficial) dose-range - for asbestos. At the hazard identification stage, this comment seems irrelevant. For regulation, it would be proper to introduce the concept of a “virtually safe dose (VSD)”.

It is important to use biologically low-toxic mediums to apply test chemicals to animals in any toxicology study. The medium we used for dispersing MWCNT has limited ability to disperse all MWCNT into individual fibers, even though, as shown in Figure 2a of our paper, there are considerable portions of dispersed fibers in the suspension. Moreover fibers are falling off from the surface of the clumps as shown in Figure 2d, indicating that this MWCNT is not hydrophobic. The asbestos and MWCNTs that were effective for induction of mesotheliomas were the free, well dispersed portion of the applied suspension. Also, there are plenty of dispersed fibers in both asbestos and MWCNT treated groups that can induce frustrated phagocytosis linked to mesotheliomagenesis. Clumps in both asbestos and MWCNT groups are well segregated from the surface mesothelial cells by granulation and fibrosis (cf. Figures 5 and 6 of our paper).

2. The use of genetically modified mice. To provide a shortcut, they used p53 defective animals. These mice were far more likely to get cancer. That's fine and an interesting model, but what we don't know is if this model is anything like the norm. It's so sensitive that anything could have been an irritant. There was no control. C60 was not a good control since it's an anti-oxidant. In short, this is an unvalidated model for mesothelioma.

**Reply:**

The nature of response of the p53<sup>+/-</sup> mouse to carcinogenic stimuli has long been characterized by groups studying the field of carcinogenesis. As a result, a special issue for this mouse, along with another transgenic mouse (Tg.AC), appears in the journal “Toxicologic Pathology” volume 26, number 4 (1998) arranged by The National Toxicology Program (NIEHS, Research Triangle Park, North Carolina, US), Department of Pathology, NIHS, Tokyo, Japan, and other toxicology groups. Three articles on the p53<sup>+/-</sup> mouse in that volume indicate that (1) only lymphomas and sarcomas are the increased spontaneous tumors, (2) the repertoire of the treatment-induced tumors are the same as those induced in the wild type mice, (3) the onset rate of the spontaneous tumors within 26 weeks for p53<sup>+/-</sup> mouse are lower than in studies for the two year wild type mouse. Therefore, it is not at all true that this

system is hypersensitive to any types of stimuli. For example, phenobarbital, a well known hepatocarcinogenic promoter did not induce liver tumors in p53 +/- mice within the 26 week time period. This negative result is equal to wild type mice in a two year study. (4) Mesothelioma is not reported to be induced spontaneously in the p53 +/- mice. (5) Mesothelioma is not reported to be induced by any treatment in the p53 +/- mice except by asbestos (by Kane et al), and, as Kane's papers show, the p53 +/- mice only exhibit earlier onset and accelerated progression of mesothelioma; no tumor types other than mesothelioma are virtually induced in relation to asbestos treatment. Tazawa's paper indicates the same property of p53 +/- mice, i.e. tumorigenesis observed in wild type mice is accelerated in the system, and the type of tumor (sarcoma) is the same.

As a whole, this p53 +/- mouse system is known to retain mechanistic specificity of the wild type mouse against carcinogenic stimuli, so that the types of induced tumors are the same. Therefore, if the test substance induces reactions including tumorigenesis identical to asbestos, it is highly plausible that the test substance shares the same mechanism with asbestos. All these basic findings of p53 +/- mice favor our conclusion that this study is valid for hazard identification of mesotheliomagenic potential due to a mechanism similar to asbestos. If the mice were to respond in non-specific fashion as the writers of the letter consider, then there should be non-mesotheliomatous tumors, such as malignant fibrous histiocytoma (a typical sarcoma seen in subcutaneous foreign body carcinogenesis), highly induced in the abdominal cavity from the granulation and scars where clumps of asbestos and MWCNT are embedded. But that is not the case. Histopathological findings clearly show that the lesions, including non-neoplastic and neoplastic nature, are qualitatively identical in asbestos and MWCNT treated mice.

3. The size of the dose. Three milligrams, or 3,000 micrograms, is 30,000 times the dose that we find to be the lowest dose that has an effect. It's one reason that the fibers clumped together, probably. They seemed to have used that dose in order to meet the fiber criteria of 10<sup>9</sup>, a dosage defined by the European Union that was in common usage for detecting the carcinogenicity of fibers in rats. But that's a rat dose, and these are mice.

**Reply:**

Our study covers the highest end of dosage used in the past, including those performed before guidelines were established. The aim of our study was to identify whether MWCNT could induce mesothelioma or not and provide information for further dose-response studies if a tumor has been induced. In such a first and limited animal number study, false-negative results should be avoided, and of course, non-specific responses should be avoided as well. As

discussed in the paper and explained in detail above, we consider that the p53<sup>+/-</sup> mouse system is valid for hazard identification of asbestos-type mesotheliomagenesis, and therefore, we think that the selection of high dose in our study is rational (peritoneal surface area of a 25 gram mouse is roughly 4.6 times smaller than for a 250 gram rat, and since about a quarter of total MWCNT was longer than 5 micrometers, this gives a number not extremely larger than  $10^9$  for a rat on a surface area basis). At this stage, what matters most is whether the mechanisms hold for the toxicity. Dose-response issue is the next step in risk assessment. Whether MWCNT reaches the pleural mesothelium when inhaled is beyond the scope of our article and, therefore should be tested elsewhere.

The argument of maximum tolerated dose does not apply to the hazard identification study phase. From the clinical observation of mesothelioma patients in Japan, maximum tolerated dose would not be the proper parameter for this type of exposure such that the clearance of the responsible particle is very slow. Instead, the cumulative dose (or body burden) is the important factor for further risk assessment. There are two issues to be mentioned in relation to this claim. Firstly, in Japan, there are, unfortunately, a group of workers exposed to a very high cumulative dose of asbestos for a considerably long time, so that severe pleural thickening and respiratory disturbance were the first clinical symptoms. Secondly, as reported elsewhere, mesotheliomagenesis by asbestos does not seem to have a threshold. Indeed, 75% of new mesothelioma patients in Japan have no pleural anomaly (plaques, fibrosis, etc) by CT/MRI or X-ray, and a recent study by the Ministry of Environment of Japan showed that 40 % of mesothelioma patients cannot identify where and when they were exposed to asbestos. We consider that it is very important to identify that a hazard of MWCNT through a mechanism which is very likely identical to asbestos could occur before mass exposure takes place in the work place and the market place. From this view point, we consider that high dose studies using a p53 <sup>+/-</sup> mouse system that show mechanistic specificity to asbestos-type carcinogenesis are valid for the first step hazard identification.

We are aware that the dosage was at the high end range in this study. Therefore, we immediately started a follow up dose-response study with MWCNT dosage 10 times (high-dose group, 300microg/animal), 100 times (medium-dose group, 30microg/animal), and 1000 times (low-dose group, 3microg/animal) lower than this study. We have just terminated the study at day 365 and macroscopic findings indicate that mesotheliomas were induced in a dose-dependent manner, including some of the low-dose group mice where no severe peritoneal adhesion was observed.

In conclusion, some of the critiques are based on misunderstanding, and we consider that our study is informative as the first step in identifying the hazard of MWCNT. Our study

confirmed that MWCNT share the mechanism of asbestos in mesotheliomagenesis by the histopathological characterization of biological responses in a mechanistically controlled p53 +/- mouse system. As the next step, a dose-response follow up study is about to be completed, and we should be able to deliver information soon for further risk assessment. We hope that Dr. Donaldson's and our information are broadly distributed to the manufacturers who are planning to use this potential material for future beneficial products, so that at least an asbestos-like disaster is effectively prevented for ever. Finally, we have to add that, as mentioned above, toxicity of nanometer-sized particles in the sample has not been assessed yet. The next main effort should be devoted to the hazard identification and risk assessment of the nanometer-sized fractions.

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