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Review

Progress of malignant mesothelioma research in basic science: A review of the 14th international conference of the international mesothelioma interest group (iMig2018)

Licun Wu[a], Irene Dell'Anno[b], Moshe Lapidot[c], Yoshitaka Sekido[d], Mei-Lin Chan[a], Mikihiro Kohno[a], Veronique Serre-Beinier[e], Emanuela Felley-Bosco[f], Marc de Perrot[a,*]

[a] Latner Thoracic Surgery Laboratories, Division of Thoracic Surgery and Princess Margaret Cancer Centre, University Health Network (UHN), Toronto, ON, Canada
[b] Department of Biology, University of Pisa, Via Derna 1, Pisa, Italy
[c] Division of Thoracic Surgery, Lung Center and International Mesothelioma Program, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA
[d] Division of Cancer Biology, Aichi Cancer Center Research Institute, Kanokoden 1-1, Chikusa-ku, Nagoya, Aichi, 464-8681, Japan
[e] Department of Thoracic and Endocrine Surgery, University Hospitals and University of Geneva, 1211 Geneva 4, Switzerland
[f] Laboratory of Molecular Oncology, University Hospital Zurich, University of Zurich, 8044, Zürich, Switzerland

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ABSTRACT

Here we summarize the most recent update of mesothelioma research in basic science presented at the 14th iMig2018 international conference. The symposium of basic science track mainly focused on the drivers of mesothelioma initiation and progression, molecular pathogenesis, and perspectives on potential therapeutic approaches. This review covers several promising fields including strategies efficiently inhibiting YAP/TAZ functions or their critical downstream targets, heparanase inhibitors, *RAN* depletion, and MIF/CD74 inhibitors that may be developed as novel therapeutic approaches. In addition, targeting mesothelioma stem cells by depleting M2-polarized macrophages in tumor microenvironment or blocking Tnfsf18 (GITRL)-GITR signalling might be translated into therapeutic modalities in mesothelioma treatment.

1. Introduction

Malignant mesothelioma is associated with long-term exposure to asbestos [1–3]. Consequently, asbestos consumption is banned or controlled in a large number of industrialized countries and should be banned worldwide to reduce the incidence of asbestos-related diseases [4–6]. However, the incidence of mesothelioma continues to go up due to the long latency period of 20–50 years between exposure and the development of mesothelioma [7,8].

Mesothelioma is a notorious cancer with poor prognosis, and most patients died within a year after diagnosis. Unfortunately, the efficacy of current treatment modalities remains limited. The first line chemotherapy drugs are composed of cisplatin and pemetrexed, which showed limited although significant survival benefit compared with cisplatin alone [9]. Surgical treatments with extrapleural phneumonectomy (EPP) and pleurectomy decortication (P/D) have been widely performed and may provide benefit in some selected patients although adequately powered randomized trials are lacking to confirm this presumption [10,11]. Quality of life is an important goal of therapy and is generally better for those who underwent P/D compared to EPP, but longer term prospective studies are required to confirm this observation beyond the first year after surgery [12]. The indication for surgery and the type of surgery remain topics of controversy [13]. Hemithoracic radiotherapy after EPP can be safely administered by adjuvant intensity-modulated radiotherapy (IMRT) and tomotherapy, but not all patients are able to complete IMRT after EPP [14]. Hence, the novel approach-Surgery for Mesothelioma After Radiation Therapy (SMART) for resectable malignant pleural mesothelioma was developed. This innovative protocol presents encouraging results and supports future studies to look at long-term outcome in patients with epithelial subtypes [15–17].

Immunotherapies using immune checkpoint inhibitors alone or in combination with other immunotherapies and conventional therapies are considered most promising nowadays [18]. The scientific advances and new frontiers in relation to mesothelioma management achieved in 2017 were reviewed elsewhere [19].

The International Conference of the International Mesothelioma Interest Group (iMig) is held every two years in different countries

* Correspondence author at: Toronto Mesothelioma Research Program, Toronto General Hospital, 9N-961, 200 Elizabeth Street, Toronto, M5G 2C4, ON, Canada.
E-mail address: Marc.DePerrot@uhn.ca (M. de Perrot).

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worldwide [20]. The 14th iMig2018 was held in Ottawa, Canada in May 2018. The multidisciplinary meeting brought together over 500 delegates from around the world and featured the most up-to-date research, diverse topics of interest, and educational sessions with leading experts. The meeting offered plenty of networking opportunities with leading scientists and researchers. Canada hosted the iMig conference for the first time in its capital city. The iMig2018 conference has gathered worldwide experts in different areas of mesothelioma including basic research and clinical management.

We are very excited to summarize the update of most advances in basic sciences on mesothelioma research. The symposium of basic science track mainly focused on the drivers of mesothelioma initiation and progression and perspectives on potential therapeutic approaches.

2. Carcinogenic potential of asbestos and asbestform fibres in mouse models

Dr. Felley-Bosco reported about the investigation of early steps of mesothelioma development after exposure of experimental animals to asbestos [21]. Mesothelioma development is associated with asbestos exposure but early steps of carcinogenesis are ill-defined. To assess the role of loss of homeostasis in the mesothelial environment during mesothelioma development, Nf2+/- mice were injected eight times with asbestos fibers every three weeks in the peritoneum and several parameters were assessed thirty-three weeks after the first exposure. Chronic exposure to intraperitoneal asbestos triggered a marked response in the mesothelium well before tumor development. Mesothelioma developed in 10% of mice, when macrophages and mesothelial precursors ($Msln^+CD90^+CD34^+$) levels significantly increased into the peritoneal cavity, accompanied by increased levels of IL-10, G-CSF, CCL2, IL-6, VEGF, CCL5, IFN-γ, GM-CSF and CXCL1. Transcriptomic profile revealed that 1260 genes were commonly upregulated in tumor and inflamed tissue, including signature of YAP/TAZ activation in inflamed mesothelium which was further activated in tumors, paralleled by increased levels of cells with nuclear YAP. *Arg1* was one of the highest upregulated genes in inflamed tissue and tumor. The presence of Arg1 positive cells and other markers associated with M2-polarized macrophages was confirmed. Inflamed tissue showed increased levels of single nucleotide variations, with an RNA-editing signature, which were even higher in the tumor samples. Subcutaneous injection of asbestos-treated, but tumor-free mice with syngeneic mesothelioma tumor cells resulted in a significantly higher incidence of tumor growth when compared to naïve mice supporting the role of the environment in tumor progression.

In this study, they comprehensively describe the alterations of tissue homeostasis prior to cancer development by analyzing the mesothelium and its environment at the histological and molecular level and they report for the first time A to G RNA editing signature, YAP/TAZ activation and a protumorigenic environment as early steps in preneoplastic lesions.

Another interesting presentation was given by Dr. Fisher from the University of Western Australia. They assessed the carcinogenic potential of asbestos and asbestiform fibers in MexTAg mouse, which is a transgenic mouse model replicating many aspects of human mesothelioma [22]. Their data demonstrate the carcinogenic potential of various types of amphibole and serpentine asbestos fibres via their capacity to induce ARD in MexTAg mice. Although ARD incidence was similar between asbestos exposed groups, disease latency was significantly shorter for amphibole exposed mice. In contrast, significantly less ARD and increased disease latency were observed following erionite exposure, suggesting a less carcinogenic profile for this asbestiform mineral in MexTAg mice.

In the recent years, emerging evidence has indicated that exposure of mice to carbon nanotubes induced similar pathogenesis with exposure to asbestos, including initial and chronic inflammation, loss of tumour-suppressor pathways and eventual development of malignant mesothelioma, suggesting that nanotubes may result in significant health risks to humans [23,24].

3. Molecular pathogenesis of mesothelioma in mouse and human

Dr. Sekido from Aichi Cancer Center Research Institute, Nagoya, Japan presented the most recent findings in his group on Taz-Nf2-Hippo signalling pathway, specifically entitled: Taz activation by Nf2-Hippo pathway dysregulation induces cytokine expression and provides growth advantage to mesothelioma.

3.1. Inactivation of the Hippo pathway in mesothelioma cells

Malignant mesothelioma (MM) is characterized by a relatively smaller number of genetic mutations than other human malignancies, most driver mutations being detected in tumor suppressor genes [25]. The *NF2* tumor suppressor gene, which encodes Merlin, a member of ezrin/radixin/moesin protein family, is inactivated in approximately 40% of MM cases. Merlin regulates the Hippo signalling pathway, which regulates organ size and tissue homeostasis [26]. Besides *NF2*,

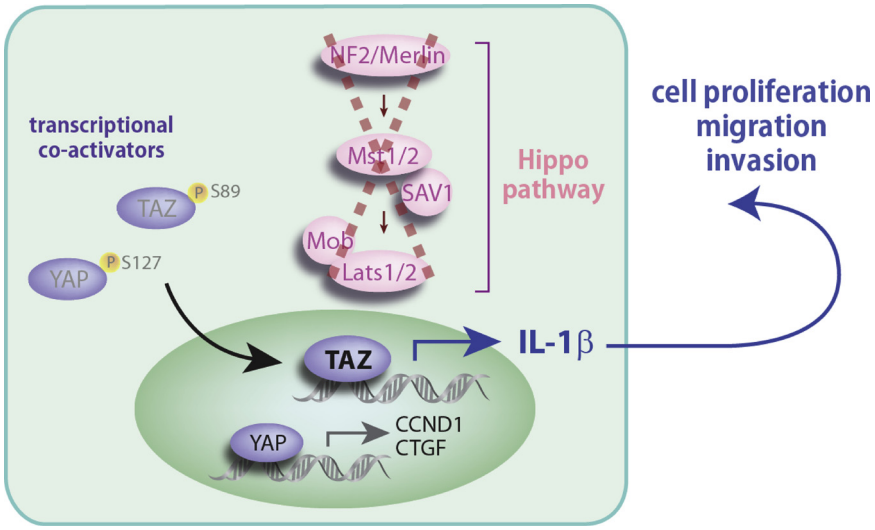


Fig. 1. The NF2-Hippo pathway is frequently inactivated in malignant mesothelioma cells.
Inactivation of the Hippo pathway constitutively activates TAZ and YAP. Activated TAZ upregulates *IL1B* and confers malignant phenotypes.

other components of the Hippo pathway are also inactivated in MM including MST1/2, LATS1/2, and SAV1 [25] (Fig. 1). Inactivation of the Hippo pathway induces constitutive hypophosphorylation (activation) of YAP transcriptional coactivator. Activated YAP enhances expressions of oncogenes including cyclin D1 (*CCDN1*) and connective tissue growth factor (*CTGF*) [27,28]. CTGF is involved in epithelial-mesenchymal transition (EMT) in mesothelioma cells and in the formation of the extracellular matrix. While YAP plays a pro-oncogenic in MM, it is yet unclear whether TAZ, a YAP paralog, also contributes to MM pathogenesis and progression.

In the mini-symposium, Sekido et al. reported the pro-oncogenic functions of TAZ in MM. Using a panel of MM cell lines, they showed that the majority of MM cell lines show more activation of TAZ compared to immortalized mesothelial cells. Upon shRNA-mediated knockdown of TAZ in MM cells upregulating TAZ, cell proliferation, anchorage-independent growth, and cell motility and invasion were strongly inhibited in vitro. Moreover, using immortalized mesothelial cells, transduction of a constitutive activated form of TAZ enhanced these phenotypes in vitro.

They further analyzed the downstream target genes of TAZ in mesothelioma cells via microarray analyses. While most upregulated genes were common between the TAZ- and YAP-activated cells, TAZ induced genes encoding cytokines and their receptors to a greater extent than YAP. Among the upregulated cytokine genes, TAZ directly bound to the promoter region of the *IL1B* gene along with TEAD transcription factors, thereby enhancing its transcription and inducing cell proliferation. In contrast, IL1B knockdown or anIL-1 receptor antagonist inhibited cell proliferation, suggesting that suppression of IL-1 signalling may have stronger inhibitory effects on MM cells actively expressing TAZ. Taken together, TAZ-IL-1-beta axis was considered essential for the acquisition and maintenance of malignant phenotypes in MM cells (Fig. 1).

Since MM cells do not harbor mutations in well-known oncogenes such as receptor tyrosine kinases, different approaches are needed to develop molecular-targeted therapies for MM. Because the Hippo pathway is frequently suppressed in MM cells, thereby inducing constitutive YAP/TAZ activation, targeting the Hippo pathway would be a reasonable potential therapeutic approach for MM [29]. Although YAP and TAZ are not kinases, thereby posing a difficulty in drug development, various strategies efficiently inhibiting YAP/TAZ functions or their critical downstream targets are currently under investigation [29].

3.2. Evaluation of RAN gene using siRNA and CRISPR/Cas9 in pleural mesothelioma

Dr. Dell'Anno from University of Pisa, Italy, reported their recent findings on the evaluation of RAN gene using siRNA and CRISPR/Cas9 gene editing in pleural mesothelioma.

Ras-related nuclear protein (RAN) is a small GTP binding protein, member of the RAS superfamily, acting as a "master regulator" of several and various cellular processes [30]. The best known is the nucleocytoplasmic transport of proteins and ribonucleoprotein molecules (called "cargo") [31]: RAN alternates between two different states, GTP- (mainly in the cytoplasm) and GDP-bound (mainly in the nucleus), resulting in a gradient across the nuclear envelope. By coupling cargo-transport receptor interactions to this cycle, RAN drives the shuttling of different cargoes through the nuclear pore complex in both import and export directions [32–34]. A similar mechanism regulates the nuclear envelope formation and mitotic spindle assembly. In this latter RAN allows the release of microtubule associated proteins (MAPs), such as TPX2, NuMA and HURP [35], from their import receptors close to the chromosomes, in order to increase their activity *in situ* and allow the formation of a proper and functional mitotic apparatus [36,37]. In the light of this brief overview it seems clear that *RAN* has a pivotal role in the physiological functioning and living of eukaryotic cells and deregulation in this sensitive RAN-network could easily result in errors and impairments, predisposing the cell to become malignant. Therefore, it is not surprising that the greatest abundance of RAN is found in multiple human tumours, among which renal cell carcinoma, pancreatic and breast cancer and that it correlates with malignant cell survival and cancer progression [38–42]. Previous studies showed *RAN* gene is up-regulated in mesothelioma tissues and cell lines [43], nevertheless little is known about its role in MPM. The purpose of this study is to explore the functional role of *RAN* and its potential involvement in the carcinogenesis of MPM. The role of *RAN* in MPM tumorigenesis was initially investigated through the RNA interference technique, on a panel of one non-malignant mesothelial cell line (Met-5 A) and four MPM cell lines (Mero-14, Mero-25, Istmes-2 and NCI-H28). To validate data from siRNA experiments, two different siRNAs, here called siRAN-1 and siRAN-2, were independently used to target *RAN* gene. The gene knockdown was monitored and confirmed at both mRNA and protein levels in all cell lines. The effect of *RAN* silencing was examined performing a phenotypic study through the Caspase-3/7 luminescence, Sulforhodamine B (SRB), Wound-Healing and Colony Formation assays. Preliminary results showed *RAN* silencing caused an increment of caspase 3/7 activity (i.e. apoptosis) in all the malignant cell lines, compared to Met-5 A, but no significant difference was observed, in any cell lines, with both siRNAs. Regarding the SRB assay, a statistically significant decrease in proliferation rate was observed eight days after the treatment in all MPM cell lines, for both siRNAs. In Met5A we did observe a significant difference between the proliferation curves of the control and the silenced The migration ability was affected in Met-5 A but not in MPM cells. Instead, the colony formation ability was strongly affected by *RAN*-knockdown in all MPM lines in a statistically significant way. Finally, cell cycle analysis was also performed by employing flow cytometry technique. *RAN* depletion induced an increase of cells in G0/G1 phase and an interesting decrease of cells in S phase, being significant in Mero-14 cell line only. In view of the above results, *RAN* gene was also knocked-out by using a lentiviral CRISPR/Cas9 system in Mero-14 cell line. gRNA and Cas9 endonuclease were transduced by two different lentiviral transfer vectors, pLX-sgRNA and pCW-Cas9, respectively. The doxycycline-regulated Cas9 induction was followed by DNA, RNA and proteins extraction. The occurrence of gene disruption has been confirmed from all the three points of view. Interestingly, *RAN*-deficient Mero-14 cells appear clearly impaired and grow slowly. In light of the important changes elicited in mesothelioma cells, compared to the treatment with siCTRL, *RAN* gene appeared to have a key role for the maintenance of the malignant phenotype. Ongoing experiments will hopefully enhance our understanding of the mechanism involving *RAN* in MPM. *RAN*-deficient Mero-14 cells will be employed as a model to confirm not only the phenotypic assays but also the suitability of *RAN* as anti-cancer targets.

4. Potent therapeutic strategies for malignant mesothelioma

Dr. Lapidot from Harvard Medical School, Boston, USA in collaboration with Technion, Israel Institute of Technology, Haifa, Israel and Langone Medical School, New York University School of Medicine, presented their recent results on a potential therapeutic strategy for malignant mesothelioma with heparanase inhibitors.

4.1. Haparanase inhibitor-a potential therapeutic strategy for malignant mesothelioma

Heparanase is an endo-β-D-glucuronidase that cleaves heparan sulfate (HS) side chains of heparan sulfate proteoglycans (HSPG). This activity is responsible for remodelling of the extracellular matrix (ECM), thereby promoting cell dissemination associated with tumor metastasis, angiogenesis and inflammation [44,45]. Heparanase expression is augmented in numerous cancers, including carcinomas, sarcomas, and hematologic malignancies [46–48]. Clinically, elevated heparanase levels are most often associated with increased angiogenesis

and metastasis, and reduced postoperative survival of cancer patients [44,45,49–51]. These aspects are to a large extent relevant to malignant pleural mesothelioma, a highly aggressive tumor characterized by rapid and diffused local growth in the thoracic cavity. The etiology of the disease involves a long latency period that is extended by durable asbestos fibers, the tumor microenvironment, and inflammatory stimuli [52,53]. The overarching hypothesis guiding this research is that heparanase is a key regulator of the aggressive phenotype of mesothelioma, and the objective of the study was to further elucidate the biological significance of heparanase as a therapeutic target in mesothelioma.

Heparanase expression and enzymatic activity was evaluated first in human pleural mesothelioma cell lines (i.e, MSTO-211H, H2052, CD484, CD487). The effects of heparanase gene silencing and inhibitors (PG545, Defibrotide) were examined in vitro and in vivo (using mouse model), for their ability to restrain mesothelioma tumor growth in comparison with conventional chemotherapy (i.e, cisplatin). Tumor growth was inspected by IVIS methodology and mortality rates were compared between the study groups. In addition, Synchronous pleural effusion and plasma samples from 63 patients with mesothelioma, other malignancies, and benign effusions were collected and analyzed for heparanase content (enzyme-linked immunosorbent assay). Biopsies from 68 patients with mesothelioma were analyzed by H-Score for the prognostic impact of heparanase using immunohistochemistry.

Analysis of the results demonstrates that heparanase is expressed and highly enzymatically active in pleural mesothelioma cell lines. Moreover, AE17 mouse mesothelioma cells developed significantly smaller tumors when inoculated subcutaneously in heparanase knockout (Hpa-KO) vs control mice. Immunostaining revealed lower proliferation and higher apoptosis rates in tumors developed in HpaKO mice associated with reduced ERK and c-Jun phosphorylation and VEGF expression. Likewise, heparanase gene silencing inhibit cell invasion in vitro and tumor xenograft growth in-vivo. Mesothelioma tumor growth, measured by bioluminescence or tumor weight at termination, was markedly attenuated by heparanase gene silencing ($P = 0.02$) and by heparanase inhibitors (PG545 and defibrotide; $P < 0.001$ and $P = 0.01$, respectively). Furthermore, heparanase inhibitor (PG545) attenuated cell invasion and anchorage independent growth of mesothelioma cell lines in vitro, and reduced mesothelioma tumor xenografts growth associating with decreased angiogenesis and Akt phosphorylation. The effect of heparanse inhibitors on the tumor microenvironment was demonstrated by collapsed tumor vasculature devoid of a typical patent lumen, leading to impaired angiogenesis and localization of macrophages. Notably heparanase inhibitors significantly increased the survival rates of mice implanted with mesothelioma cells, beyond cisplatin treatment ($p = 0.0012$) (Fig. 2). Clinically, heparanase levels in patients' pleural effusions could distinguish between malignant and benign effusions, and a heparanase H-score above 90 was associated with reduced patient survival (hazard ratio = 1.89, 95% confidence interval = 1.09–3.27, $P = 0.03$).

In summary, these results strongly imply that heparanase is clinically relevant in mesothelioma development. The preclinical and clinical data, demonstrate that heparanase appears to be an important mediator of mesothelioma tumor progression, thus encouraging further development and testing in clinical trials of heparanase inhibitors as a new therapeutic modality for this malignancy.

Another interesting study was reported by Dr. Busacca from Leicester Cancer Centre, University of Leicester, UK. She presented a small molecular transcriptional suppression of PRMT5 to induce synthetic lethality in MTAP-negative mesothelioma [54]. This transcriptional suppressor of PRMT5 is synthetic lethal in the context of MTAP loss and functions through inhibiting c-JUN. Their data reveal a potential path for developing PRMT5 as a therapeutically actionable target in MTAP-negative mesothelioma.

4.2. Experimental model of human malignant mesothelioma in athymic mice for in vivo evaluation of novel therapies

Dr. Serre-Beinier reported about an *in vivo* orthotopic xenograft model of human mesothelioma [55]. Reliable *in vivo* preclinical models are required to validate new therapeutic target for mesothelioma. Several asbestos-induced and genetically engineered murine mesothelioma models have been developed. But their use is limited by a low take rate, a long latency in tumor development, and a high cost. Most preclinical studies on MPM rely on subcutaneous or peritoneal xenotransplants of human mesothelioma cell lines in immunodeficient mice. But the tumor environment is different from the *in situ* thoracic pleural mesothelioma environment. To assess the human mesothelioma development in a tumor microenvironment close to that of the original human tumor, a human mesothelioma cell line was injected into the pleural space of partially immunodeficient athymic mice.

A new human MPM cell line, the H2052/484, was obtained from the dissociation of a pleural tumor formed after NCI-H2052 cell injection in athymic mouse (Fig. 3). H2052/484 cells conserved their mesothelioma phenotype and most characteristics of the parental H2052 cells, and demonstrated faster tumor development. Sizable intrathoracic tumor masses were identified by ($[^{18}F]$FDG)-PET/CT imaging within 2 weeks in nearly all injected mice (86%) and the mice were found to tolerate tumor burden over a two-week time course (median survival of 31 days), without euthanasia requirement due to distress. Except for the lack of T lymphocytes, most other immune cell types are present in the athymic mice, and we observed an increase in the immune cell population in the pleural fluid with the increase of tumor development [56]. The proportion of B lymphocytes ($CD19^+B220^+$) and NK cells ($CD49b^+$) did not change in tumor-bearing mice compared to mice without tumor. Interestingly, as previously shown in an immunocompetent mouse model of mesothelioma [57], an increase in the proportion of myeloid-derived suppressor cells ($CD11^+Gr^+$), monocytes ($CD19^-CD11b^+$), and macrophages ($CD19^+CD11b^+F4/80^+$), especially M2 macrophages ($CD206^+$), were observed during MPM development.

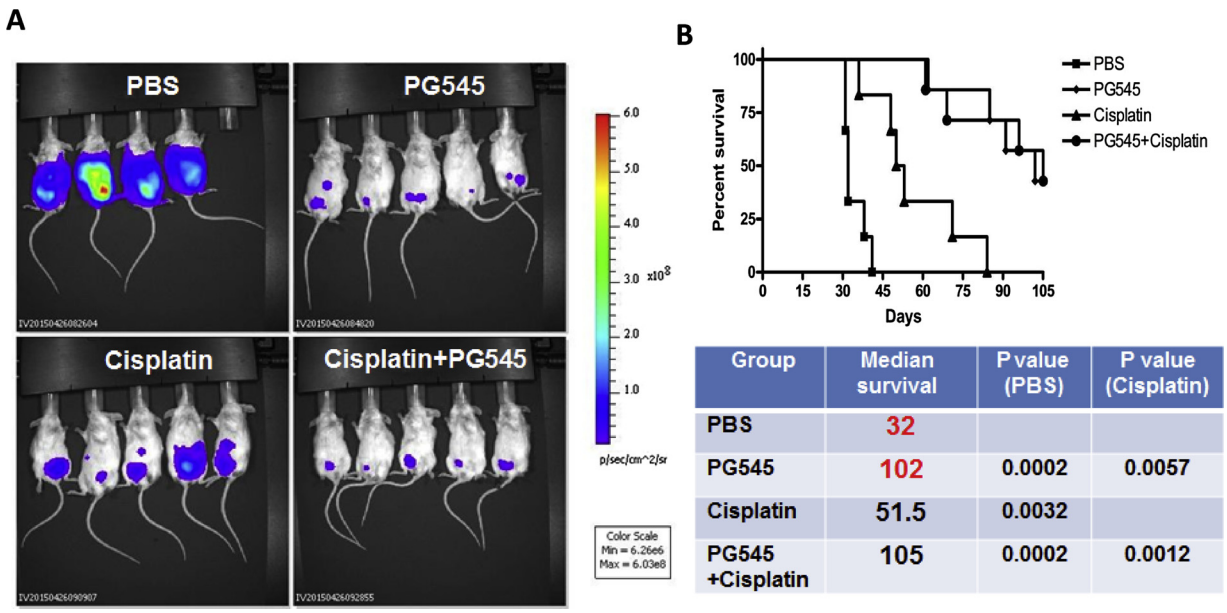
Thus the orthotopic xenotransplantation model of H2052/484 MPM cells in athymic mice is a reproducible model to evaluate the anticancer effects of new treatments and possible tumor relapse and resistance due to subpopulations of cells that might escape therapy. They assessed the inhibition of MIF/CD74 pathway, alone or in combination with cisplatin using this xenograft model, and their preliminary results demonstrate for the first time that treatment with MIF/CD74 inhibitor shows enhanced latency in mesothelioma development compared with cisplatin treatment alone.

Three presentations were delivered from Dr. de Perrot's group, Latner Thoracic Surgery Research Laboratories, Division of Thoracic Surgery, Toronto General Hospital and Princess Margaret Cancer Center, Toronto, Canada.

4.3. Depleting M2-polarized tumor-associated macrophages can decrease mesothelioma cell stemness and tumorigenesis in murine model

Previous study showed that macrophages and mesothelial precursor cells (MPC) accumulate in the peritoneal lavage during mesothelioma development after long-term exposure to asbestos in Nf2 heterozygous mice, suggesting that mesothelioma-associated macrophages (MAM) might be critical to promote tumor growth [21,58,59]. RN5 cell line was hence developed [60]. We aimed at demonstrating a link between MAM and mesothelioma stem cells (MSC).

RN5 mouse malignant mesothelioma (MM) cells were injected i.p. into mice to investigate the changes of MAM in association with MSC at different time points after tumor challenge. Depletion of total macrophages was achieved by using clodronate liposomes (CL), while transgenic mice LysM-Cre/Dicer1lox/lox ($D^{-/-}$) in comparison with LysM-Cre mice were employed to selectively deplete M2-polarized



Group	Median survival	P value (PBS)	P value (Cisplatin)
PBS	32		
PG545	102	0.0002	0.0057
Cisplatin	51.5	0.0032	
PG545 +Cisplatin	105	0.0002	0.0012

Fig. 2. PG545 inhibits mesothelioma tumor growth and significantly increases survival rates.
A)Luciferase-labeled MSTO-211H human mesothelioma cells (2×10^6) were inoculated i.p in SCID mice. Mice were treated with PG545 (400 mg/mouse, once a week), Cisplatin (once/2 weeks; 3 mg/kg), PG545 and Cisplatin combination (Cispaltin + PG545) or control vehicle (PBS) and tumor development was inspected by IVIS imaging. Quantification of the luciferase intensities is shown graphically; B) The effect of PG545 and Cisplatin on mice survival are represented by Kaplan-Meier curves.

macrophages [61]. The characteristics of MAM and MSC were identified using flow cytometry, immunofluorescence staining and RT-PCR. The impact of global vs M2-selective macrophage depletion on tumorigenesis was evaluated by the intraperitoneal spheroid assay and analysis of tumor development on the diaphragm.

The percentage of both MAM and MPC in the peritoneal lavage was significantly decreased after treatment with CL compared with PBS liposomes. Also the number of spheroids considerably dropped from 50.7 ± 4.8 to 9.4 ± 1.3 and from 60.8 ± 4.0 to 9.8 ± 1.0 at 4 and 6 weeks, respectively. In $D^{-/-}$ mice, where M2-polarized macrophages were selectively depleted, the number of spheroids was significantly lower than that in the control LysM-Cre mice. Tumor cell invasion into the muscular tissue of the diaphragm was clearly visible in LysM-Cre mice at 2 weeks after RN5 tumor cell challenge, while in $D^{-/-}$ mice almost no tumor cell invasion was detected in diaphragms even at 4 weeks after tumor challenge. Of interest, in both models of macrophage depletion (CL and in $D^{-/-}$ mice), once tumor spheroids had developed, there were no significant differences in tumor characteristics including the expression of WT1, pan-CK, Laminin, Ki67 and CD31 staining, as well as tumor morphology, suggesting that MAM is more important at the early phase of mesotheliom tumorigenesis.

They concluded that accumulation of MAM and MPC after exposure to asbestos or RN5 challenge facilitate spheroid formation and tumorigenesis, while systemic or selective depletion of MAM results in dramatic reduction of MPC, leading to eventual consequences of limiting sphere formation and tumorigenesis, which suggest that MPC may function as mesothelioma stem cells (MSC) to initiate mesothelioma development and further progression. More interestingly, MAM were found to share some phenotypes with MPC/MSC, indicating that a subpopulation of MAM may adopt MPC/MSC property and play critical roles in mesothelioma tumorigenesis. Putative MSC with more resistance to chemoradiation may be the key factor in cancer cell repopulation leading to treatment failure [62,63]. Further identification of selected genes of interest may be used to evaluate prognosis and to design novel target therapies against MSC so as to eliminate cancer cell repopulation in mesothelioma (Fig. 4).

4.4. GITRL-GITR system promotes proliferation of malignant mesothelioma

Using microarray analysis to compare the gene expression profile of untreated murine mesothelioma cell line (RN5), RN5 treated with cisplatin, RN5 treated with gamma ray irradiation, and enriched mesothelioma stem cell (RN5-EOSPuro2), we found 41 genes potentially linked to cell stemness that were over 2 folds up- or down- regulated in chemotherapy and radiotherapy compared with the untreated RN5 cell lines [62]. Among those 41 genes, Tnfsf18 (also known as GITRL) was one of the cell surface markers. We therefore decided to analyze the presence and role of this receptor in human mesothelioma cell lines.

Two human mesothelioma cell lines (CRL5915, CRL5946) were used in this study. They were treated with cisplatin and Cs-137 irradiator respectively. The RT-PCR and Western Blot were used to evaluate the GITRL and GITR expression level at different time points. In order to evaluate the effect of GITRL-GITR system in mesothelioma cell lines we design in vitro and in vivo model for it. A neutralizing monoclonal antibody was used to break the GITRL-GITR system in vitro and in vivo. In the in vitro model, mesothelioma cells were seeded into 96 well plates and the MTT test was used to check cell viability 4 days after seeding. In the in vivo model we injected the mesothelioma cells into the peritoneal cavity of the NOD/SCID mice. We then sacrificed the mice 4 weeks later and checked tumor growth. Results: Both mesothelioma cell lines demonstrated increased expression of Tnfsf18 (GITRL) and GITR at an mRNA and protein levels after treatment with cisplatin or Cs-137 irradiation. Blocking the conjugation of GITRL to GITR with neutralizing monoclonal antibody decreased cell growth and survival rate in both cell lines after chemotherapy or radiotherapy. In vitro cell viability test, the MTT test results with OD value (at 540 nm wavelength) of cisplatin-treated group versus cisplatin + GITR blocking group was 0.54 vs 0.47 in CRL5946 cell line (n = 6/group, p = 0.014); 0.52 versus 0.39 in CRL5915 cell line (n = 6, p100 μm) in cisplatin-treated group was 30.2 versus 4.5 in cisplatin + GITR blocking group (n = 5, p = 0.0021). The inhibiting effect of GITR neutralizing monoclonal antibody could be demonstrated in vitro and in our in vivo model.

Their results demonstrate that the GITRL-GITR system plays an important role in mesothelioma cells growth and survival after

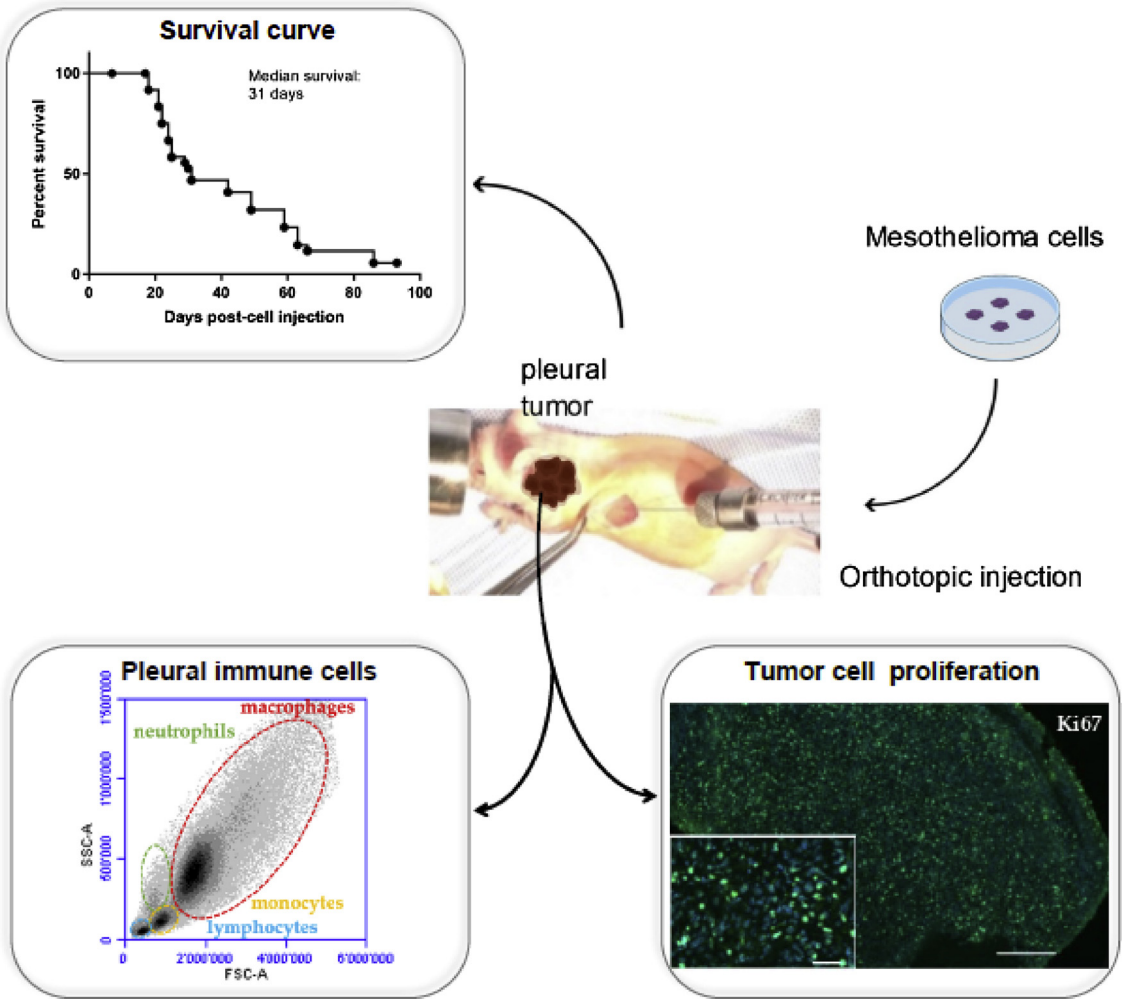


Fig. 3. A new in vivo orthotopic xenograft model.
This model recapitulates human pleural mesothelioma and allows to assess the tumor development and tumor-dependent patterns of inflammation. This model may be used to study the effect of new anti-cancer agents on human mesothelioma development.

chemotherapy or radiotherapy.

4.5. Time course of myeloid-derived suppressor cells and M2 macrophages in murine malignant pleural mesothelioma microenvironment

Solid tumors contain a significant population of tumor-infiltrating myeloid cells that include myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAM) [64,65]. However, the role of MDSCs and TAMs in tumor development and the mesothelioma microenvironment has not yet been fully addressed. We have developed an intrathoracic murine mesothelioma model to investigate the tumor immunity locally in the pleural space. This model has the advantage to provide the pleural effusion as a good surrogate of the tumor microenvironment. Our aim in this study is to elucidate the infiltration of MDSCs and TAMs in pleural effusion at different time points after tumor cell injection, providing insights on the dynamics and kinetics of the myeloid cell recruitment process.

Balb/c mice were injected with 0.5×10^6 of AB12 murine malignant mesothelioma cell line into the pleural cavity. Pleural effusion was collected on days 0 (before tumor cell injection), 3, 7, 10 and 14 after tumor cell injection. On days 0 and 3 before pleural effusion formation, thoracic exudate cells were harvested by washing the thoracic cavity with 1 mL of sterile phosphate-buffered saline. The proportion of MDSCs and macrophages were determined by flow cytometry. Results: Both monocytic MDSCs (M-MDSCs, $CD11b^+Ly6C^{high}Ly6G^-$) and polymorphonuclear MDSCs (PMN-MDSCs, $CD11b^+Ly6C^{low}Ly6G^+$) increased significantly in the pleural effusion from day 3 after tumor cell injection. The peak of M-MDSC accumulation was on day 7, while PMN-MDSCs continued to increase gradually over time. The levels of M-MDSC were significantly lower than that of PMN-MDSCs at all-time points. $F4/80^+CD68^+$ macrophages increased gradually up to day 10 before falling on day 14. On the other hand, the level of $F4/80^+CD68^+CD206^+$ M2 macrophages was stably low up to day 7 and increased dramatically on day 10 and 14. Interestingly, they distinguished two coexisting macrophage subsets: $F4/80^{high}$ cells and $F4/80^{low}$ cells. The percentage of $F4/80^{high}$ cells in live cells was relatively stable up to day 10 (14.8 ± 1.8% on day 0 vs. 15.3 ± 4.0% on day 10), but $F4/80^{low}$ cells rapidly increased on day 3 (2.5 ± 0.5% on day 0 vs. 25.2 ± 8.1% on day 3). The proportion of $CD206^+$ in $F4/80^{high}$ cells was relatively stable over time (22.2 ± 3.2% on day 0 vs. 12.1 ± 1.8% on day 10), but the proportion of $CD206^+$ in $F4/80^{low}$ cells dramatically increased on day 10 and 14 (12.5 ± 1.6% on day 7 vs. 77.6 ± 3.8% on day 10 and 66.2 ± 8.3% on day 14). Furthermore, the proportion of $CSF1R^+$ in $F4/80^{low}$ cells dramatically increased on day 10 and 14 (14.0 ± 1.4% on day 7 vs. 80.0 ± 7.1% on day 10 and 73.3 ± 5.1% on day 14).

The time course of tumor-infiltrating myeloid cells is highly variable depending on the sub-population. M-MDSCs peak early on, while PMN-MDSCs continue to rise throughout the tumor development. $F4/80^{low}$ macrophages contribute to a large proportion of M2 macrophages.

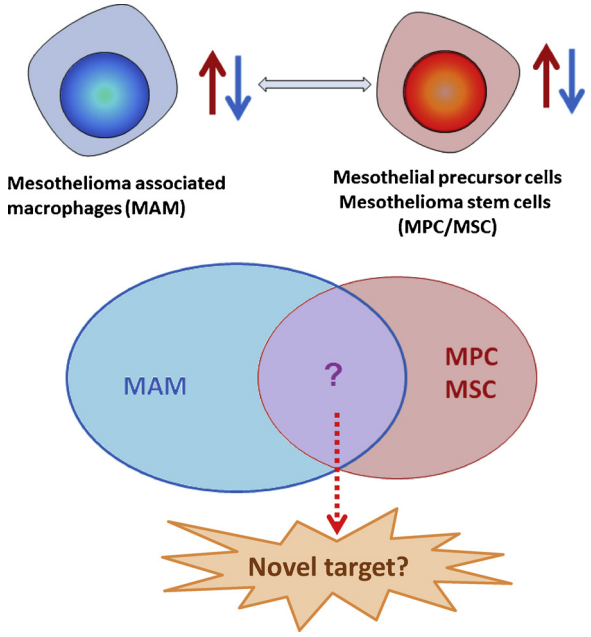


Fig. 4. A subpopulation of tumor-associated macrophages may adopt mesothelioma stem cell property and become a novel target to control cancer cell repopulation.
This model may be useful in search of novel therapeutic approaches to inhibit cancer cell repopulation. Tumor-associated macrophages (TAM) promote cancer survival and progression through a wide variety of mechanisms. Mesothelioma-associated macrophages (MAM) and mesothelial precursor cells (MPC) are found to increase in parallel after exposure asbestos and tumor challenge, and depletion of MAM results in dramatic reduction of MPC, leading to eventual consequences of limiting sphere formation and tumorigenesis, suggesting that MPC may function as mesothelioma stem cells (MSC) to initiate mesothelioma development and further progression. There might be a subpopulation of MAM adopting MPC/MSC phenotypes. Putative MSC with more resistance to chemoradiation is the key factor in cancer cell repopulation leading to treatment failure. Therefore, the subpopulation of MAM may be a novel target to inhibit cancer cell repopulation.

5. Concluding remarks

Better understanding the underlying mechanisms of mesothelioma development and progression would make it possible to design novel therapeutic approaches. Most recent progresses include molecular pathogenesis induced by carcinogenic asbestos and asbestform fibres in mouse and human, tumor infiltrating components in mesothelioma microenvironment and various potential therapeutic approaches. Potential strategies efficiently inhibiting YAP/TAZ functions or their critical downstream targets are currently under investigation. Heparanase inhibitors, suitability of *RAN* as anti-cancer target, and MIF/CD74 inhibitors have shown promising antitumor effect. Targeting mesothelioma stem cells by depleting M2-polarized macrophages or blocking Tnfsf18 (GITRL)-GITR signalling might be new therapeutic modalities in mesothelioma treatment.

Conflict of interest

All authors have read and approved submission of the manuscript. No conflict of interest is associated with this review.

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