A panel of emerging EMT genes identified in malignant mesothelioma

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Malignant mesothelioma (MESO) is a highly aggressive cancer with poor prognosis. Epithelial–mesenchymal transition (EMT) is a critical process in malignancies involved in tumor angiogenesis, progression, invasion and metastasis, immunosuppressive microenvironment and therapy resistance. However, there is a lack of specific biomarkers to identify EMT in MESO. Biphasic MESO with dual phenotypes could be an optimal model to study EMT process. Using a powerful EMTome to investigate EMT gene signature, we identified a panel of EMT genes COL5A2, ITGAV, SPARC and ACTA2 in MESO. In combination with TCGA database, Timer2.0 and other resources, we observed that overexpression of these emerging genes is positively correlated with immunosuppressive infiltration, and an unfavorable factor to patient survival in MESO. The expression of these genes was confirmed in our patients and human cell lines. Our findings suggest that these genes may be novel targets for therapeutics and prognosis in MESO and other types of cancers.

Malignant mesothelioma (MESO) is a rare cancer associated with poor prognosis. Clonal structure in mesothelioma may be a critical prognostic indicator. Histologically, MESO is divided into three major subtypes: epithelioid, biphasic and sarcomatoid. Considerable evidence has shown that biphasic and sarcomatoid subtypes are associated with worse prognosis than the epithelioid subtype, most likely indicating that MESO with dual phenotypes such as biphasic mesothelioma could be an optimal model to study the epithelial-mesenchymal transition (EMT) process, a process through which epithelial cells adopt mesenchymal features.

The EMT is crucial not only to embryonic development but also to carcinogenesis, cancer progression, invasion and metastasis. Under normal conditions, epithelial stem cells play critical roles in tissue repair and regeneration through self-renewal and differentiation capacity. Activation of EMT process has been implicated in normal and neoplastic epithelial stem cells during tissue regeneration and repair.

Cellular heterogeneity and plasticity generated by EMT programs may contribute to our understanding of cancer stem cell biology and cancer metastasis. However, the links between EMT and cancer cell stemness are still elusive. Cancer cells can reactivate EMT programs by increasing their aggressiveness. EMT is associated with enhanced stemness to drive cancer metastasis, recurrence and therapy resistance. While undergoing EMT, most tumor cells adopt some mesenchymal features and maintain some epithelial characteristics. Mesothelioma is characterized by epithelial and mesenchymal diversity, which may make it particularly useful to study EMT. Partial EMT can drive distinct migratory properties and enhance the epithelial-mesenchymal plasticity of cancer cells. Characterization of EMT can be important to determine prognosis and, also, potentially predict response to immunotherapy. Increasing evidence demonstrates that EMT is associated with changes in the tumor immune microenvironment, suggesting that EMT could become important biomarkers in the context of immunotherapy.

Epithelial cells may lose their polarity and cellular adhesions to migrate and invade stroma. EMT frequently takes place at an intermediate state between epithelial and mesenchymal features. EMT is often activated during cancer cell migration, invasion and metastasis. A direct link between the EMT and the gain of epithelial stem cell properties has been investigated previously. Once a metastatic tumor grows, cancer cells will trigger a reverse process of mesenchymal–epithelial transition (MET). Epithelial cells may be triggered to differentiate into a

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A variety of EMT gene signatures have been identified dependent on different cancer types\(^{20,21}\). Each individual cancer may have a particular signature; nevertheless, some cancers may share common gene signatures\(^{22}\). Yet it remains open to clarify the most representative gene signature to each type of cancer, especially mesothelioma. The EMT signature of interest from different sources might be biased towards epithelial or mesenchymal genes, due to cancer type and gene identification method. To avoid that bias, ETomE selected Top50 Epithelial and Top50 Mesenchymal genes to assess how they correlated with each phenotype\(^{23}\).

In this study, we screened EMT-involved hallmark gene sets using murine mesothelioma models, explored EMT-related genes in 32 pan-cancer cohorts, and further analyzed the EMT interactome, EMT score, and correlations with patient survival and immune enrichment in TCGA database to identify EMT specific genes of importance in MESO. These genes could have implications in other tumor types as well.

Results

Overlaps of up-regulated genes in mesothelioma microenvironment from microarray and scRNA-Seq data sets. First, we screened all up-regulated genes in the mesothelioma microenvironment using peritoneal lavage. The up- or down-regulated genes with significant changes over time were analyzed using microarray from single cells collected from peritoneal lavage of RN5-bearing mice. The total number of genes changed over time after tumor challenge (> twofold change, \(p < 0.05\)) (Fig. 1A–C). The overlaps of all up-regulated genes at different time points were determined by GeneVenn. In total, 429 genes were found to be overexpressed at all time points compared with naive controls (Fig. 1D). Peritoneal lavage from naive mice (N) was used as controls.

Cultured mesothelioma RN5 cells were then used to differentiate genes originating from tumor cells (defined as "tumor genes") from those originating from the tumor microenvironment itself (defined as "non-tumor genes") (Fig. 1E). A total of 3637 genes were up-regulated in RN5 cells compared to the peritoneal lavage cells of naive mice.

Single cell RNA-sequencing from the peritoneal lavage of tumor bearing mice was performed at 4 weeks. The 4-week time point was selected as the most representative time point to perform scRNA-Seq in comparison with microarray analysis of peritoneal lavage from naive control since the tumor was always well established and the mice were still healthy. scRNA-Seq demonstrated that 255 genes were up-regulated in intraperitoneal tumor microenvironment at 4 weeks of tumor-bearing mice compared to naive mice (Fig. 1F).

The overlaps between the 429 up-regulated genes derived from the microarray analysis of peritoneal lavage, the 3637 genes from the microarray analysis of cultured RN5 cells, and the 255 genes from scRNA-Seq from peritoneal lavage single cells of tumor-bearing mice identified 55 non-tumor cell genes and 72 tumor cell genes by GeneVenn from the three data sets (Fig. 1G).

The selected lists of 55 non-tumor cell genes and 72 tumor cell genes are presented in "Supplementary data" (Table 1S). The differentiation of tumor genes from non-tumor genes was confirmed in the scRNA-Seq of tumor bearing mice. The non-tumor cell genes were found to be expressed by tumor cells as well in TME ("Supplementary data", Fig. 1S). The identified EMT genes are positively correlated each other in mRNA expression ("Supplementary data", Fig. 2S).

Epithelial–mesenchymal transition (EMT) is the major hallmark gene set in both tumor and non-tumor genes determined by GSEA. We then explored what hallmark pathways these selected genes were involved. Among the list of 72 tumor cell genes and 55 non-tumor cell genes, 12/72 tumor cell genes (FDR \(q\)-value 7.9E – 14, \(p = 1.58E – 15\)) and 11/55 non-tumor cell genes (FDR \(q\)-value 8.59E – 14, \(p = 1.72E – 15\)) are involved in the EMT pathway, identified by MSigDB ("Supplementary data", Table 2S). Top hallmark gene sets are presented in bar graphs (Fig. 2A,B). Gene expression of selected lists is shown in heatmap: 12 tumor cell genes and 11 non-tumor cell genes (Fig. 2C,D). Hallmark EMT was the top pathway involved in both gene sets.

EMTomE: both gene sets were correlated with epithelial and mesenchymal phenotypes. Top genes were identified to be highly correlated with EMT signature. Since EMT is the most out-
Figure 2. Epithelial–mesenchymal transition (EMT) is the top hallmark gene set in both tumor and non-tumor cell genes determined by GSEA. (A, B) 12/72 tumor genes and 11/55 non-tumor genes are involved in epithelial mesenchymal transition (EMT) pathway, identified by http://www.gsea-msigdb.org/gsea/msigdb. Top hallmark gene sets were presented in bar graphs. (C, D) Gene expression in heatmap: 12/72 tumor genes and 11/55 non-tumor genes. Gene set enrichment analysis (GSEA) was computed to identify the overlaps with hallmark gene sets in Molecular Signatures Database (MSigDB) Collections, with FDR q-value less than 0.05.
standing hallmark pathway in both gene lists, we were interested to know which genes played the most predominant roles in this process. EMT score and EMT interactome were thus performed by importing each individual gene of interest.

Each gene was correlated with Epithelial top 50 and Mesenchymal top 50 genes in pan-cancer cohorts. R scores from all genes of interest were plotted in bar graphs of both tumor and non-tumor genes, p values for the mesenchymal phenotypes were also indicated (Fig. 3A). The correlation plot demonstrated positive correlation with the mesenchymal markers and negative correlation with the epithelial markers, thus characterizing the mesenchymal phenotype (Fig. 3B). Summary of top four selected genes (COL5A2, ITGA V, SPARC and ACTA2) which were correlated with EMT signature in 32 pan cancer cohorts was presented in radar graphs, and the well-known CDH1 (Epithelial) and VIM (Mesenchymal) genes were included as controls (Fig. 3C).

Network of OMICS profile of interest for the most significantly correlated genes (COL5A2, ITGA V, SPARC and ACTA2) were presented (“Supplementary data”, Fig. 3S). OMICS includes mRNA, miRNA, copy number alteration (CNA), methylation, and reverse phase protein analysis (RPPA) correlation were also analyzed. Significant correlation was considered between genes (mRNA expression) in TCGA cohort MESO with expression if FDR < 0.05.

The mRNA expression of COL5A2, ITGA V, SPARC and ACTA2 genes correlated with mesenchymal phenotype in epithelioid vs non-epithelioid (biphasic and sarcomatoid) subtypes in MESO cohort. Patients with epithelioid subtype (n = 62) and biphasic and sarcomatoid subtypes (n = 23 and n = 2, respectively) were included. Gene expression of COL5A2, ITGA V, SPARC and ACTA2 (RNA expression in Transcripts Per Million-TPM) was significantly different between epithelioid and non-epithelioid subtypes in TCGA Cohort MESO (p < 0.05 for all comparisons). All four genes had significantly higher mRNA expression in non-epithelioid subtypes compared to epithelioid subtypes as shown in violin graphs (Fig. 4A).

Malignant mesothelioma therefore appears to be an optimal model to study the EMT process. The TCGA database contains only two patients with sarcomatoid MESO, thus limiting our ability to look at significant differences between biphasic and sarcomatoid MESO. Despite this limitation, the trend in mRNA expression of these four genes of interest clearly increased from epithelioid to biphasic and then sarcomatoid MESO (Fig. 4B).

In TCGA MESO cohort, gene expression of COL5A2, ITGA V, SPARC and ACTA2 correlated with the hallmark EMT gen set as shown in scatter plots (Fig. 4C). The expression of each gene was positively correlated with hallmark EMT regardless of tumor stages, all p values for each comparison were less than 0.05, suggesting that these genes are EMT markers in MESO independent of tumor stages. It appears that along with tumor progression, q values tended to be bigger while p values tended to be smaller and far less than 0.05.

Confirmation of EMT gene expression in human mesothelioma tissues and cell lines. In our scRNA-Seq data from the biopsy specimens of naive MESO patients, the patient (SMTR02T0) with biphasic subtype had the highest expression of COL5A2, ITGA V, SPARC and ACTA2 genes compared with other cases who were confirmed epithelioid subtypes (Fig. 4D). Dotplot was shown to compare the five cases of these four genes of interest clearly increased from epithelioid to biphasic and then sarcomatoid MESO (Fig. 4B).

Fluorescent immunostaining of human mesothelioma cell lines showed that sarcomatoid CRL-5946 cells expressed COL5A2 and SPARC dramatically higher compared to epithelioid CRL-5915 cells, while CRL-5915 cells expressed epithelial marker EpCAM specifically (Fig. 4E).

The mRNA expression of top genes COL5A2, ITGA V, SPARC and ACTA2 is associated with overall survival in epithelioid subtype and all MPM patients in TCGA database. High expressions of tumor cell genes COL5A2 and ITGA V and non-tumor cell genes SPARC and ACTA2 are both positively correlated with poor overall survival and disease-free survival in MESO cohort (Fig. 5). The results indicate that overexpression of these genes of interest is unfavourable prognostic factors in mesothelioma patients.

Tumor cell genes COL5A2 and ITGA V expression correlated with overall survival in MESO cohort. Median survival is 13.61mon in COL5A2 ≥ and 24.89mon in COL5A2<, respectively, hazard ratio: 2.318, 95% CI of ratio 1.424–3.774; Median survival is 15.02mon in ITGA V< and 24.07mon in ITGA V≥, respectively, hazard ratio: 1.791, 95% CI of ratio 1.116–2.875 (Fig. 5A).

Non-tumor cell genes SPARC and ACTA2 expression correlated with overall survival in MESO cohort. Median survival is 13.61mon in SPARC< and 26.14mon in SPARC≥, respectively, hazard ratio: 2.337, 95% CI of ratio 1.441–3.791; Median survival is 14.17mon in ACTA2< and 24.36mon in ACTA2≥, respectively, hazard ratio: 2.035, 95% CI of ratio 1.266–3.270 (Fig. 5B).

Tumor genes COL5A2 and ITGA V expression also correlated with overall survival in the epithelioid subtype, demonstrating that epithelioid MESO can acquire EMT characteristics affecting survival before being categorized as biphasic. Median survival is 15.22mon in COL5A2≥ and 28.37mon in COL5A2<, respectively, hazard ratio: 2.823, 95% CI of ratio 1.566–5.088; Median survival is 19.43mon in ITGA V< and 25.94mon in ITGA V≥, respectively, hazard ratio: 1.946, 95% CI of ratio 1.107–3.422 (Fig. 5C).

Similarly, non-tumor genes SPARC and ACTA2 expression correlated with overall survival in epithelioid subtype. Median survival is 17.95mon in SPARC< and 27.06mon in SPARC≥, respectively, hazard ratio: 2.179, 95% CI of ratio 1.230–3.862; Median survival is 14.76mon in ACTA2< and 27.16mon in ACTA2≥, respectively, hazard ratio: 2.148, 95% CI of ratio 1.213–3.803 (Fig. 5D).
Figure 3. EMTome: both gene sets correlated with epithelial and mesenchymal phenotypes were analysed respectively. Top genes were identified to be highly correlated with EMT signature. EMT score and EMT interactorome were performed by importing each individual gene of interest. (A) The correlation of each gene with Epithelial top 50 and Mesenchymal top 50 genes in pan-cancer cohorts. R scores from all genes of interest were plotted in bar graphs of both tumor and non-tumor genes, p values with mesenchymal phenotypes were also indicated. (B) Correlation plot from genes of interest demonstrated positive correlation with mesenchymal markers and negative correlation with epithelial markers. (C) Summary of 4 top selected genes correlated with EMT signature in 32 pan cancer cohorts was presented in radar graphs, and the best-known CDH1 (Epithelial) and VIM (Mesenchymal) genes were included as controls. Network of OMICS profile of interest with significant correlation between gene of interest (RNA expression) in TCGA cohort MESO with OMICS profile were shown in “Supplementary data” (“Supplementary data”, Fig. 3S).

Gene expression of COL5A2, ITGAV, SPARC and ACTA2 correlated with immune enrichment in MESO cohort of TCGA database. To assess the correlation of gene expression with the immune enrichment by EMTome, we imported these genes into the TCGA pan cancers. COL5A2, ITGAV, SPARC and ACTA2 genes had similar patterns in positive correlation with activated CD4 T cells, macrophages, monocytes, mast cells, Th2 and Treg cells, while these genes had negative correlation with Th17 and NKT cells. The representative genes COL5A2 and SPARC were presented in Fig. 6. Heatmaps included 32 cancers (COL5A2 in Fig. 6A; SPARC in Fig. 6B). Correlation with immune enrichment in MESO cohort was shown in bar graphs (Fig. 6C). The most outstanding changes of gene expression were positively correlated with immunosuppressive enrichment including macrophages, Th2, and Treg cells, while negatively correlated with immune enrichment NKT and Th17 cells. These genes were shown quite similarly (“Supplementary data”, Figs. 5S, 6S and 7S).

More strikingly, Timer 2.0 gene module showed strong positive correlation between gene expression and cancer associated fibroblasts (CAF), immunosuppressive infiltrates of MDSC and Th2 cells in MESO (“Supplementary data”, Fig. 8S). Clinical relevance of tumor immune subsets with gene expression showed that either the infiltration of CAF, Th2 cells or EMT gene expression was significantly associated with survival of MESO cohort. Dual high levels of immunosuppressive infiltration and EMT gene expression dramatically increased the risk of poor prognosis, compared to dual low variables. Cox proportional hazard model ran Cox regression and presented clinical relevance of the normalized coefficient infiltrates and correlated with COL5A2, ITGAV, SPARC and ACTA2 gene expression. Kaplan–Meier curves display gene expression associated with clinical outcome in MESO cohort, corresponding to each gene of interest and immune infiltrates of CAF, Th2, and MDSC (Fig. 6D), and M2 macrophage and Treg cells (“Supplementary data”, Fig. 9S).

Discussion

Our previous studies and that of others have indicated that non-epithelioid mesothelioma cells are more aggressive and have higher capacity to form mesospheres in vitro and in vivo. Compared with epithelioid subtype, non-epithelioid mesothelioma cells were more resistant to chemoradiation, and the surviving cells had enhanced stemness. Our findings in this study imply that EMT may be a critical process to predict cancer cell invasiveness and stemness. Nowadays the linkage between the EMT and cancer cell stemness, metastasis and resistance to therapy has been driving efforts to develop novel therapeutic targets. EMT occurs through distinct intermediate states. Biphasic mesothelioma characterized by dual histology could thus best mimic the intermediate hybrid state of EMT stage while transitioning from epithelial to partially and completely mesenchymal states. A recent study revealed that in mouse and human squamous cell carcinoma, loss of function of FAT1 could promote tumor initiation, progression, invasion, stemness and metastasis through inducing a hybrid EMT state. EMT genes can be novel potential targets to interrupt cancer invasion and metastasis. EMT is driven by the SNAIL and ZEB transcriptional repressors of epithelial genes. TGF-β is a potent inducer of EMT specifically working with RAS-MAPK signaling pathway. Our findings identify previously less well recognized genes that promote EMT in MESO. Reversal of EMT into MET process may reduce the capacity of invasiveness, cancer cell stemness and metastasis of mesothelioma and augment their sensitivity to therapy. The genes COL5A2, ITGAV, SPARC and ACTA2 play critical roles resulting in EMT in MESO. Therefore, these genes may be potential therapeutic targets as well as prognostic indicators in mesothelioma.

A previous study investigating the relationship between collagen type V alpha 2 chain (COL5A2) expression and clinical outcome in bladder cancer patients observed that patients with lower expression of COL5A2 had better survival than those with higher expression. Recently, a study evaluated the mutational profile of a panel of 34 genes including COL5A2 gene in MESO. They found that BAP1 mutation was related to a prolonged survival of patients treated with platinum/pemetrexed regimens. Mutations in COL5A2 was observed in 6% of the patients. However, the expression level of COL5A2 was not analyzed and therefore its impact on outcome could not be directly addressed.

It is well known that integrin signaling drives multiple cancer cell functions, including tumor initiation, epithelial plasticity, metastasis, and resistance to targeted therapies. Integrin alpha V (ITGAV), a transmembrane glycoprotein, has been found to enhance tumor progression. ITGAV expression is associated with shortened overall survival in esophageal adenocarcinoma. Genes ITGAV, FN1, and ITGB1 were shown to be the targets of miR-9-3p, which could inhibit proliferation and metastases of nasopharyngeal carcinoma by downregulating FN1, ITGB1, and ITGAV, thus inhibiting the EMT process.

SPARC (Secreted protein acidic and rich in cysteine) gene was up-regulated specifically at the early stage of lung adenocarcinoma consistently with TCGA transcriptome database when EMT markers were screened in a cellular model and validated in lung adenocarcinoma. There was a proteomics-based study to identify SPARC
its immunosuppressive impact43–45. In cancer immunotherapy, therefore, targeting EMT could improve the outcome of immunotherapy by restricting tumour immunosuppressive microenvironment and EMT plasticity. The immunosuppressive TME is a critical hurdle to the efficacy of immune cells release a wide variety of inflammatory mediators and growth factors to facilitate immunosuppressive environment in cancerous cells. The interaction between EMT and immunosuppression promotes tumor progression42. Tumor-infiltrating NKT cells, supporting a notion that EMT process may abrogate the immune response against tumor. Previous studies have shown that EMT transcriptional factors lead to immunosuppressive cell infiltration, resulting in a tumor immunosuppressive microenvironment. The immunosuppressive cells in turn promote EMT in tumor cells. The interaction between EMT and immunosuppression promotes tumor progression42. Tumor-infiltrating immune cells release a wide variety of inflammatory mediators and growth factors to facilitate immunosuppressive microenvironment and EMT plasticity. The immunosuppressive TME is a critical hurdle to the efficacy of cancer immunotherapy, therefore, targeting EMT could improve the outcome of immunotherapy by restricting its immunosuppressive impact43–45.

Our study may be the first time to demonstrate that strong positive correlation of panel EMT genes overexpression with poor clinical outcome, and immunosuppressive enrichment in MESO, indicating that these newly identified genes are most likely powerful drivers of EMT process in MESO. Therefore, regulation of each individual gene expression and its signalling pathways might potentially control EMT process thus leading to development of novel strategies for MESO therapeutics. SPARC protein has been observed upregulated in MESO cell lines46. Transcriptional studies have identified that EMT-linked genes may contribute towards the resistance of chemotherapy and immunotherapy, suggesting that targeting EMT genes may be potentially applied to treat MESO patients47. This work has important implications for targeting nonimmune components in TME to improve the efficacy of chemotherapy and boost the responses to immunotherapy of cancer patients.

In conclusion, utilising transcriptional and EMTome analysis, we identified these EMT genes that are overexpressed in tumor microenvironment of mouse and human mesothelioma. These genes strongly correlate with an EMT signature and prognosis in MESO. Besides a few sporadic studies, this may be the first study to identify COL5A2, ITGAV, SPARC and ACTA2 as a panel of emerging EMT genes in mesothelioma. Furthermore, these genes may be universal EMT markers for most cancer types, as well as prognostic indicators for overall survival across cancer types. Due to a lack of biomarkers to predict survival in epithelioid subtype, it is difficult to further stratify this subgroup of MESO. Encouragingly, this study demonstrated that overexpression of these four genes...
in epithelioid mesothelioma was associated with poor prognosis, indicating that these genes could potentially serve as independent prognostic indicators in this subtype of mesothelioma.

More importantly, overexpression of these genes is associated with an immunosuppressive microenvironment promoted by the EMT process. Therefore, these genes could be potential biomarkers and targets to select appropriate immunotherapy.

**Materials and methods**

The schema of experimental design was depicted in Fig. 1A. Briefly, murine mesothelioma cells were injected intraperitoneally (ip) into wild-type mice, and peritoneal lavage was collected at different time points to investigate gene expression profile in tumor microenvironment (TME). Naïve mice, and cultured tumor cells were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin at 37 °C in an atmosphere containing 5% CO2. Cells were treated with prophylactic 5 µg/ml Plasmocin™

Patients with mesothelioma. Five naïve patients were confirmed with a diagnosis of malignant pleural mesothelioma. Tumor biopsy tissues were processed freshly for scRNA-seq analysis. This study was approved by Ethics Board (REB#19-5858) and all patients signed the consent form.

**Single cell RNA sequencing (scRNA-Seq).** Fresh single cells obtained from peritoneal lavage were processed by Princess Margaret Genomic Centre, University Health Network (UHN), following the standard protocol (www.pmgenomics.ca). Loupe Cell Browser v5.0.0 provided by 10x Genomics was used to analyze single cell gene expression in clusters. A reference mouse genome (mm10) was selected to set threshold of mitochondrial expression in clusters. A reference mouse genome (mm10) was selected to set threshold of mitochondrial expression in clusters. A reference mouse genome (mm10) was selected to set threshold of mitochondrial expression in clusters. A reference mouse genome (mm10) was selected to set threshold of mitochondrial expression in clusters.

Analytical tools for our data sets. The online analytical tools such as Transcriptome Analysis Console (TAC) Software, Loupe Cell Browser https://support.10xgenomics.com/single-cell-gene-expression, https://crescent.cloud/, Gene set enrichment analysis (GSEA) http://www.gsea-msigdb.org/gsea/msigdb/index.jsp, GeneVenn http://genevenn.sourceforge.net/. The Cancer Genome Atlas (TCGA) https://www.cziportal.org/.
Figure 6. EMT gene expression and immune cell infiltration in correlation with clinical outcome in MESO cohort of TCGA database. After importing these genes into EMTome, the correlation of gene expression with the immune enrichment was analyzed in the TCGA pan cancers. COL5A2 and SPARC genes had similar patterns characterized by positive correlation with the activated CD4 T cells, macrophages, monocytes, mast cells, Th2 and Treg cells, and negative correlation with Th17 and NKT cells. (A) Heatmaps included 32 cancers of COL5A2; (B) Heatmaps included 32 cancers of SPARC. (C) Correlation with immune enrichment in MESO cohort was shown in bar graphs of COL5A2 and SPARC. The most outstanding changes of gene expression were positively correlated with immunosuppressive enrichment including macrophages, Th2, and Treg cells, while negatively correlated with immune enrichment NKT and Th17 cells. These genes were shown quite similarly ("Supplementary data", Figs. S5, S6 and 7S). Clinical relevance of tumor immune subsets with gene expression was explored using Timer 2.0 (D). The infiltration of cancer-associated fibroblasts (CAF), Th2 cells and MDSC either alone or in correlation with EMT gene expression increased the risk of MESO. Cox proportional hazard model ran Cox regression and presented clinical relevance of the normalized coefficient infiltrates and correlated with COL5A2, ITGA5, SPARC and ACTA2 gene expression. Kaplan–Meier curves display gene expression associated with clinical outcome in MESO cohort, corresponding to each gene of interest and immune infiltrates of CAF, Th2, and MDSC, and M2 macrophage and Treg cells ("Supplementary data", Fig. 9S). For all integrative survival curves of gene expression and immune enrichment: 1. Blue curve: Low gene expression + Low immune enrichment; 2. Light blue curve: Low gene expression + High immune enrichment; 3. Orange curve: High gene expression + Low immune enrichment; 4. Red curve: High gene expression + High immune enrichment.

EMTome http://www.emtome.org/, and Timer2.0 http://timer.comp-genomics.org/ were employed to identify the EMT genes and signatures in correlation with clinical outcome in mesothelioma.

Overlaps of all genes with significant change. After transcriptomic analysis with TAC and Loupe Cell, the overlaps of all gene lists were determined by GeneVenn. The final overlaps were compared by three major gene lists: all time points up-regulated genes (All wks up), culture RN5 cells up-regulated genes (RN5vsN up) and scRNA-Seq up-regulated genes at 4 weeks (scRNASeq up).

To compute hallmark genesets for selected genes. By importing the gene list from each comparison, GSEA was able to compute and identify the hallmark gene sets in Molecular Signatures Database (MSigDB) Collections. The cut-off value was selected with FDR q-value of 0.05.

EMT genes and signatures of interest analyzed by EMTome. This online program was developed by Dr. S. Mani Team, MD Anderson Cancer Center, Houston TX. The database EMTome collected EMT and MET core gene signatures from publications to identify their interaction with miRNA, transcription factors and proteins using cell lines (CCLE), TCGA, and other datasets. The EMTome acts as resource and a platform to interrogate EMT signatures across cancer types49.

Fluorescent immunostaining in human mesothelioma cell lines. Human mesothelioma cell lines CRL-5915 (characterized with more epithelioid) and CRL-5946 (more sarcomatoid), which were provided by ATCC, were used to stain COL5A2 and SPARC proteins in cultured cells. Cells were fixed with 1% PFA for 15 min, and 1% BSA was to block endogenous enzymes. Triton X-100 (0.25%, 5 min) was added for permeabilization. Primary antibodies rabbit polyclonal to COL5A2 (ab134800) and SPARC (ab14174) were used 1:100 for 1 h at RT, and the secondary antibody anti-rabbit IgG1 conjugated with Alexa-555 was 1:1000 for 1 h at RT. Anti-human EpCAM-FITC monoclonal antibody was applied 1:100. Mount medium containing DAPI was applied to display nuclei.

Analysis of immune infiltrates, gene expression and clinical outcome in MESO by Timer 2.0. TIMER is a comprehensive resource for systematic analysis and visualization functions of tumor infiltrating immune cells, and their association with gene expression and clinical outcome (http://timer.cistrome.org/)51.

Statistical analysis. Transcriptomic analysis was made according to the filter criteria: Fold Change > 2 or < − 2, P < 0.05. The numbers of genes are differentially expressed genes that pass through the filter criteria.

Gene expression (mRNA in TPM) in the violin graph was plotted using Prism 8.0. Unpaired two-tailed t test, with a p-value less than 0.05 was considered a significant difference.

Significant correlation was considered between genes (mRNA expression) in TCGA cohort MESO with expression if false discovery rate (FDR) < 0.05.

Log-rank (Montel–Cox) test was used to compare survival curves. A p-value less than 0.05 was considered a significant difference. Group cutoff median was selected to determine the cutoff-high (50%) and cutoff-low (50%) of gene expression, approximately 50%. Hazard ratio was calculated based on Cox PH Model with 95% Confidence Interval (95% CI).

Ethics approval and informed consent of participants. At University Health Network (UHN), research involving humans conducted within the jurisdiction or under the auspices of UHN must be reviewed and granted written approval by the UHN Research Ethics Board (REB) prior to commencement of research (https://www.uhnresearch.ca/). This applies to research involving humans that is conducted by a UHN Principal
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**Author contributions**

L.W. and M.P. contributed to the overall design of this work, results discussion, data analysis and manuscript preparation. S.M. was invited to discuss about the results presentation, interpretation and the manuscript preparation; L.W. and H.Y. conducted the animal experiments, and molecular work. S.A. joined results discussion, data analysis and manuscript editing. All authors reviewed the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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