Mesothelial Signature in Mesenchymal Stem/Stromal cells derived from High-Grade Serous Ovarian Cancer marks their Identity

Settore scientifico-disciplinare BIO/13

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ANNO ACCADEMICO 2012/2013
Mesenchymal Stem/Stromal Cells (MSCs) are the precursors of various cell types that compose both normal and cancer tissue microenvironments. In order to support the widely diversified parenchymal cells and tissue organization, MSCs are characterized by a large degree of heterogeneity, although available analyses of molecular and transcriptional data do not provide clear evidence. Moreover a wealth of studies has demonstrated a significant role of the microenvironment and MSCs in tumor growth. In the course of the years MSCs were isolated by different groups from different tissues, both healthy and cancerous. The laboratory in which I conducted my PhD project was able to purify MSCs from different healthy tissues (N-MSCs) and, using an adapted protocol, from High-Grade Serous Ovarian Carcinomas (HG-SOC-MSCs). It was possible to shown that these cells do not possess gross chromosomal aberrations and are not tumorigenic in vivo. To better characterize these cells, an integrative bioinformatics analysis was conducted using the deep-CAGE-derived expression profiles obtained from HG-SOC-MSCs, N-MSCs and the FANTOM5 large comprehensive primary cells and tissues dataset. When compared to the other cells and tissues, HG-SOC-MSCs showed a correlation with mesothelial cells and cells hypothesized to have a mesothelial origin, such as smooth muscle cells and fibroblasts. It is known that mesothelial cells in culture can alternate between epithelioid and fibroblastoid morphologies and express high levels of either keratin or vimentin or both depending on their state of growth and the presence of EGF. When cultivated in the absence of EGF, which is known to induce a morphological switch in mesothelial cells, HG-SOC-MSCs switch from a fibroblast-like to an epithelial-like shape. N-MSCs in the same culture conditions, instead, do not change morphology. Moreover, in absence of EGF, HG-SOC-MSCs but not N-MSCs raise the levels of Keratin 7 both in protein and in mRNA. Starting from the list of up-regulated genes in HG-SOC-MSCs compared to N-MSCs a list of mesothelial-related genes was generated. This mesothelial-related gene list was compared to high-throughput gene expression datasets of MSCs derived from other tissues. Such analysis revealed that the mesothelial-related signature is specific to HG-SOC-MSCs. Moreover, Kaplan-Meier survival analysis conducted on a comprehensive SOC microarray dataset showed that patients with higher levels of the mesothelial-related gene signature displayed shorter progression-free survival time. Such correlation was rather specific for HG-SOC given that its performance was either statistically non-significant in the case of lung cancer or correlated with good prognosis in the case of breast cancer. Altogether, the study allowed us to assign a specific identity for MSCs derived from high-grade serous ovarian cancer. We demonstrated a cell-type specific transcriptional activity associated with HG-SOC-MSCs, which identifies them compared to N-MSCs from other districts and position them close to primary mesothelial and mesothelial-derived cells within the FANTOM5 dataset.
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INTRODUCTION

Ovarian cancer is the sixth more common tumor in women (Vargas, 2014). It remains the most lethal gynecological disease and the overall survival rate has not changed in the past 50 years despite the studies carried out in the last few years (Kurman, 2013). Recent molecular genetic studies have illuminated our understanding of ovarian carcinogenesis in unexpected ways and challenged the conventional wisdom regarding their origin and development. One of the major problems is that the ovarian cancer is not a single disease, as it was treated up until a few years ago, but is, in fact, a heterogeneous disease composed of different types of tumors with different features and behaviors. On the basis of morphologic and molecular genetic studies Kurman and colleagues propose a dualistic model that groups various types of epithelial ovarian cancer in two different categories named type I and type II (Shih et al., 2004; Kurman et al., 2011). Type I tumors consist of low-grade serous, low-grade endometrioid, mucinous and clear cell carcinomas. These tumors typically have a large mass confined in one ovary, and are associated to the mutation of KRAS, BRAF, PTEN, PI3KCA, CTNNB1, ARID1A and PPP2R1A (Shih et al., 2004; Kurman et al., 2011; Wiegand et al., 2011). The alteration of the cell signaling due to the abovementioned mutation results in morphological changes which are reflected by a step-wise progression from benign, through varying degrees of atypia (borderline tumor), to noninvasive and then invasive low-grade carcinoma, similar to the progression firstly described in the colon cancer. Type II tumors include high-grade serous, high-grade endometrioid, undifferentiated carcinomas and malignant-mixed mesodermal tumors. They are aggressive and present at an advanced stage, which contributes to their fatality (Kurman et al., 2011). Type II tumors show marked chromosomal aberration in their initial stages which remains stable over the course of the disease while type I tumors are
relatively genetically stable. Since the High-Grade Serous Ovarian Cancer (HGSOC) is the most common, accounting for the majority of deaths, it is also the most studied. The present study focuses specifically on this neoplasy among the ovarian cancer heterogeneity and the cells characterized and described here have been purified from it. It was believed that all the ovarian cancers arise from the Ovarian Surface Epithelium (OSE) or the Cortical Inclusion Cysts (CIC) but a convincing precursor of HGSOC has not been found. In the last few years there has been an increase in prophylactic salpingio-oophorectomies for women at high risk of ovarian cancer due to germ line mutation of BRCA1 and 2. Pathologists therefore began to section the ovaries and the fallopian tubes in search of a lesion precursor of the tumor, but surprisingly they found none in the ovary itself instead they found some occult noninvasive and invasive lesion in the fallopian tube (Piek et al., 2001; Gorss et al., 2010). A gene expression study demonstrated that the expression profiles of HGSOC are more similar to the Fallopian Tube Epithelium (FTE) than the OSE (Piek et al., 2003). Moreover, a study on women with pelvic (ovarian, fallopian tubes and primary peritoneal) HGSOC reported tubal intraperitoneal carcinomas in 48% (Kindelberger et al., 2007) the authors suggested designating this type of carcinoma serous tubal intraepithelial carcinoma (STIC). In another study STICs were found in 61% of the HGSOC (Przybycin et al., 2010). The 92% of the STICs harbors a TP53 mutation and, interestingly enough, the mutation are identical to the one found in the concordant HGSOC (Kuhn et al., 2012). Moreover STICs have shortened telomeres with respect to the correlated HGSOC. It is known that shortened telomeres are one of the earliest molecular changes in cancer cells that lead to genetic instability, which is a feature of the HGSOC (Kuhn et al., 2010). A further observation supporting the hypothesis that the HGSOC do not arise from OSE but from FTE, is the fact that in the 10-15% of the fallopian tube removed prophylactically in women with high risk to develop ovarian cancer, STICs are present but not ovarian cancer (Kurman et al., 2013). It is possible that some HGSOCs arise from the CICs (Kurman et al., 2010). In particular, two types of CICs are known: one lined by flattened epithelium similar to the OSE and the other one
lined by ciliated columnar epithelium like the one in the FTE (Banet et al., 2015). This second type of cyst contains, like FTE, not only ciliated and secretory cells but also leukocytes population including CD3+ and CD8+ T lymphocytes and CD68R+ macrophages. These immune cells are located just above the basement membrane in both the FTE and the columnar-type CICs. So a subset of HGSOCs could arise from the CICs, which are in turn derived from the tubal epithelium. Recent studies carried out on the two types of CICs proposed two different origins for these. In particular it has been proposed that flattened CICs derive from an invagination of the OSE that could occur also in a premenharcal period during the ovarian development, while columnar like CICs arise postmenharcally and have a histology that resembles the FTE. Therefore it has been proposed that they derive from a part of the fimbriae that came in direct contact with the site of ovarian rupture during the ovulation. Moreover the majority of the ciliated cysts express PAX8, a transcription factor that plays an important role in the development of a number of organs including the ones derived from the müllarian duct, such as the fallopian tube (Ozcan et al., 2011a; Ozcan et al., 2011b). Flattened CICs, instead, express calretinin, which is a reliable marker of tissue of mesothelial origin such as OSE (Banet et al., 2015). A research conducted on a mouse model of ovarian cancer, obtained disabling Dicer and Pten in the reproductive tract using an anti-müllerian hormone receptor type 2-directed Cre (DKO mice). These mice always develop early serous carcinomas in the fallopian tube when ovaries are still distinguishable from tumors and do not show any gross or histologic evidence of tumors. In DKO mice the fallopian tube tumors spread to envelope the ovaries, and aggressively metastasize in through the abdominal cavity. All the DKO mice die from the metastatic cancer. These tumors histologically resemble the human HGSOC and are reproducible in vivo when cells from these tumors are passed in immunocompromised mice. Interestingly enough, the DKO mice tumors resemble the human HGSOC not only at a histological level but also at a molecular one. From the comparison of the gene expression profiles between the mouse fallopian tube carcinomas and the human HGSOC emerges a list of known up-regulated genes shared by both types of cancers, such as Spp1, CA125, Folr1, Ccl8, Cxcl9 and 10 (Kim et al., 2012) To further confirm the fallopian tube origin of these
tumors, ovaries were bilaterally removed from DKO mice. It was found that these mice without ovaries still developed tumor, from fallopian tube similar to their counterpart with both the ovaries. If both fallopian tubes were removed from the DKO mice but the ovaries were left intact, none of the mice develop tumors (Kim et al., 2012). In another work Karst and colleagues immortalize in vitro fallopian tube cells by expressing viral oncogenes or inhibiting the expression of p53 and Rb. The cells immortalized in both ways, demonstrated in vivo, in mouse models, tumorigenicity. The tumors so generated resemble the human HGSOC from a histological point of view. (Karst et al., 2011)

**Mesenchymal Stem Cell**

Stem cells are defined as having the capacity for extensive self-renewal and for originating at least one type of highly differentiated descendant (Watt and Hogan, 2000). Post-natal tissues have reservoir of specific stem cells, which contribute to maintenance and regeneration. Examples include epithelial stem cell in epidermis and intestinal crypts (Slack, 2000), neural stem cell in the central nervous system (McKay, 1997), and satellite cells in muscle (Chargé and Rudnicki, 2004). The adult bone marrow shelters different types of stem cells, including hematopoietic (Weissman, 2000) and mesenchymal (Prockop, 1997; Da Silva Meirelles et al., 2006) stem cells. Experiments using bone marrow cells have raised the issue of phenotypic plasticity (Herzog et al., 2003; Wagers and Weissman, 2004) since they have shown the consequent generation of specialized cells derived from bone marrow in the central nervous system (Eglitis and Mezey, 1997; Mezey et al., 2000), skeletal muscle (Ferrari et al., 1998), liver (Petersen et al., 1999) and heart (Orlic et al., 2001). It was observed that Mesenchymal Stem Cells (MSC) can differentiate into specific cell types in vivo and in vitro (Woodbury et al., 2000; Kopen et al., 1999; Sato et al., 2005), and have a tendency to acquire tissue-specific characteristics when co-cultured with specialized cells types or exposed to tissue extracts in vitro (Houghton et al., 2004; Choi et al., 2005; Lange at al., 2005). In addition, the capacity to differentiate into mesodermal (Pittenger et al., 1999), ectodermal (Kopen et al.,
and endodermal (Sato et al., 2005) cell lineages characterizes MSCs as pluripotent cells. (Beyer and da Silva Meirelles, 2006)

**Mesenchymal stem cells: sites of origin**

After the discovery of bone marrow (BM)-derived MSCs many studies find out that population of cells with similar characteristic can be generated from a number of different tissues. In fact, it has been demonstrated that MSCs could be derived from multiple organs such as brain, liver, lung, spleen, kidney, muscle, thymus, pancreas, heart and adipose tissue (da Silva Meirelles et al., 2006; Beltrami et al., 2007). Despite the different sources these cells share common characteristics such as markers expression, differentiation potential and morphology. But there are some differences in the MSCs derived from different tissues; in particular there is a great discrepancy about the surface markers expressed from the cells and in the effectiveness of the differentiation potential. Some of these differences could be due to the different microenvironment from the cells derived or to the different technique of selection and purification used among the different groups. That is why it was important to define the minimal characteristic that a cell population should have in order to be assessed as MSCs. The International Society for Cellular Therapy (ISCT) issued a statement defining the minimal characteristics that a cell population needs to have to be defined as MSC. These criteria are:

- Adherence to plastic
- Specific surface antigen (Ag) expression
- Multipotent differentiation potential

MSC must be plastic adherent when maintained in standard culture conditions using tissue culture flasks. Surface Ag expression, which allows for rapid cell population identification, is a standard technique in immunology and hematology. To avoid that MSCs are confused with other cell population it is important that these cells lack the expression (≤ 2% positive) of some cell surface markers, in particular hematopoietic
markers such as CD45, CD34, CD14, CD11b, CD97α, CD19 and HLA class II; in fact, the MSC population must express (≥95% of positivity), CD105, CD73 and CD90. In addition, the cells must be able to differentiate to osteoblast, adipocytes and chondroblasts under standard, *in vitro* tissue culture-differentiating conditions (Dominici et al., 2006).

**MSC in normal physiology**

As mentioned above, MSCs are normally present in a large variety of tissues. Studies associate MSCs with pericytes, which are present in the surrounding of the blood vessels, meaning that MSCs are present quite everywhere in the organism (da Silva Meirelles et al., 2006). Several studies have demonstrated that MSCs are active players in the tissue in which they are present, in particular they concur to tissue homeostasis, through generation of precursor cells and wound healing. After transplantation into the bone marrow of non-obese diabetic-severe combined immunodeficiency (NOD-SCID) mice, MSC have been shown to differentiate into pericytes, myofibroblasts, bone-marrow stromal cells, osteocytes, osteoblasts, and endothelial cells, all of which constitute the functional components of the Hematopoietic Stem Cells (HSCs) niche that support hematopoiesis (Muguruma et al., 2006). The developing hematopoietic cells are retained in a quiescent state in the bone marrow until, after appropriate stimulation, they differentiate and are then released in the sinusoidal vascular system. In the bone marrow, the niche stromal cells surround the HSC and their progeny (Schofield, 1978). Stromal cells in the niche provide a sheltering microenvironment that supports the maintenance and self-renewal of HSCs by shielding them from differentiation and apoptotic stimuli that would otherwise challenge the stem-cell reserves. Moreover, the niche also controls the proliferation and differentiation of HSCs and the release of the mature progeny in the vascular system. The regulation of HSCs quiescence, through the maintenance of HSCs in the G0 phase of the cell cycle in the endosteal niche, and the control of HSCs proliferation, differentiation and recruitment in the vascular niche can be ascribed to the bone marrow stromal cells (Wilson and Trumpp, 2006; Kieal
and Morrison, 2008). The immunomodulatory effect of MSCs has only been described recently, following the observation that bone marrow derived MSCs suppress T-cells proliferation (Di Nicola et al., 2002; Bartholomew et al., 2002). These studies direct the attention on the possible immunoregulatory effect of the MSCs. In fact, MSCs have been shown to inhibit the maturation of monocytes, and cord-blood and CD34+ hematopoietic progenitor cells into dendritic cells (DC) in vitro (Jiang et al., 2005; Nauta et al., 2006; Ramasamy et al., 2007; Li et al., 2008). Furthermore, plasmacytoid DCs (pDCs), which are specialized cells for the production of high level of type I IFN in response to microbial stimuli, up-regulate production of the anti-inflammatory cytokine IL-10 after incubation with MSCs (Aggarwal and Pittenger, 2005). Natural killer (NK) cells are important effectors of innate immunity and they have a key role in anti-viral and anti-tumor immune responses owing to their cytolytic activity and production of pro-inflammatory cytokines (Moretta, 2002). MSCs can inhibit the cytotoxic activity of resting NK cells by down-regulating expression of NKP30 and natural killer group 2, member D (NKG2D), which are activating receptors that are involved in NK-cell activation and target-cell killing (Spaggiari et al., 2006). NK cells proliferate and acquire strong cytotoxic activity after culture with IL-2 or IL-15. However, when NK cells are incubated with these cytokines in the presence of MSCs, NK-cells proliferation and IFN-γ production were almost completely abrogated (Spaggiari et al., 2006; Spaggiari et al., 2008). Similarly, pre-activated NK cells have decreased proliferation, IFN-γ production and cytotoxicity after culture with MSCs in vitro (Spaggiari et al., 2006; Spaggiari et al., 2008; Sotiropoulou et al., 2006; Selmani et al., 2008). Neutrophils are another important cell type of innate immunity that, in the core of bacterial infection, are rapidly mobilized and activated to kill microorganisms. After binding to bacterial products, neutrophils undergo a process known as the respiratory burst. MSCs have been shown to dampen the respiratory burst and to delay the spontaneous apoptosis of resting and activated neutrophils through an IL-6-dependent mechanism (Raffaghello et al., 2008). Studies have established a link between the down-regulation of the respiratory burst and an increase in the life span of neutrophils (Fadeel et al., 1998). MSC-mediated preservation of resting
neutrophils might be important in those anatomical sites where large number of mature and functional neutrophils are stored, such as the bone marrow and lungs (Craddock et al., 1960). After T-cell receptor (TCR) engagement, T cells proliferate and exert several effector functions, including cytokine release and, in the case of CD8+ T cells, cytotoxicity. The proliferation of T cells stimulated with polyclonal mitogens, allogenic cells or specific antigen is inhibited by MSCs (Di Nicola et al., 2002; Bartholomew et al., 2002; Aggarwal and Pettinger, 2005; Meisel et al., 2004; Zappia et al., 2005; Rasmusson et al., 2005; Krampera et al., 2003; Glennie et al., 2005; Sato et al., 2007; Benvenuto et al., 2007). MSC-mediated inhibition of T-cell proliferation depends on the arrest of T cells in the G0/G1 phase of the cell cycle (Glennie et al., 2005; Benvenuto et al., 2007). MSSs do not promote T cell apoptosis, but instead support the survival of T cells that are subjected to overstimulation through the TCR and are committed to undergo CD95-CD95-ligand-dependent activation-induced cell death (Benvenuto et al., 2007). The MSC-mediated anti-proliferative effect on T cells is associated with the survival of T cell in a state of quiescence that can be partially reversed by IL-2 stimulation (Zappia et al., 2005). Inhibition of T-cell proliferation by MSCs has been reported to lead to decreased IFN-γ production both in vitro and in vivo (Aggarwal and Pettinger, 2005; Zappia et al., 2005) and to increased IL-4 production by T helper 2 (Th2) cells, which indicates a shift in T cells from a pro-inflammatory (IFN-γ-producing) state to an anti-inflammatory (IL-4-producing) state (Aggarwal and Pettinger, 2005). Regulatory T cells are a specialized subpopulation of T cells that suppress activation of the immune system and thereby help to maintain homeostasis and tolerance to self-antigens. MSCs have been reported to induce the production of IL-10 by pDCs, which, in turn, triggered the generation of regulatory T cells (Aggarwal and Pettinger, 2005; Spaggiari et al., 2006). In addition, after co-culture with antigen-specific T cells, MSCs can directly induce the proliferation of regulatory T cells through release of the immunomodulatory HLA-G isoform HLA-G5 (Selmani et al., 2008). These findings indicate that MSCs can modulate the intensity of an immune response by inhibiting antigen-specific T-cell proliferation and cytotoxicity and promoting the generation of regulatory T cells. The second main cell type involved in adaptive immune
responses are B cells, which are specialized for antibody production. Studies of the interactions between MSCs and B cells have produced different outcomes, possibly as a result of the experimental conditions used (Glennie et al., 2005; Krampera et al., 2006; Corcione et al., 2006; Traggiai et al., 2008). Many published works indicate that MSCs inhibit B-cell proliferation in vitro (Glennie et al., 2005; Corcione et al., 2006). MSCs can also inhibit B-cell differentiation and the constitutive expression of chemokine receptors (Corcione et al., 2006). These effects seem to depend on the release of soluble factors (Corcione et al., 2006) and on cell–cell contact, possibly mediated by interactions between programmed cell death 1 (pD1) and its ligands. However, other in vitro studies have shown that MSCs could support the survival, proliferation and differentiation to antibody-secreting cells of B-cells from normal individuals (Traggiai et al., 2008; Rasmusson et al., 2007) and from pediatric patients with systemic lupus erythematosus (Traggiai et al., 2008). Regardless of the controversial in vitro effects, it should be emphasized that B-cell responses are mainly T-cell dependent and therefore the final outcome of the interaction between MSCs and B-cells in vivo might be significantly influenced by the MSC-mediated inhibition of T-cell functions. Paradoxically, despite their broad immunosuppressive activities, it is possible that MSCs could function as non-professional antigen-presenting cells (ApCs). Low concentrations of IFNγ up-regulate the expression of MHC class II molecules by MSCs, which indicates that they could act as ApCs early in immune response when the levels of IFNγ are low. However, this up-regulation of MHC expression by MSCs, together with the ApCs function, was progressively lost as IFNγ concentrations increased. Such a mechanism could allow MSCs to function as conditional ApCs in the early phase of immune response and later switch their function to immunosuppression (Staag et al., 2006). Most of the immunomodulatory activities of MSCs described here have been documented by in vitro experiments. As MSCs are derived from stromal progenitor cells that reside in the bone marrow, their potential role in the control of physiological immune responses is unknown, despite the fact that the bone marrow might be a site for the induction of T-cell responses to blood-borne antigens (Feuerer et al., 2003; Uccelli et al., 2008). MSCs
are also implicated in the regenerative system suited to functionally repair tissues opposed to the damage filling process known as scarring (Caplan, 2007).

**MSCs and cancer**

A wealth of studies demonstrates that the tumor is more than a homogenous bulk of cancer cells, since it incorporates several other cells such as fibroblasts, immune cells, smooth muscle cells and endothelial cells from the blood vessels (Marx, 2008). Numerous studies have highlighted that human MSCs enhance tumor growth and/or metastatic progression in neoplasias. Growing tumors continuously remodel local tissue architecture and generate a state similar to chronic inflammation evoked by wounds. It is believed that MSCs migrate into tumor the same way they migrate into injured tissues. Endogenous MSCs have been recovered from the stroma of both experimental xenografts and human tumors, suggesting that cancer development could be somehow related to the presence of MSCs. In recent years cancer cells were finely studied and now it is generally recognized that they have some properties that make them cancer cells. These properties are known as hallmarks of cancer and are: Sustaining proliferative signaling, Evading growth suppressor, Avoiding immune destruction, Enabling replicative immortality, Activating invasion and metastasis, Inducing angiogenesis, Resisting cell death and Deregulating cellular energetics. Several studies have elucidated that MSCs could help tumor cells to acquire many of these hallmarks.

Sustaining proliferative signaling

MSCs can be recruited and induced to differentiate into myofibroblast defined by the expression of alpha smooth muscle actin (αSMA) (Paunescu et al, 2011), or into adipocytes defined by the expression of fatty acid binding protein 4 (FABP4) (Rosen and MacDougal, 2006). In literature these kinds of stromal cells are normally referred to as Cancer Associated Fibroblasts (CAFs)(Hanahan and Weinberg, 2011). Each of these CAFs subtypes could contribute with a variety of stimuli to promote different hallmarks capabilities. CAFs can produce signaling mitogenic proteins such
as epithelial growth factor (EGF), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), stromal cell-derived factor (SDF-1/CXCL12), and a variety of other growth factors with the capability to enhance cancer cells proliferation. (Cirri and Chiarugi, 2011; Erez et al., 2010; Franco et al., 2010; Kalluri and Zeisberg, 2006; Orimo et al., 2005; Räsänen and Vaheri, 2010; Rosen and MacDougald, 2006; Spaeth et al., 2009). Moreover CAFs could induce epithelial-to-mesenchymal transition (EMT) via secretion of transforming growth facto beta (TGF-β) (Chaffer and Weinberg, 2011), which can affect also other hallmark properties. The immunomodulatory role of MSCs in normal condition is well known; in the tumor microenvironment CAFs can recruit, by secreting a wide spectrum of pro-inflammatory cytokines, infiltrating immune cells, which can provide cancer cells with other mitogenic stimuli. (Celis et al., 2005; Dirat et al., 2011; Erez et al., 2010),

Evading growth suppression

Co-culture experiments have demonstrated that normal fibroblasts are able to impair cancer cells proliferation, in a process requiring cell-cell contact. CAFs can contribute to this particular hallmark capability with what they have lost from their normal counterpart during the process of “activation” as CAFs. Moreover it has been hypothesized that CAFs can produce protease and other paracrine factors that disrupt normal epithelial architecture relieving the growth suppression induced by epithelial cell-cell adhesion (Bissell and Hines, 2011; Flaberg et al., 2011).

Resisting Cell Death

A number of studies have implicated CAFs in the capability to limit the impact on tumor growth and progression of cancer cell apoptosis (Kalluri and Zeisberg, 2006; Loeffler et al., 2006; Pietras and Ostman, 2010). One modality involves the secretion of paracrine survival factors such as IGF-1 and IGF-2. A second one relates to synthesis of Extra Cellular Matrix (ECM) and ECM-remodeling proteases that contribute to the formation of neoplastic ECM, different from normal tissue stroma, which provides survival signals, such as ligands for antiapoptotic integrins; it was
also found that CAF-derived ECM can modulate cancer cell survival (Lu et al., 2011). Activated adipocytes showed the ability to confer radioresistant phenotype to breast cancer cells via IL-6 secretion (Bochet et al., 2011).

Inducing angiogenesis

There is great evidence that CAFs are involved in angiogenesis in both wound healing and tumor progression. CAFs can produce a number of proangiogenic signaling proteins including VEGF, FGF2 (and other FGFs), IL-8/CXCL8 and PDGF-C. It is worth noting that PDGF-C may recue angiogenesis in some anti-VEGF resistant tumors (Crawford et al., 2009). As said above, CAFs are able to secrete ECM-remodeling enzymes and the degradation of ECM may cause the releasing of some angiogenic factors, such as bFGF, VEGF and TGF-β, which are normally embedded in the matrix itself thus rendering them bioavailable (Kalluri and Zeisberg, 2006; Pietras and Ostman, 2010; Räsänen and Vaheri, 2010). Moreover CAFs can recruit proangiogenic macrophages, neutrophils and other myeloid cells, thereby orchestrating tumor angiogenesis, as well as directly stimulating recruitment of endothelial precursor cells via CXCL12 (Räsänen and Vaheri, 2010; Vong and Kalluri, 2011; Orimo and Weinberg, 2007).

Activating invasion and metastasis

There is increasing evidence that CAFs can modulate the capability of cancer cells to invade locally or establish secondary tumor at distant metastatic sites. One of the most important CAF-derived effector of this hallmark capability is the production of HGF which can stimulate c-Met signaling thus improving both invasiveness and proliferation. TGF-β is also involved in inducing EMT in cancer cells, at least in some cancers, enabling the invasion and metastasis capabilities (Chaffer and Weinberg, 2011). It has been proved that CAFs could be involved in different contexts, for example, secretion of CXCL5 stimulates breast cancer metastasis (Karnoub et al., 2007). Moreover CAFs can produce a characteristic repertoire of ECM protein as well as a variety of ECM remodeling enzymes that modify the tumor
microenvironment which becomes more supportive to cancer cell invasion, both proximal to the CAFs and in the adjacent tissue (Chaffer and Weinberg, 2011; Cirri and Chiarugi, 2011; Kalluri and Zeisberg, 2006; Pietras and Ostman, 2010). Notably, inhibiting either TGF-β, its type I receptor, or the PDGF receptors (Kano et al., 2007; Sounni et al., 2010, Pietras et al., 2001) similarly reduces interstitial fluid pressure in certain tumors, resulting in improved hemodynamics and more favorable biodistribution of drugs, thus reducing the abundance of ECM component of hyaluronic acid in the tumor microenvironment (Provenzano et al., 2012).

Evading immune destruction

As said above, MSCs, and therefore CAFs, have the capability to regulate the immune response, in particular they are able to blunt cytotoxic T-cells, NK cells and B-cells activities (Stover et al., 2007)

Reprogramming energy metabolism

There is interesting evidence that CAFs, induced by reactive oxygen species released by cancer cells, can switch to aerobic glycolysis, secreting lactate and pyruvate that can serve as fuel to cancer cells (Rattigan et al., 2012; Sotgia et al., 2012). In other cases, the symbiosis is opposite: cancer cells switch on aerobic glycolysis, utilizing glucose and exporting lactate, which the CAFs use as energetic substrate to drive their tumor-promoting activities (Balliet et al., 2011; Ertel et al., 2012; Martinez-Outschoorn et al., 2012; Sotgia et al., 2012)

It has also been demonstrated that MSCs could induce resistance to standard chemotherapy. In fact, if stimulated with platinum-based treatment but not with other chemotherapeutic agent such as 5-FU or irinotecan, they are able to produce fatty acids that, in a systemic way, can protect cancer cells from chemotherapy (Roodhart et al., 2011; Hanahan et al., 2012).
MSC and ovarian cancer

As in the other kinds of cancer, also in ovarian cancer the role of MSC is emerging as an important feature in cancer progression and metastasis. The roles of MSCs in ovarian cancer progression are various and spam from supporting the Ovarian Cancer Stem Cells (OCSCs), to the protection from apoptosis or chemotherapy. A study demonstrates that while α-SMA staining, which is a marker for CAFs, is absent in normal ovarian tissues, it becomes present in benign and borderline tumors and abundant in epithelial ovarian carcinomas. Moreover the presence of mesenchymal cells correlate with the tumor stage, lymph node metastasis, omentum metastasis, lymphatic vessel and micro vessel density. (Zhang et al., 2011) The omentum, a large fat pad that extends from the stomach and covers the bowel, functions as an endocrine organ and storage site for energy-dense lipids. Ovarian cancer metastasis to the omentum transforms this soft pad tissue, mainly composed of adipocytes, into a solid tumor devoid of adipocytes. If metastasis were a random event, all the organs touched by the peritoneal fluid would be equally affected. However ovarian cancers prefer to metastasize into the adipose tissue. It has been observed that omentum-derived adipocytes, or their conditioned media, can stimulate migration and invasion in OCCs, while adipocytes derived from other tissues do not exert this ability. Moreover, omentum adipocytes can induce OCCs homing by secretion of a variety of cytokines such as IL-6, IL-8, MCP-1, TIMP-1 thus promoting the early steps of metastasis to the omentum. Adipocytes help cancer growth by transferring lipids to the cancer cells in order to give them an energy-dense source of fuel (Nieman et al., 2011). After culture with MSCs ovarian cancer cells becomes resistant to carboplatin treatment, showing a decrease in the chemotherapy-induced apoptosis. This escape is due to the secretion of some unknown factor able to induce the phosphorylation of Akt and one of its downstream target XIAP. It is therefore possible that MSCs could activate the antiapoptotic PI3K/Akt signaling pathway. In fact, if the ovarian cancer cells were cultivated in presence of PI3K inhibitor or treated with siRNA specific for XIAP, they do not show any protection from carboplatin-induced apoptosis (Castells et al., 2013). Recent experimental work has
demonstrated that some of the MSCs secreted factors are able to induce peculiar characteristics in the ovarian cancer cells. In particular when ovarian cancer cells are co-cultivated with MSC, they acquire a more invasive phenotype, depending of the expression of TGF-β and the cell-cell contact. In fact if the TGF-β is added to the culture, the OCCs do not increase their motility measured by migration assay, but this goal is only achieved if OCCs are co-cultured with MSCs (Yeung et al., 2013). IL-6, a cytokine already associated with tumor progression and aggressiveness, is found to be over-expressed by MSCs in ovarian cancer and this expression stimulates OCCs to migrate and act as homing stimulus (Touboul et al., 2013) The communication between MSCs and OCCs is not unidirectional, in fact the MSCs could induce some behavior in OCCs but the system functions also all the way around. In several cases the OCCs can secrete factors to activate the surrounding MSCs inducing them to give rise to a permissive microenvironment for tumor growth. For instance the OCCs can stimulate the surrounding microenvironment through the production of HOXA9 (Ko et al., 2012). After the stimulation the MSCs become permissive to tumor growth, and begin to provide tumor with stimuli that help the tumor growth. The tumor microenvironment can also recruit MSCs from other tissues. In fact it has been reported that MSCs migrate toward the site of injuries, and the tumor is a “wound that never heals” (Spaeth et al., 2008; Dvorak 1986). After the recruitment the MSCs become “activated” and assume characteristics similar to TAF of CAF. It has been shown that MSCs injected in animal model admixed with OCCs (Skov-3), give rise to tumors with a larger and more complex perivascular network. Moreover the activated MSCs secrete a panel of growth factor prompting the tumor growth (Spaeth et al., 2009). Activated MSCs in ovarian cancer, have been reported to protect the OCCs from hyperthermic chemotherapy (Lis et al., 2011), and also promote stemness (McLean et al, 2011) and metastasis (lis et al., 2012; Tuoboul et al., 2013).
Clinical application of MSC

Wound healing is a complex process that involves mitosis, inflammation, angiogenesis, synthesis and remodeling of the extracellular matrix (Caplan et al., 2011). MSCs are very versatile and promote pro- and anti-inflammatory response along with angiogenesis (Bonfield, 2013). Research has been performed on the effect of using MSCs in the treatment of wounds, both with indirect and direct delivery to the wound site. With indirect delivery, the MSCs are infused systematically into the circulatory system. Studies have demonstrated that MSCs home to the site of injury and provide therapeutic impact. Once the MSCs reach the wound, they exit from vasculature in the connective tissue stromal region (da Silva Meirelles et al., 2009). The MSCs respond to the specific tissue microenvironment while at the same time contributing to the microenvironment through the secretion of biomolecules (Murphy, 2013). This peculiar interaction between the MSCs and the tissue defines the efficacy, potency and overall therapeutic impact of the MSCs themselves. The problem with the intravenous delivery of the MSCs is that they localize in the lungs. In fact it has been demonstrated that the MSCs in the lungs fail to engraft and die in a bulk as a result of complement activation; the surviving cells entrapped in the lung microcirculation are cleared within 72h (Bianco, 2014). Moreover the intravenously injected cells could go off route and go to other sites reducing the number of cells that arrive to the site of injury, impairing the effectiveness of the treatment. Due to this reason direct application of MSCs has been proposed as an alternative method, injecting the cells directly in the site of injury. Several studies demonstrate that injection of MSCs in or near the site of injury improves tissue function and reduces the scarring (Stoff et al., 2009; Falanga et al., 2007). It is known that MSCs secrete factors that promote bone regeneration (Knight and Hankenson, 2013) so the use of these cells in an orthopedic setting was studied. MSCs based cartilage repair in rabbit model with full thickness cartilage defect showed improved healing. After six months the MSCs showed effectiveness in chondrocyte transplantation, as well as tissue regeneration (Shafiee et al., 2011). In another study MSCs were used in a foot-ankle reconstruction. The cells are used in
*vivo*, because of their osteogenic potential. MSCs grafted during the surgery improved healing and interval to partial weight-bearing (Scott et al., 2011). MSCs have the potential to successfully aid in Hematopoietic Stem Cells (HSCs) engraftment and prevent rejection with their immune-suppressive properties. They can also generate cytokines that aid hematopoiesis and could enhance the efficacy of MSCs in bone marrow recovery after chemotherapy and/or radiation (Giordano et al., 2007). In a study performed on patients who had severe idiopathic anemia, MSCs were infused in combination with HSCs. After the infusion most of the medical problems were resolved, although there was no recovery of hematopoietic tissue. A phase I clinical trial resulted in hematopoietic recovery for most patients with the 50% of them that did not develop Graf versus Host disease (GvHD) (Giordano et al., 2007). MSCs have demonstrated their ability to differentiate into neurons and astrocytes (Pittenger et al., 1999). Therefore, MSCs were infused in a mice model of sphingomyelinase, a neurodegenerative disease, showing a delay in the start of neurological abnormalities and improving overall survival in the mouse model (Giordano et al., 2007). A study was also performed to determine the effectiveness of MSCs transplantation into human patients with amyotrophic lateral sclerosis, a disease that causes degeneration of motor neurons and muscle functionality. After the injection of the MSCs into the spinal cord of the patients a trend of improvement in muscle was detected, although there was no preliminary data to state how long the effect could be sustained (Pittenger et al., 1999). Central nervous system (CNS) injury situation can be caused by stroke, trauma, or an underlying neurological condition. In CNS, neural MSCs (NMSC) and MSCs are used to regeneration purposes to create new cells to replace those that were lost (Fabbio et al., 2013; Chao et al., 2009). However, this process has not been completely effective due to oxidative stress and toxic by-products, which can affect MSC transplantation. This causes slowing of tissue regeneration, as well as reduced longevity. Currently, carbon nanotubes (CNT) are being used to support MSC differentiation. CNT/MSC composites were used to improve neurite growth after CNS damages. In both the *in vitro* and the *in vivo* settings, biocompatibility was demonstrated for CNT with MSCs and NSCs. BM-MSCs have also been used as a therapeutic for metachromatic
leukodystrophy. After undergoing successful bone marrow transplantation from human leukocyte antigen (HLA) identical siblings, patients suffering from metachromatic leukodystrophy were given BM-MSCs from their sibling donors by injection. In some patients there was significant improvement in nerve conduction velocities (Koc et al., 2002). Diabetes is defined as a person's inability to maintain proper blood insulin levels. With the shortage of insulin producing cells in diabetes, pancreas islet transplantations have been performed to eliminate the need for insulin injections on regular basis (Zhao et al., 2007). The issue is that pancreas and islets are scarce and are often rejected by the recipient after the implantation. So autologous MSCs becomes a good alternative because it eliminates the rejection risk. A possible source is the peripheral blood, which also contains the normal human insulin producing cells (Zhao et al., 2007; Koc et al., 2002). It was found that peripheral blood insulin producing cells could be isolated and preserved for future insulin production because they have the ability to adhere to a plastic tissue culture dish, and they showed transcription and insulin production at protein and mRNA level. With this method it is possible for patients to generate their own insulin producing cells. A clinical trial on newly diagnosed type 1 diabetes patients showed prolonged insulin independence in most participants after transplantation of MSCs (Voltarelli et al., 2011). MSCs have the potential to impact damage or inflamed lung areas by repairing the tissue or stimulating the host tissue to regenerate itself. In lung condition, involving fibrotic disease, MSCs would be involved in reversing extracellular matrix disposition and collagen synthesis (Bonfield et al., 2010; Brody et al., 2010; Tzouveleki et al., 2011). In the situation of idiopathic pulmonary fibrosis (IPF), lung fibrosis results in scarring and terminal pulmonary insufficiency (Tzouveleki et al., 2011, Sukpat et al., 2013; Toonkel et al., 2013). It has been shown that MSCs administration after bleomycin treatment, a model for IPF, in the animal model can reduce both collagen deposition and inflammation (Tzouveleki et al., 2011; Toonkel et al., 2013; Nueringer et al., 2006). Acute lung injury (ALI) is a devastating disease with a high mortality rate and significant morbidity (Murphy et al., 2013; Matthay et al., 2010). Common effects are damage to both the alveolar epithelium and the vascular endothelium. MSCs treatment reduces pro-
inflammatory cytokines, but resolution response and anti-inflammatory cytokine levels are increased (Nueringer et al., 2006; Krasnodembskaya et al., 2010). If patients with chronic obstructive pulmonary disease (COPD) are infused with MSCs, they will show a significant decrease in circulatory reactive protein (Weiss et al., 2013). Cystic Fibrosis (CF) is a genetically inherited disease that results in mutation in the cystic fibrosis transmembrane regulator (CFTR) gene. The mutation impacts almost every organ but the major cause of morbidity and mortality is the inability to control lung infection and inflammation. A study was performed on a mouse model of CF. These mice lose weight without resolution and succumb to infection. MSCs treatment on these animal models resulted in weight increase reaching level similar to the control, with improved gross lung pathology and decreased cellular recruitment into the lungs (Bonfield et al., 2010). Asthma is a chronic inflammatory disease that causes airway inflammation and reactivity, which can ultimately result in lung injury (Bonfield et al., 2010). It has been observed that when MSCs are given to an asthma mouse model there is a significant decrease in airway hyper-responsiveness and eosinophil level in bronchoalveolar lavage fluid (Weiss et al., 2008).
MATERIAL AND METHODS

Human samples

Human samples were obtained from the Azienda Ospedaliero-Universitaria of Udine (AOU) and collected after informed consent in accordance with the declaration of Helsinki and with approval by the Bioethics Committee. The pathology records of the samples are reported in the following tables.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>AGE</th>
<th>DIAGNOSIS</th>
<th>MITOSIS (SCORE)</th>
<th>GRADING (S)</th>
<th>pTNM</th>
<th>FIGO ST</th>
</tr>
</thead>
<tbody>
<tr>
<td>sample 12</td>
<td>56</td>
<td>HIGH-GRADE SEROUS ADENOCARCINOMA</td>
<td>79/10HPF(3)</td>
<td>3 (8)</td>
<td>pT3b,Nx,Mx</td>
<td>IIIB</td>
</tr>
<tr>
<td>sample 17</td>
<td>75</td>
<td>HIGH-GRADE SEROUS ADENOCARCINOMA</td>
<td>48/10HPF (3)</td>
<td>3 (8)</td>
<td>pT3c,Nx,Mx</td>
<td>IIIC</td>
</tr>
<tr>
<td>sample 19</td>
<td>47</td>
<td>HIGH-GRADE SEROUS ADENOCARCINOMA</td>
<td>46/10HPF (3)</td>
<td>3 (9)</td>
<td>pT3c,Nx,Mx</td>
<td>IIIC</td>
</tr>
<tr>
<td>sample 26</td>
<td>64</td>
<td>HIGH-GRADE SEROUS ADENOCARCINOMA</td>
<td>37/10HPF (3)</td>
<td>3 (8)</td>
<td>pT3c,Nx,Mx</td>
<td>IIIC</td>
</tr>
<tr>
<td>sample 31</td>
<td>23</td>
<td>HIGH-GRADE SEROUS ADENOCARCINOMA</td>
<td>17/10HPF (2)</td>
<td>2(6)</td>
<td>pT3c,N1,M1(cute)</td>
<td>IV</td>
</tr>
<tr>
<td>sample 33</td>
<td>70</td>
<td>HIGH-GRADE SEROUS ADENOCARCINOMA</td>
<td>46/10HPF (3)</td>
<td>3 (9)</td>
<td>pT3c,Nx,Mx</td>
<td>IIIC</td>
</tr>
<tr>
<td>sample 34</td>
<td>51</td>
<td>HIGH-GRADE SEROUS ADENOCARCINOMA</td>
<td>60/10HPF (3)</td>
<td>3 (9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sample 36</td>
<td>52</td>
<td>HIGH-GRADE SEROUS ADENOCARCINOMA</td>
<td>48/10HPF (3)</td>
<td>3 (9)</td>
<td>pT2c,Nx,Mx</td>
<td>IIC</td>
</tr>
<tr>
<td>sample 38</td>
<td>68</td>
<td>HIGH-GRADE SEROUS ADENOCARCINOMA</td>
<td>68/10HPF (3)</td>
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<td>pT3c,N0,Mx</td>
<td>IIIC</td>
</tr>
<tr>
<td>sample 39</td>
<td>30</td>
<td>BORDER SEROUS ADENOCARCINOMA</td>
<td>0/10HPF(1)</td>
<td>1(5)</td>
<td>pT1C,N0,Mx</td>
<td>IC</td>
</tr>
<tr>
<td>sample 40</td>
<td>55</td>
<td>HIGH-GRADE SEROUS ADENOCARCINOMA</td>
<td>28/10HPF (3)</td>
<td>3 (9)</td>
<td>pT2b,Nx,Mx</td>
<td>IIB</td>
</tr>
<tr>
<td>sample 41</td>
<td>39</td>
<td>HIGH-GRADE SEROUS ADENOCARCINOMA</td>
<td>66/10HPF (3)</td>
<td>3 (9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sample 43</td>
<td>83</td>
<td>HIGH-GRADE SEROUS ADENOCARCINOMA</td>
<td>50/10HPF (3)</td>
<td>3 (9)</td>
<td>pT3b,Nx,Mx</td>
<td>IIIB</td>
</tr>
<tr>
<td>sample 48</td>
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<td>BORDER SEROUS ADENOCARCINOMA</td>
<td>12/10HPF (2)</td>
<td>2(6)</td>
<td>pT1A,N0,Mx</td>
<td>IA</td>
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<tr>
<td>sample 49</td>
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<td>HIGH-GRADE SEROUS ADENOCARCINOMA</td>
<td>166/10HPF (3)</td>
<td>3 (8)</td>
<td>pT3a,Nx,Mx</td>
<td>IIA</td>
</tr>
<tr>
<td>sample 57</td>
<td>63</td>
<td>HIGH-GRADE SEROUS ADENOCARCINOMA</td>
<td>36/10HPF (3)</td>
<td>3 (9)</td>
<td>pT3c,Nx,Mx</td>
<td>IIIC</td>
</tr>
</tbody>
</table>
Isolation and in vitro expansion of HG-SOC-MSC

Primary serous ovarian cancer samples were obtained from the Azienda Ospedaliero-Universitaria di Udine (AOU) in accordance with consent procedures of the Bio-Ethic Committee. Surgical biopsies were freshly collected from patients undergoing surgery, washed several times in PBS solution and then mechanically disaggregated by mincing it with razor blades. Further dissociation was carried out by enzymatic digestion (20µg/ml collagenase IV (StemCell Technologies)) for 10 minutes at 37 °C. Single cell suspensions were obtained by filtering the disaggregated tissue through a nylon mesh with 70µm pores (Cell Strainer, BD Falcon). Recovered cells were cultured in MyeloCult Medium (StemCell Technologies) containing 25% of serum (12.5% horse serum and 12.5% of fetal bovine serum (both StemCell Technologies)). After colony formation, the cells were detached with 0.25% trypsin-EDTA (Sigma-Aldrich) and re-plated at a density of 2.5x10^3/cm^2 onto fibronectin (Sigma-Aldrich) coated 100mm dishes (Sacco-Falcon), in an expansion medium composed as follows: 60% low glucose DMEM (Invitrogen),

<table>
<thead>
<tr>
<th>MSC line</th>
<th>Sex</th>
<th>Age</th>
<th>Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSLIM-16</td>
<td>F</td>
<td>71</td>
<td>Adipose</td>
</tr>
<tr>
<td>HSLIM-26</td>
<td>F</td>
<td>46</td>
<td>Adipose</td>
</tr>
<tr>
<td>HSLIM-85</td>
<td>F</td>
<td>50</td>
<td>Adipose</td>
</tr>
<tr>
<td>BM-21</td>
<td>M</td>
<td>56</td>
<td>Bone Marrow</td>
</tr>
<tr>
<td>BM-29</td>
<td>F</td>
<td>56</td>
<td>Bone Marrow</td>
</tr>
<tr>
<td>BM-37</td>
<td>M</td>
<td>70</td>
<td>Bone Marrow</td>
</tr>
<tr>
<td>HH-337-AD</td>
<td>M</td>
<td>27</td>
<td>Heart tissue</td>
</tr>
<tr>
<td>HH-354-AD</td>
<td>M</td>
<td>70</td>
<td>Heart tissue</td>
</tr>
<tr>
<td>HH-394-AD</td>
<td>M</td>
<td>57</td>
<td>Heart tissue</td>
</tr>
<tr>
<td>HH-421-AD</td>
<td>M</td>
<td>54</td>
<td>Heart tissue</td>
</tr>
<tr>
<td>SOC-19-01</td>
<td>F</td>
<td>47</td>
<td>Serous Ovarian Carcinoma - Left Ovary</td>
</tr>
<tr>
<td>SOC-19-02</td>
<td>F</td>
<td>47</td>
<td>Serous Ovarian Carcinoma - Right Ovary</td>
</tr>
<tr>
<td>SOC-41-01</td>
<td>F</td>
<td>39</td>
<td>Serous Ovarian Carcinoma - Left Ovary</td>
</tr>
<tr>
<td>SOC-41-02</td>
<td>F</td>
<td>39</td>
<td>Serous Ovarian Carcinoma - Right Ovary</td>
</tr>
<tr>
<td>SOC-43-01</td>
<td>F</td>
<td>83</td>
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</tr>
<tr>
<td>SOC-43-02</td>
<td>F</td>
<td>83</td>
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</tr>
<tr>
<td>SOC-57-01</td>
<td>F</td>
<td>63</td>
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</tr>
<tr>
<td>SOC-57-02</td>
<td>F</td>
<td>63</td>
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</tr>
<tr>
<td>SOC-57-03</td>
<td>F</td>
<td>63</td>
<td>Serous Ovarian Carcinoma - Right Ovary</td>
</tr>
</tbody>
</table>
40% MCDB-201, 1mg/mL linoleic acid-BSA, 10⁻³M dexamethasone, 10⁻⁴M ascorbic acid-2 phosphate, 1X insulin-transferrin-sodium selenite (all from Sigma-Aldrich), 2% fetal bovine serum (StemCell Technologies), 10ng/mL hPDGF-BB, 10ng/mL hEGF (both from Peprotech EC). Medium was replaced with fresh one every 3-4 days.

**Flow cytometry and immunofluorescence**

For flow cytometry assays, N-MSCs and HG-SOC-MSCs grown in expansion medium at passage P3 were detached from the culture substrate through a short incubation in a trypsin-EDTA solution (0.5 g/l trypsin, 0.2 g/l EDTA, Sigma-Aldrich) and incubated with the properly conjugated antibody. Antibodies used were: CD117, CD90, CD49b, CD34, CD73, CD44, CD29, CD49a, (BD-Biosciences), CD133 (Miltenyi Biotec), HLA-DR (BD-Biosciences), CD105 (Serotec, UK). The analysis was performed either by FACS-Calibur (BD-Biosciences) or by CyAn (Dako).

For immunofluorescence studies, cells were grown on fibronectin-coated glass coverslips, or 96-well black clear-bottom plates, fixed with 4% paraformaldehyde (Merk) for 20 min at room temperature and permeabilized with 0.1% Triton X-100/PBS (Calbiochem) for 5 min before incubation with specific or control antibodies. Antibodies used were: KRT7 (Dako), KRT18 (Dako), VWF (Dako), SMA (Dako), Conexin 43 (Santa Cruz Biotechnology), α-actin (Sigma Aldrich), β3-tubulin (Abcam), Collagen I (Abcam), lectin UEA1 (Molecular Probes). For the 96-well format, antibodies used were KRT7 (Abcam) and VIM (Abcam).

Epifluorescence and phase contrast images were obtained utilizing a live cell imaging dedicated system consisting of a Leica DMI 6000B microscope connected to a Leica DFC350FX camera (Leica Microsystems) and equipped with a 63X oil immersion objective (numerical aperture: 1.40) or a 40X oil immersion objective (numerical aperture: 1.25). Adobe Photoshop software was utilized to compose, overlay the images and to adjust contrast (Adobe). For the 96-well format, fluorescence images were acquired using an ImageXpress Micro automated high-
content screening fluorescence microscope (96-well plates); a total of 9 images were acquired per well corresponding to ca. 500-1,000 cells analyzed per well; multiple replicate wells were imaged for each experimental condition. Image analysis to quantify the percentage of cells expressing KRT7 or Vimentin was performed using the ‘Multi-Wavelength Cell Scoring’ application module implemented in MetaXpress software (Molecular Devices).

**Cell proliferation assays**

N-MSCs and HG-SOC-MSCs were plated on 6-well plates at a density of 1000, 2000, and 3000 cells/cm². Cells were harvested and counted, with a Neubauer chamber, up to ten days after plating.

**Multilineage differentiation**

Osteoblast differentiation was obtained plating cells at a density of 2x10⁴/cm², in DMEM supplemented with 5% FBS, 10mM beta-glycerophosphate, 10⁻⁷M dexamethasone and 0.2 mM ascorbic acid (all from Sigma-Aldrich); medium was changed every 3 to 4 days for 28 days. In some experiments, Tetracycline (Sigma-Aldrich), at a final concentration of 40ug/ml, were added to the culture medium at the end of osteoblast differentiation.

Endothelial cell differentiation was obtained plating cells at a density of 1x10⁴/cm² onto fibronectin-coated dishes in a serum-free medium containing 10ng/mL VEGF (PeproTech EC).

Hepatocytic differentiation was induced by growing cells for two weeks at a density of 1.5x10⁴/cm² onto fibronectin coated dishes in a medium containing 0.5% FBS, 10 ng/ml FGF-4 and 20 ng/ml HGF (both from PeproTech EC). After this period, FGF-4 and HGF were substituted for 20 ng/ml Oncostatin-M for further 14 days (PeproTech EC). Muscle cell differentiation was achieved plating 1x10⁴/cm² cells in expansion medium containing 5% FCS (Sigma-Aldrich), 10 ng/mL bFGF, 10 ng/mL
VEGF, and 10 ng/mL IGF-1 (all from PeproTech EC), but without EGF. Cells were allowed to become confluent and cultured for up to 16 weeks with medium exchanges every 4 days.

Adipogenic differentiation was induced seeding 5x10^3/cm^2 cells in a culture medium supplemented with 0.5 mM isobutylmethylxanthine, 50 µM indomethacin and 0.5 µM dexamethasone (all from Sigma-Aldrich). Cells were cultured for up to 2 weeks with medium exchanges every 4 days. At the end of every treatment, cells were fixed either with 4% buffered Paraformaldehyde or with methanol/acetone (1/1). Undifferentiated cells were stained in parallel as negative controls, and did not show any positive signal.

**Functional assays**

**PAS (Periodic Acid Shiff) staining for glycogen detection.**

Slides were fixed for 4 minutes in a 1:9 solution of 40% formalin and absolute ethanol (both Sigma-Aldrich). After washing in distilled water (dH2O), slides were incubated in 1% periodic acid (Sigma-Aldrich) for 10 minutes. Slides were then washed in dH2O, dried and incubated in the dark for 20 minutes with the Shiff’s reagent (Sigma-Aldrich). Afterward, slides were immersed in a sulphurous (potassium metabisulfite 10% in dH2O (Sigma-Aldrich) solution for 1 minute, rinsed in dH2O for 5-10 minutes, stained with Mayer’s hematoxylin (Sigma-Aldrich) for 1 minute, and rinsed in dH2O. Diastase digestion was performed by treating the sections with 1ug/ml preheated diastase solution (Sigma-Aldrich) at 37ºC for 15 minutes.

**Dil-Acetylated-LDL up-take.**

After differentiation, the endothelial cell property to actively uptake acetylated LDL was assessed incubating the cells (2x10^4) for 4 hours in 100µl differentiation medium (Iscove’s Medium with 2% FBS) additioned with Dil-Acetylated-LDL (Life Technologies) at a final concentration of 25mg ug/ml. Cells were then washed and
acetylated LDL red fluorescence was imaged utilizing an inverted fluorescence microscope. After fixation with 4% paraformaldehyde endothelial cell differentiation was subsequently confirmed by expression of von Willebrand Factor.

**Von Kossa staining.**
Cells were fixed in 4% PFA (Sigma) for 30 minutes at room temperature. After washing, slides were exposed to UV light for 30 minutes in a solution of 5% Silver Nitrate (Merck). Reaction was stopped after the salt of calcium turned black brown. Slides were rinsed in dH2O, fixed with 5% sodium thiosulphate (Merck) for 2 minutes. Nuclear Fast Red (BDH) was used as contrast staining. Cells were counterstained with Nuclear Fast Red (Sigma) for 1 minute and rinsed in tap water.

**Alcian Blue Assay.**
Cells induced to differentiate into chondroblasts were firstly fixed with 4% PFA (Sigma), washed with dH2O and then colored with Alcian Blue (Sigma) for 1 minute.

**Oil Red-O assay**
The accumulation of lipid droplets indicating adipogenic differentiation was detected staining the cells in a solution of 0.5% Oil Red-O (Sigma-Aldrich). After incubation for 3 minutes with Propylene glycol (Sigma-Aldrich), cells were added with Oil Red preparation for 30 minutes. Subsequently, cells were incubated in 80% and in 50% Propylene glycol for 3 minutes each. Finally, cells were counterstained with hematoxylin for 10 minutes.

**High-resolution genotyping analysis**
Genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen) and analyzed using HumanCNV370-Quadv3_C SNP platform (Illumina). Data were first analyzed in Bid-Studio (Illumina) to generate raw genotype information. Copy number information was derived normalizing each tumor or each HG-SOC-MSC sample to its matched normal. Regions of copy number gains and losses were visually identified and confirmed by interrogation of the raw copy number log2 ratios from BAF
segmentation (http://baseplugins.thep.lu.se/wiki/se.lu.onk.BAFsegmentation). For the call histograms the datasets were analyzed using crlmm package (Carvalho et al., 2007).

Sample Ontology

Samples were annotated and divided into functional groups using class three of a modified version of the sample Ontology developed by an internal member of the FANTOM5 Consortium (Dr Tom Freeman, University of Edinburgh).

This hierarchical ontology goes from the most generic attributes of class one (i.e. Cell line, Tissues, Primary cells) to the most specific ones of class four (acinar cells, small airway lung cells, etc).

FANTOM5 data matrix and data access

Robust transcriptional (RLE) activity peaks (n=184,827) from 889 human CAGE libraries for were obtained from FANTOM5 project.

Cluster analysis

The RLE-TPM FANTOM5 data matrix was used for cluster analysis.

For obtaining the hierarchical clustering of the peaks data generated by deep-CAGE sequencing of HG-SOC-MSCs and N-MSCs for the differentially expressed genes, we started from the RLE-TPM matrix (sample n=889, peaks n=184828), after gene median centering, features whose standard deviation were less than 0.55 were filtered out, obtaining the RLE-TPM filtered matrix (sample n=889, peaks n= 26266). For qRT-PCR validation of selected genes differentially expressed between HG-SOC-MSCs and N-MSCs, starting from the RLE-TPM matrix we selected only the HG-SOC-MSCs and N-MSCs differential peaks (see below, samples n=19, peak=1035). Unsupervised hierarchical cluster analysis (average-linkage method) was then performed using Cluster software (Eisen et al, 1998). Cluster results were then visualized using Java TreeView (Saldanha et al, 2004).
**Differential expression analysis**

The robust transcriptional (RLE) activity peaks count FANTOM5 data matrices (TPM or counts) were used for the differential transcriptional analysis (a, b, count Matrix), relationship analysis (c, TPM matrix) and correlation analysis (d, TPM matrix). EdgeR (Robinson et al, 2010) package for R/Bioconductor Environment (Gentleman, et al 2005) was used for statistical analysis.

a) Direct comparison between HG-SOC-MSCs and N-MSCs.

We considered genes with a differential transcriptional activity with log fold change at least greater/lower than 2/-2 respectively (p<10⁻⁵, FDR corrected).

b) HG-SOC-MSCs and N-MSCs vs. other samples.

The purpose of this analysis was to obtain specific gene profiles of HG-SOC-MSCs and N-MSCs with respect to all other FANTOM5 project samples. However, it would be totally inappropriate to perform such comparison by pooling all the other samples together (for biological and statistical reasons) since performing such comparison will bias specific groups of samples. To this aim we created 100 randomly selected samples of similar sizes of that of the HG-SOC-MSCs (n=9) and N-MSCs (n=10) subsets. After performing all the contrasts, we considered only the genes showing a differential transcriptional activity (p<10⁻⁴, FDR corrected) in at least 90% of comparisons between HG-SOC-MSCs or N-MSCs and random samples subsets. The log fold change is the average value of all comparison log fold changes.

c) Relationship analysis.

We compared HG-SOC-MSCs against all other samples divided in biological classes (Class 4_Class1, n=220) with respect to previously described samples ontology, thus obtaining the lists of differential genes. We then ranked the biological groups on the basis of the number of differential genes with
different cutoff of p-values (p<10^{-10}, p<10^{-15}, p<10^{-20}, FDR corrected). The biological groups most similar HG-SOC-MSCs (less than 20 genes) where reorganized into biological categories despite the tissue of origin.

d) Correlation analysis.

MSC samples data was extract from the TPM matrix, log transformed and center normalized. Pearson Correlation matrix among the different MSC groups was computed and graphical represented using gplots (Warnes et al, 2010) package for R/Bioconductor Environment(Gentleman, et al 2005).

Differential gene peak usage analysis

Starting from the RLE-TPM matrix (sample n=889, peaks n=184828), we considered each peak active in a sample if the corresponding TPM was greater than 15. The number of active peaks for each gene in each sample was then calculated. We then performed the following analysis:

a) Direct comparison between of HG-SOC-MSCs and N-MSCs.

Since in this analysis we wanted to select genes with differential global peak activity, we excluded from the analysis the genes with no active peaks in at least 50% of the samples in each group.

Then we compared the total number of peaks activated in each genes between the two the groups using Welch’s t test. Finally we considered differential genes with at least pValue less than 0.05.

b) Relationship analysis.

We compared HG-SOC-MSCs against all other samples divided in biological classes (Class 4_Class1, n=220) with respect to previously described samples ontology, using the same approach used in direct comparison between of HG-SOC-MSCs and N-MSCs thus obtaining the lists of global peak activity analysis. We then ranked the biological groups on the basis of the number of differential peaks with p<10^{-5}. The biological groups most similar HG-SOC-
MSCs (less than 200 genes) where reorganized into biological categories despite the tissue of origin.

**Real-time quantitative RT-PCR**

Total RNA was extracted from N-MSCs and HG-SOC-MSCs using the TRIZOL Reagent (Invitrogen) and quantified using Nanodrop ND-100 spectrophotometer. The QuantiTect Reverse Transcription Kit (Qiagen) was used to generate cDNA starting from 500ng of total RNA, according to the manufacturer’s instructions. Real-time quantitative RT-PCR was performed using the SYBR Green Master Mix (Applied Biosystems) on a StepOnePlus Real-Time System. Thermocycle conditions included initial denaturation at 95°C (10 min each), followed by 40 cycles at 95°C (15 s) and 60°C (1 min). The expression level of each target gene was normalized to beta-actin.

<table>
<thead>
<tr>
<th>GENE</th>
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<th>Primer Reverse</th>
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<td>Gene</td>
<td>5' Promoter Sequence</td>
<td>3' Promoter Sequence</td>
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AIM OF THE STUDY

Epithelial ovarian cancer (EOC) is the gynecological disease with the highest death incidence. Despite relatively low morbidity, EOC presents a high case-fatality ratio and the overall 5-year survival is still less than 30%. The death rate for this disease has not changed much in the last 50 years. EOC is a morphologically and biologically heterogeneous disease, which has likely contributed to difficulties in defining the molecular alterations associated with its development and progression. Unfortunately, almost 70 percent of women with the common epithelial ovarian cancer are not diagnosed until the disease is advanced in stage. The 5-year survival rate for these women is only 15 to 20 percent, whereas the 5-year survival rate for stage I disease patients approaches 90 percent and for stage II disease patients approaches 70 percent. In the last few years it has become clear that not only the cancer cells themselves are responsible for tumor growth and progression, but also the microenvironment has a great role in helping the tumor growth. A permissive microenvironment could allow tumor cells to attach and start to proliferate giving rise to a neoplasia or a metastasis, while a non permissive one can impair tumor start or growth. Mesenchymal stem cells, a population of adult stem cells first isolated in the ’70 in the bone marrow, seem to have an important role in supporting or impairing the tumor. It has been proved that MSCs can help tumor in a variety of ways such as, secreting factors that help tumor growth, giving the tumor mechanical substrate, abolishing the immune response, secreting factors that promote chemoresistance, generating a niche for cancer stem cells an so on. The laboratory team with which I carried out my PhD project successfully isolated a subpopulation of MSCs derived from high-grade serous ovarian carcinoma (HG-SOC-MSC) and such cells appeared to be normal and have no tumorigenic potential. In order to better characterize these cells, their surface markers were analyzed, and their gene expression profiles were compared to the FANTOM5 large comprehensive database. Moreover biochemical analyses were performed to properly identify HG-SOC-MSCs within the mesenchymal stem cells heterogeneity.
RESULTS

HG-SOC-MSCs are multipotent cells that do not possess gross chromosomal alteration and are not tumorigenic in vivo.

The laboratory in which I have done my PhD project had already reported a protocol for the derivation of multipotent stromal cells from normal human adult bone marrow, heart, liver and adipose tissues (N-MSCs) that maintain the ability to differentiate along various lineages (Beltrami et al., 2007). The same protocol was adapted and used to isolate a population of mesenchymal stem cells from primary high-grade serous ovarian carcinomas (HG-SOC-MSCs) from patients undergoing surgery. Despite a similar morphology HG-SOC-MSCs show a specific growth phenotype when compared to N-MSCs. In vitro the cell population doubling times are 30±3 h for the HG-SOC-MSC and 46±15 h for the N-MSC. Moreover HG-SOC-MSCs show the capacity to increase population density compared to N-MSCs. The maximum cell expansion was achieved, for HG-SOC-MSCs, with a seeding density of 1000 cells/cm², while, for N-MSCs, the same parameter was reached with 500 cells/cm² or less. In order to classify HG-SOC-MSC as mesenchymal stem cells, assays were performed to fulfill the minimal criteria for defining multipotent mesenchymal stromal cells, stated by the international society for cellular therapy (ISCT)(Dominici et al., 2006). HG-SOC-MSCs have a mesenchymal phenotype and are adherent to standard tissue culture plastics as well as the normal counterpart. Cytofluorimetric assays were conducted on both N- and HG-SOC-MSCs, in order to investigate their surface markers expression. Both cell populations show a typical MSC-like cell surface marker profile, with high levels of the mesenchymal markers such as CD37, CD90 CD59, CD13 and CD49a. HG-SOC-MSCs have a reduced expression of CD105 compared to N-MSCs. As expected both cell populations resulted negative for the expression of the hematopoietic system such as CD34 and CD45. When compared to N-MSCs, HG-SOC-MSCs, show an alteration in the expression of the surface adhesion molecule, in particular a lower expression of
fibronectin-binding integrins such as CD29, CD49a and CD49d, and a higher expression of CD146/MCAM, marker of endothelium. The last criterion stated by the ISCT for defining MSCs is the ability to differentiate through different lineages. HG-SOC-MSCs differentiation was induced using established protocols (Beltrami et al., 2007) and evaluated by immunostaining and functional assays. 70-90% of cell populations were able to differentiate into endodermic and mesodermic derivatives. As shown in Figure 2, HG-SOC-MSC populations were able to differentiate into hepatocyte-like cells (panels A, B and C), smooth muscle-like cells (panels D, E and F), endothelial-like cells (panels G, H and I), osteoblast-like cells (panels J and K), chondroblast-like cells (panel L) and adipocyte-like cells (panels M and N). It is to be noted that endothelial differentiation turned out to be the most efficient since at least 90% of the initial population resulted positive for the selected markers, coupled with the ability to organize into capillary-like structures actively up-taking acetylated low-density lipoprotein (LDL). It is widely reported in literature that stromal tissue supporting the tumor in breast and ovarian cancer originates from normal cells and does not show evident genomic alterations. So it was decided to analyze the genomic status of HG-SOC-MSCs in order to clarify if these cells were normal or derived from the tumor. A set of HG-SOC-MSCs, SOC primary tissue and peripheral blood mononuclear cells derived from the same patient was analyzed; three of these sets were analyzed using an Illumina Infinium SBP BeadChip. It was clear from the high-resolution genotyping analysis that there was no SNPs difference between HG-SOC-MSCs and PBMCs, while genome wide aberration was found between PBMCs and HG-SOCs. To test the tumorigenic potential of HG-SOC-MSCs, after in vitro culture, they were injected in NOD-SCID mice, in parallel to SKOV3 cells, chosen as a tumorigenic ovarian cancer derived cell line. In the animal model 1x10^6 of cells were injected, either HG-SOC-MSCs or SKOV3. After the injection the SKOV3 inoculated mice developed tumor in two weeks while the ones injected with HG-SOC-MSCs did not develop any kind of tumor after twelve weeks.
HG-SOC-MSCs (A) and N-MSCs (B) display a similar morphology when cultured in standard condition. The two cell populations show a different growth kinetic, HG-SOC-MSCs (red line) grow faster compared to N-MSCs. Numbers of cells are measured by counting with a Neubauer chamber.
Panels A, B, and C show the capacity of HG-SOC-MSCs to differentiate into hepatocytes, the cells are stained for KRT7 (panel A), KRT18 (panel B) and positivity to the PAS reaction (panel C). Panels D,E and F shows the capacity of HG-SOC-MSCs to differentiate into myogenic derivatives, the cells are stained for αSMA (panel D), CX43 (panel E), α-ACT. Panels G, H, and I shows the capacity of HG-SOC-MSCs to differentiate into endothelial-like cells, staining for UEA1 (panel G), vWF (panel H) and capacity to uptake LDL (panel I). Panels J and K shows the capacity of HG-SOC-MSCs to differentiate into osteoblast-like cells, staining for collagen I (panel J) and von Kossa (panel K). Panel L shows the capacity of HG-SOC-MSCs to differentiate into chondroblast-like cells, staining for alcin Blue pH1. Panels M and N show the capacity of HG-SOC-MSCs to differentiate into adipocytes, staining for PPAR-gamma (panel M), Oil Red-O highlighting intracytoplasmatic lipid droplets.
Figure 3: HG-SOC-MSCs do not possess gross chromosomal aberration and are not tumorigenic in vivo.

Application of the segmentation strategy (BAF estimates) to the matched three samples belonging to the same patient (SOC-43-01): Peripheral Blood Mononuclear Cells (PBMCs), HG-SOC whole tumor sample and HG-SOC-MSCs derived from the tumor sample independently hybridized on Affymetrix 450k SNP arrays. Top panel shows BAF estimates and the lower panel copy number estimates for PBMCs versus HG-SOC-derived-MSCs (A) and PBMCs versus HG-SOC (B). The table in panel C shows the trend of the in vivo experiment. Six mice were injected with three different type of HG-SOC-MSCs and none of them show any sign of tumor after twelve weeks while the mice injected with SKOV-3 show neoplastic mass after two weeks.
Transcriptional identity of HG-SOC-MSC.

To better characterize the HG-SOC-MSCs transcriptional identity they were compared to the large comprehensive collection of samples of the FANTOM5 project. Ten N-MSC cell lines and nine HG-SOC-MSC cell lines were compared. This analysis showed that 625 genes were more highly expressed in HG-SOC-MSCs and 450 more highly expressed in N-MSCs (p-value <10^{-5}) as shown in figure 4. Some differentially expressed genes were validated by qRT-PCR analysis and the results showed an agreement between the expression analysis based on CAGE-seq peak activity and qRT-PCR results. Next HG-SOC-MSCs and N-MSCs profiles were compared to the FANTOM5 phase 1 dataset, which included 899 samples from healthy and cancerous tissues and cell lines. The obtained results were subjected to annotation functional analysis (AFA). HG-SOC-MSCs and N-MSCs shared activation of common core biological functions and transcriptional regulators and the difference resided in the level of their activation. Among the most up-regulated biological themes, in HG-SOC-MSCs compared to N-MSCs, the following emerged as significant: cancer-related and developmental functional terms, extracellular matrix, cell adhesion and cell motility. These data suggest that globally the differences between HG-SOC-MSCs and N-MSCs are quantitative more than qualitative, with similar core functions activated but at different levels.
Figure 4: transcriptional signatures of N-MSCs and HG-SOC-MSCs

A. Hierarchical clustering of the peaks generated by deep-CAGE sequencing of HG-SOC-MSCs and N-MSCs for the differentially expressed genes. Red bars represent up-regulated genes compared to the average of all samples while green bars represent down-regulated genes. B. Validation of same of differentially expressed genes by qRT-PCR. C. HeatMap of qRT-PCR confirmed genes
HG-SOC-MSCS are close relative to mesothelial cells

In order to continue and ameliorate the characterization of HG-SOC-MSCs, their transcription levels were compared to the ones included in the FANTOM5 project dataset. FANTOM5 project is, to date, the largest transcription start site based atlas obtained from the majority of mammalian cells and tissues. The expression levels of HG-SOC-MSCs were compared to the complete set of primary cells, cell lines and tissue (n=899) in search of the closest cell/tissue relatives. When first examined all the samples aggregated in 4 major groups: cluster 1 blood and immune relate cells, cluster 2 adult tissues in particular of neuronal origin, cluster 3 mesenchymal and primary cells, cluster 4 cancer cell/tissue and cancer cell lines. HG-SOC-MSCs and N-MSCs are both included in cluster 3. It was interesting to note that when MSCs are compared between each other the MSCs derived from the heart (H-MSCs) are the most similar to HG-SOC-MSCs. When compared to the other types of cell/tissue contained in the FANTOM5 project dataset, in terms of differentially expressed genes, HG-SOC-MSCs resulted very similar to primary mesothelial cells and several cell types such as, smooth muscle and fibroblasts, which are thought to derive from mesothelial precursor (Rinkevich et al., 2012).
Figure 5: A. Hierarchical clustering of the peaks data generated by deep-CAGE sequencing of the primary cells and tissues of the FANTOM5 project belonging to the phase1 sample dataset (n=889). Red and green bars represent peaks up-regulated or down-regulated compared to the average value of all samples. Colored boxes highlight the four main identified clusters. Cluster 1: Blood and Immune. Cluster 2 Adult Tissues. Cluster 3 Mesenchymal. Cluster 4 Cancer.
HG-SOC-MSCs but not N-MSCs show EGF-dependent phenotypic and gene expression alteration.

It is known that, in *in vitro* culture, the mesothelial cells alternate between epithelioid and fibroblastoid morphologies, and express high levels of either keratin or vimentin or both. Mesothelial cells have, *in vivo*, high levels of keratin, which fall often to undetectable levels, when they start to rapidly grow *in vitro*. They can be subcultured many times in the low keratin state but when their growth slows, the levels of keratin begin to rise. The turnover of the keratins is very similar and very slow, so the levels are defined by rate of synthesis and the dilution resulting after cell division. Also the levels of vimentin are regulated but not in the same way as the levels of keratins: in fact, once in culture, the mesothelial cells begin to raise the levels of vimentin, reaching levels similar to those in the fibroblasts, regardless the rapidity or slowness of the growth. Another factor that influences the fibroblastoid vs. epitheliod morphology in the mesothelial cells is epidermal growth factor (EGF). In fact, when EGF is added to the mesothelial cell growth medium they shift from a flat epidermal like morphology to a fibroblast like morphology. Along with the change in morphology, the presence or absence of EGF in the culture medium influences the levels of mesothelial keratins. In fact, in absence of EGF the levels of keratin rise (Connel et al., 1983). HG-SOC-MSCs and N-MSCs were normally cultured in a medium containing hEGF (10 ng/ml final concentration). In order to test if the EGF has the same effect on MSCs as it has on the mesothelial cells, HG-SOC-MSCs and N-MSCs were cultured in presence or absence of EGF. After five days of culture in absence of EGF, HG-SOC-MSCs but not N-MSCs, when compared to the respective controls, displayed a switch from a fibroblastoid morphology to an epitheliod one. The switch involved the majority of the cell population, although not all of it. Moreover, this switch was marked by an increase in the expression of Keratin 7
(KRT7) observed and quantified by immunofluorescence. Very low levels of KRT7 were observed in N-MSCs cultured with or without EGF. The levels of vimentin (VIM) were not subjected to any variation in correlation with the presence or absence of EGF and remained high in both cell types with a 100% positive staining observed in immunofluorescence. The expression levels of both KRT7 and VIM were also evaluated by qRT-PCR and after five days of EGF deprivation, KRT7 levels had strongly increased in HG-SOC-MSCs compared to the N-MSCs (p-value <0.05), while, as it was expected from the immunofluorescence data, VIM levels had not changed.
Figure 6: HG-SOC-MSCs but not N-MSCs show phenotypic alteration after EGF deprivation.

A. Expression of KRT7 in HG-SOC-MSCs and N-MSCs after 5 days of culture in presence (10 ng/ml) or absence of EGF. B. Expression of VIM in HG-SOC-MSCs and N-MSCs after 5 days of culture in presence (10 ng/ml) or absence of EGF. (A and B scale bar 100µm). Graphics in C and D quantification of positively stained cells for KRT7 (C) or VIM (D) in the immufluorescence.
HG-SOC-MSCs express a specific mesothelial related gene signature correlating with serous ovarian cancer prognosis.

Starting from the lists of genes differentially expressed between HG-SOC-MSCs and N-MSCs a literature search was performed using both manual (PubMed, Protein Atals (Pontén et al., 2008)) and automated (Ingenuity Pathway Analysis tool or Molecular Signatures Database (Liberzon et al., 2011)) in order to find mesothelial related genes. Among them a list of six genes, already reported as mesothelial lineage markers, (CALB2, SPP1, KRT7, MSLN, CNN1, LRRN4) and four mesothelial/mesothelioma-related genes (TLR2, FN1, MCAM/CD146, KRT8) were selected (Connel et al, 1983, Barberis et al 1997, Tigrani et al., 2007, LaRocca et al., 1984, Kachali et al., 2006, Taniguchi et al., 2001, Kanamori-Katayama et al 2011, Park et al, 2007, Ksiazek et al., 2009, Bidlingmaier et al., 2009, Sato et al., 2010). qRT-PCR analyses were conducted to confirm the differential expression of the chosen genes in HG-SOC-MSCs compared to N-MSCs (Figure 7). Starting from this analysis a HG-SOC-MSC gene signature composed of 9 genes CALB2, SPP1, KRT7, MSLN, CNN1, FN1, MCAM/CD146, TLR2, KRT8) was identified. This signature was compared to high-throughput gene expression datasets (GSE23066 for non-small cell lung cancer; GSE29270 for breast cancer and GSE36474 for myeloma) of MSCs derived from cancerous tissues or healthy tissues counterpart. The comparison demonstrated that this signature is specific for the MSCs derived from SOC and it is not expressed in the MSCs derived from other tissues. The next issue was if the mesothelial-related gene signature could be somewhat correlated with the ovarian cancer prognosis. To this purpose several microarray datasets of SOC consisting in more than 900 patients (Gyorffy et al., 2012) were analyzed. Kaplan-Meier survival analysis of this data shows that the high expression of the mesothelial-related gene signature in the SOC patients induces a shorter progression free survival time (p=2.5-05), as shown in Figure 7. In order to assess the specificity of the mesothelial-related gene signature for the HG-SOC-MSC, analyses on other microarray dataset of different kind of tumor were performed. To this purpose dataset from breast cancer (Györffy et al., 2010) and lung cancer (Györffy et al., 2013) were analyzed. It was
observed that in the breast cancer the higher expression of the mesothelial-related gene signature induced a longer progression free survival time \((p=7.1^{-05}\text{ in Figure 7})\), while in the lung cancer there was no significant correlation between the expression of the mesothelial-related gene signature and the free progression survival time \((p=0.06\text{ in Figure 7})\). This data suggest that the mesothelial-related gene signature has a specific role in the SOC prognosis and a possible role of the signature itself in the promotion of tumor growth.
Figure 7 Survival analyses

A. qRT-PCR analysis of the mesothelial-related gene signature, the bars show the expression levels of the selected gene in HG-SOC-MSCs compared to the N-MSCs. B. Survival curves are shown for serous ovarian cancer patients with high (red) and low (black) expression of the selected gene signature. PFS time (PFS< 96 months) has been selected as clinical outcome. C. Survival curves are shown for breast cancer patients with high (red) and low (black) expression of the selected genes. PFS time (PFS< 96 months) has been selected as clinical outcome. D. Survival curves are shown for lung cancer patients with high (red) and low (black) expression of the selected genes. FP time (FP< 96 months) has been selected as clinical outcome.
Epithelial ovarian cancer (EOC) is the gynecological disease with the highest death incidence. It presents a high case-fatality ratio and five-year survival is less than 30%. In the last 50 years the death ratio for this type of cancer has not changed significantly. Unfortunately most cases of EOC were diagnosed at advanced state when the tumor had already spread to the upper abdomen (stage III) or even beyond (stage IV). An early diagnosis is very important: in fact when the EOC is diagnosed at low stage, i.e. stage I or II, the five-year survival is nearly 70% while if the diagnosis occurs at high stage, i.e. stage III or IV, survival drops below 20%. A wealth of studies has demonstrated the importance of microenvironment in developing cancers: in particular, stromal cells and their precursors have shown several features that could assist tumor homeostasis/development. Besides the intrinsic malignant properties of tumor cells, the micro-environmental changes may regulate progression to invasion and metastasis. It has been proved that a population of mesenchymal stromal/stem cells (MSCs), originally isolated from the bone marrow in the '70, can be found in almost every tissue. The capability of these cells to differentiate in endodermal, mesodermal and ectodermal cells lineages marks MSCs as pluripotent cells. The laboratory in which I have done my PhD project was able to isolate Mesenchymal Stem Cells (MSCs) from several healthy tissues. With the same protocol it was possible to isolate MSCs form High-Grade Serous Ovarian Carcinoma (HG-SOC-MSC). These cells are able to differentiate in vitro into various cell types including cells of the mesodermal lineages such as osteoblasts, chondrocytes and adipocytes, previously reported to discriminate MSCs from terminally differentiated fibroblasts (Wagner et al., 2005; Dominici et al., 2006). Moreover, HG-SOC-MSCs do not possess gross chromosomal aberration when compared to the matched tumor tissue and are not tumorigenic in vivo. When compared to MSCs derived from normal tissues (N-MSCs), such as adipose tissue, bone marrow and heart, HG-SOC-MSCs show a significant difference in terms of
immunophenotype, growth rate and population density. HG-SOC-MSCs, compared to N-MSCs, show a lower expression of CD105, a component of the TGF-beta receptor complex, in this case lacking a correlation with limited differentiation potential. This could be explained by hypothesizing that this is a marker of a specific subpopulation of MSCs. In fact it has been recently demonstrated that adipose-derived murine MSCs with high CD105 expression have grater capacity to differentiate in adipocytes osteocytes and are able to inhibit T cell proliferation in vitro with high efficiency (Anderson et al., 2013). Another interesting observation is the higher expression of the product of the MCAM gene, CD146, an adhesion molecule, in HG-SOC-MSCs compared to N-MSCs. Recently it has been demonstrated that CD146 is involved in various cell processes such as, development, signaling transduction, cell migration, angiogenesis, immune response, and it has also been identified as a cancer biomarker (Wang et al., 2013; Zeng et al., 2012; Aldovini et al., 2006). The higher expression of this adhesion molecule has been correlated with a grater differentiation potential in MSCs. Moreover, in the bone marrow resident MSCs there is a subpopulation expressing CD146 that is committed toward vascular smooth muscle differentiation lineage (Espagnolle et al., 2014). The expression of CD146 was also found in perivascular MSCs and it differentiates perivascular (normoxic) from endosteal (hypoxic) localization of non-hematopoietic bone marrow MSCs (Crisan et al., 2008; Tormin et al., 2011). HG-SOC-MSCs were submitted to the FANTOM5 project, one of the largest compendium of sample dataset. Using the deep-CAGE-seq analysis it was possible to compare the gene expression levels between HG-SOC-MSCs and other ovarian cancer-derived MSCs showing a 75% of gene expression concordance. Intriguingly, analyzing the CAGE-peaks across the entire gene length, the differential gene list, which was so obtained, suggests a possible specific epigenetic-based mechanism to explain their global CAGE-peak pattern. Recent studies have described identities in MSCs based on their tissue of origin, both in mouse models (Pelekanos et al., 2012) and in human. Different Hox codes or Hox expression fingerprints have been associated to human MSCs population from different anatomical sources (Sági et al., 2012). Surprisingly, this specificity seems to be correlated with the functional capabilities of MSCs: in
fact, it has been reported that bone marrow-derived MSCs cannot replace MSC-like cells of pro-epicardial origin in myocardial infarction (Chong et al., 2011). It has been shown that MSCs from different origin have different healing performances in cardiac regeneration (Gaebel et al., 2011), implying that MSCs are not a homogenous population spread all over the body, but different subtypes that have common features and specific properties. Data from the FANTOM5 project suggest that HG-SOC-MSCs are close relative to mesothelial cells, smooth muscle cells and fibroblasts. It is known that cultured mesothelial cells could switch between an epitheliod and a fibroblastoid–like morphology and this switch is triggered by EGF (Connell et al., 1983). HG-SOC-MSCs but not N-MSCs are able to operate this switch in a EGF dependent manner. In particular, it is possible to observe the morphological change and the expression of specific markers such as KRT7, indicating a connection between HG-SOC-MSCs and mesothelium, and a specificity of these cells among the other MSCs. Intriguingly, with the CAGE technology it was possible to find some difference among the N-MSCs: in particular, heart-derived MSCs compared to N-MSCs from other sources, such as bone marrow and adipose tissue, show a mesothelial signature similar to the one found in HG-SOC-MSCs. In order to find out the potential role of the HG-SOC-MSCs in serous ovarian cancer, a gene signature composed of nine elements was derived. The list includes five well-known mesothelial markers and four mesothelial-related genes. By interrogating large SOC meta-dataset it was observed that the expression of the abovementioned signature has a statistically significant correlation with patients with bad prognosis, implying a role of the HG-SOC-MSCs in tumor growth/promotion. A similar interrogation was done using other meta-dataset in order to verify the ovarian specificity of the mesothelial signature. Interestingly, the signature shows no statistically significant correlation with prognosis in lung cancer, and a statistically significant correlation with breast cancer in patients with good prognosis. Comparing the genes present in the signature, it is worth noting that some of them could be related with genes already found to be involved in the MSCs-ovarian interaction. In fact the expression BMP2 (Lai et al., 2002) is reported to be connected to the up-regulation of SPP1 and FN1. TLR2 and SPP1 are, instead, correlated to the expression of MMP2 and secreted
growth factor involved in the fibrovascular network formation (Varoga et al., 2006; Cho et al., 2007; Philip et al., 2001; Xu et al., 2005). This suggests a relationship between the HG-SOC-MSCs and the MSCs previously reported in ovarian cancer (REF). It is possible to hypothesize that HG-SOC-MSCs could originate from the ovarian mesothelium or the other local mesothelium. In fact, it has been reported in mouse models that the mesothelium of several organs contains the precursor of smooth muscle cells and fibroblasts (Rinkevich et al., 2012). Another possibility is that the MSCs are recruited from the tumor and that the microenvironment is able to reprogram the MSCs toward a mesothelial-like signature. This hypothesis was put forward by Ko and colleagues in a study showing that the tumor microenvironment in a HoxA9 depended manner, could reprogram bone marrow and adipose-derived MSCs to become cancer associated fibroblasts (Ko et al., 2012). Recently Bianco raised new issues in this field, sustaining that there is a lot of confounding elements in the research on MSCs. First of all the name, because in some papers the acronym MSC stands not only for mesenchymal stem cell but also for other meanings (such as multipotent stroma cells, mesenchymal stromal cells, medicinal signaling cells). But most importantly, he asserts that only the cells from bone marrow can be called MSCs and that the majority of the abilities referred to MSCs, such as pluripotency, are indeed culture artifacts (Bianco, 2015). In conclusion, a cell type-specific transcriptional activity has been demonstrated for HG-SOC-MSCs compared to N-MSCs from other districts and position them close to primary mesothelial and mesothelial-derived cells within the FANTOM5 dataset. Moreover, other tests, especially, in vivo, are needed to better define the role of HG-SOC-MSCs in the formation and progression of serous ovarian cancer.
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Specific Mesothelial Signature Marks the Heterogeneity of Mesenchymal Stem Cells From High-Grade Serous Ovarian Cancer

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Key Words. Mesenchymal stem cells • Tissue-specific stem cells • Genomics • Cancer • Adult stem cells • Cell biology

ABSTRACT

Mesenchymal stem/stromal cells (MSCs) are the precursors of various cell types that compose both normal and cancer tissue microenvironments. In order to support the widely diversified parenchymal cells and tissue organization, MSCs are characterized by a large degree of heterogeneity, although available analyses of molecular and transcriptional data do not provide clear evidence. We have isolated MSCs from high-grade serous ovarian cancers (HG-SOCs) and various normal tissues (N-MSCs), demonstrated their normal genotype and analyzed their transcriptional activity with respect to the large comprehensive FANTOM5 sample dataset. Our integrative analysis conducted against the extensive panel of primary cells and tissues of the FANTOM5 project allowed us to mark the HG-SOC-MSCs CAGE-seq transcriptional heterogeneity and to identify a cell-type-specific transcriptional activity showing a significant relationship with primary mesothelial cells. Our analysis shows that MSCs isolated from different tissues are highly heterogeneous. The mesothelial-related gene signature identified in this study supports the hypothesis that HG-SOC-MSCs are bona fide representatives of the ovarian district. This finding indicates that HG-SOC-MSCs could actually derive from the coelomic mesothelium, suggesting that they might be linked to the epithelial tumor through common embryological precursors.

INTRODUCTION

Mesenchymal stem/stromal cells (MSCs) reside in almost all types of tissues and are believed to play a central role in tissue regeneration, wound repair, and maintenance of tissue homeostasis [1, 2]. MSCs are increasingly appreciated to be highly heterogeneous and dynamic, in order to support the wide diversified parenchymal cells and tissue organization they control during both embryogenesis and in the adulthood [3]. The most characterized “mesenchymal stem cells” are the ones derived from the bone marrow where their microvascular location and mural cell identity point to their function in the regulation and organization of the hematopoietic niche with a stringently associated, restricted, and more specialized role as osteoprogenitors [4–7].

Mesenchymal interactions with epithelial parenchyma are essential for organogenesis and consequently critical elements in cancer progression [8]. MSCs are in fact present in the tumor microenvironment, where they could be recruited via the blood stream from the bone marrow thus exerting multiple and complex roles in the crosstalk with cancer cells. Notwithstanding their critical role as precursors for various cell types of the tumor stromal microenvironment and their exact lineage relationship with cancer-associated fibroblasts (CAFs), comprising both fibroblasts and myofibroblasts, have not been clearly assessed [9]. Within the tumor microenvironment, MSCs provide more than scaffolding/structural elements. Thus, beyond MSCs acting as precursors for the various mesenchymal cell types regulating the system-level and dynamic homeostasis ecosystem of the cancer stem cell niche and tissue, their secretome includes a wide range of bioactive molecules. These molecules have both immunomodulatory and general trophic functions, including angiogenesis/vasculogenesis regulators, thus establishing a
regenerative environment responding to local inflammatory cues [10, 11]. Tripartite interactions of MSCs with macrophages and cancer cells have pointed to the existence of a complex cellular scenario requiring instructional and epigenetic crosstalk for tumor development and homeostasis [12].

Exploring the presence of MSCs in human tumors as well as characterizing their functional and prognostic significance has therefore become an important area of investigation [13–24]. The heterogeneity intrinsic to both MSCs and tumor types calls for the analysis of large datasets to investigate the level of identity of MSCs with respect to heterogeneity and their potential roles in the specific tumor.

In this study, we isolate and characterize MSCs from high-grade serous ovarian cancer (HG-SOC-MSCs) and compare them to the comprehensive panel of primary cells and tissues of the FANTOM5 project, applying the deep-CAGE technology. Our results show that MSCs derived from HG-SOC, when compared with similarly derived normal tissues MSCs, possess a specific tissue identity showing a relationship with mesothelial-derived cells. This suggests their derivation from a mesothelial progenitor, which could be common also to the epithelial ovarian cancer cells. Finally, we observed an interesting correlation between HG-SOC-MSCs gene expression signature and the clinical outcome in this specific tumor.

This work is part of the FANTOM5 project. Data downloads, genomic tools, and copublished manuscripts are summarized at http://fantom.gsc.riken.jp/5/.

**Materials and Methods**

Human Samples

Human samples were obtained from the Azienda Ospedaliero-Universitaria of Udine and collected after informed consent in accordance with the declaration of Helsinki and with approval by the Bioethics Committee. The pathology records of the samples are reported in the Supporting Information Tables S1A and S1B.

Cell Isolation and Culture

N-MSCs were isolated from normal human adult tissues (bone-marrow, heart, and adipose tissues) and cultured under uniform conditions as described in Beltrami et al. [25]. HG-SOC-MSCs were isolated from primary HG-SOC and cultured under uniform conditions as previously described [15, 25] with minor modifications (Supporting Information Materials and Methods). Peripheral blood mononuclear cells (PBMCs) were isolated from HG-SOC-bearing patients through density gradient centrifugation (Biocoll; Biochrom, Berlin, Germany).

Flow Cytometry and Immunofluorescence

N-MSCs and HG-SOC-MSCs grown in expansion medium at passage P3 were subjected to flow cytometry analysis and immunofluorescence studies as described in Supporting Information Materials and Methods.

Cell Proliferation Assays

N-MSCs and HG-SOC-MSCs were plated on six-well plates at a density of 1,000, 2,000, and 3,000 cells per square centimeter. Cells were harvested and counted up to 10 days after plating.

**Induction and Assessment of Multilineage Differentiation**

Differentiating media used and protocols followed to differentiate HG-SOC-MSCs (at passage P3) into multilineages and the analysis of the expression of lineage-specific markers by immunofluorescence, histochemistry, or functional assays were performed as previously described [25, 26] and detailed in the Supporting Information Materials and Methods.

**High-Resolution Genotyping Analysis**

Genomic DNA was extracted using QIAamp DNA Mini Kit (Qia-gen, Hilden, Germany) and analyzed using HumanCNV370-Quadv3_C SNP platform (Illumina, San Diego, CA). Copy number information was derived normalizing each tumor or each HG-SOC-MSC sample to its matched normal counterpart using B allele frequency (BAF) segmentation and crlmm package [27].

**FANTOM5 Data Matrix and Sample Ontology**

Briefly CAGE tags were counted under the RLE (robust transcription expression) set of peaks (n = 184,827) identified in FANTOM5 across 889 human CAGE libraries and then normalized (as tags per million (TPM), generating a TPM data matrix) or used as counts (count data matrix). Samples were divided into functional groups using a modified version of the Sample Ontology developed by a member of the FANTOM5 Consortium (Dr. Tom Freeman, University of Edinburgh). Data can be accessed from http://fantom.gsc.riken.jp/5/suppl/Verardo_et_al.

Cluster Analysis

The TPM data matrix was filtered (standard-deviation peaks >0.55, see Supporting Information Materials and Methods for details) and then used for cluster analysis (Fig. 3A). For Figure 3B, we selected only the HG-SOC-MSCs and N-MSCs differential peaks (see below, samples n = 19, peaks = 1,035). Unsupervised hierarchical cluster analysis (average-linkage method) was then performed using Cluster software [28] and then visualized using Java TreeView [29].

Differential Expression Analysis

The count data matrix was used to perform the following analyses: (a) direct comparison between HG-SOC-MSCs and N-MSCs, (b) HG-SOC-MSCs and N-MSCs versus all other samples (100 randomly selected groups of samples), and (c) relationship analysis. See Supporting Information Materials and Methods for details.

Annotation of Functional Analysis

Functional analysis was performed using the Ingenuity Pathway Analysis tool (QIAGEN Silicon Valley, Redwood City, CA, www.ingenuity.com) and the DAVID/EASE tool [30] using default parameters and procedures. To perform Analysis of Functional Annotation (AFA) a combined dataset of the functional analysis results were created, where the significance of each biological functional/transcriptional regulators was transformed as the −log(p-value). Hierarchical clustering analysis of the resulting datasets was performed and visualized as shown in Figure 3C.

Real-Time Quantitative RT-PCR

Total RNA was extracted using the TRIZOL Reagent (Life Technologies, Carlsbad, CA) and cDNA was produced using the
QuantiTect Reverse Transcription Kit (Qiagen). Real-time quantitative RT-PCR was performed using the SYBR Green Master Mix (Applied Biosystems, Life Technologies, Carlsbad, CA) on a StepOnePlus Real-Time System (Applied Biosystems).

Survival Analysis
We performed Kaplan-Meier survival analysis considering progression-free survival (PFS) time of ovarian cancer patients, relapse-free survival time of breast cancer patients, and first

Figure 1. Multilineage differentiation potential of mesenchymal stem/stromal cells (MSCs) obtained from high-grade serous ovarian cancer (HG-SOC-MSCs). The first row of panels shows the ability of HG-SOC-MSCs (~80% of cells at passage P3) to differentiate into hepatocyte-like cells (positive staining for KRT7 and KRT18 in panels A and B, respectively, while panel C reveals positive PAS reaction). The second row of panels shows the ability of HG-SOC-MSCs (~80% of cells at P3) to differentiate into a myogenic derivative (positive staining for alpha-Smooth Muscle Actin in panel D, for Connexin-43 in panel E and for a-actinin in panel F). The third row of panels shows the ability of approximately 90% of HG-SOC-MSCs at P3 to differentiate into endothelial-like cells (positive staining for lectin UEA1 in panel G, for von Willebrand factor in panel H, and the ability to uptake acetylated low-density lipoprotein in panel I). The fourth row of panels shows the ability of HG-SOC-MSCs (~80% of cells at P3) to differentiate into osteoblast- (positive staining for collagen I in panel J, and for von Kossa in panel K) and chondroblast-like cells (positive staining for Alcian Blue, pH1 in panel L). The last row of panels shows the ability of HG-SOC-MSCs (~70% of cells at P3) to differentiate into PPAR-gamma-positive adipocytes (yellow fluorescence, panel M), displaying intracytoplasmic lipid droplets (red staining of oil red O, panel N).
progression (FP) time of lung cancer patients. Samples were classified according to the expression of the HG-SOC-MSCs gene signature (HSM-GS) using KMplotter web tool [31–33].

RESULTS

MSCs From HG-SOC: Isolation and In Vitro Characterization

We have previously reported a protocol for the derivation of multipotent stromal cells from normal human adult bone marrow, heart, liver, and adipose tissues (hereafter referred to as N-MSCs) that maintain the ability to differentiate along various lineages [25, 34]. We have adapted this protocol to obtain HG-SOC-MSCs (Supporting Information Table S1A), freshly isolated from patients undergoing surgery (as described in Materials and Methods). Adherent HG-SOC-MSC, between population doubling level (PDL) 20 and 25, exhibit a mesenchymal morphology similar to the normal counterpart at the same PDL (Supporting Information Fig. S1). Nevertheless, the cancer-derived cell population reveals distinctive in vitro growth kinetics when compared with N-MSC populations obtained from several normal human adult tissues (Supporting Information Fig. S2). In fact, the in vitro growth of HG-SOC-MSCs revealed a marked ability to increase population density when compared with N-MSCs. The cell population doubling times (calculated at PDL 20–22) were 30±3 and 46±15 hours for the HG-SOC-MSCs and the N-MSCs, respectively. The maximum cell expansion was achieved, for the HG-SOC-MSCs, with a seeding density of 1,000 cells per square centimeter, while, for N-MSCs, the same parameter was reached with 500 cells per square centimeter or less.

We then evaluated the surface immunophenotype of HG-SOC-MSCs by flow cytometry and compared it to that of similarly derived N-MSCs. Both cell types displayed a typical MSC-like cell surface marker profile (high levels of the mesenchymal markers CD73, CD90, with the only exception being the reduced expression of CD105 in the HG-SOC-MSCs) (Supporting Information Table S2), and were strongly positive for CD59, CD13, and CD49b, but negative for the markers of the hematopoietic system (CD34 and CD45). When compared with N-MSCs, HG-SOC-MSCs displayed a clear alteration of the surface adhesion molecule setting (lower expression of the FN binding integrins CD29, CD49a, and CD49d), and a higher expression of CD146/MCAM.

In order to evaluate the multipotency of the HG-SOC-MSCs, lineage-specific differentiation was induced using established protocols [25] and evaluated by immunostaining and functional assays. 70%–90% of cell populations were able to differentiate into endodermic and mesodermic derivatives. As shown in Figure 1, HG-SOC-MSC populations were able to differentiate into hepatocyte-like cells (panels A, B, and C), smooth muscle-like cells (panels D, E, and F), endothelial-like cells (panels G, H, and I), osteoblast-like cells (panels J and K), chondroblast-like cells (panel L), and adipocyte-like cells (panels M and N). It is to be noted that endothelial differentiation turned out to be the most efficient since at least 90% of the initial population resulted positive for the selected markers, coupled with the ability to organize into capillary-like structures actively up-taking acetylated low-density lipoprotein (see Supporting Information Materials and Methods for details).

HG-SOC-MSCs Do Not Possess Gross Chromosomal Aberrations and Are Not Tumorigenic In Vivo

Stromal tissues supporting the tumor mass in breast and ovarian cancers are composed of cell populations with no evident genomic alterations [35], thus originating from normal adult tissue stem cells. We therefore decided to assess the genomic status of the HG-SOC-MSCs by high-resolution genotyping analysis using the Illumina Infinium SNP BeadChips. The investigated dataset was composed of matching HG-SOC primary tissues, HG-SOC-MSCs, and PBMCs, each triplet from three patients. As clearly shown in Figure 2A, 2B and in Supporting Information Figure S3, no differences were detected between PBMC and HG-SOC-MSC genomes, while genome-wide
Figure 3. Transcriptional signatures of N-MSCs and HG-SOC-MSCs. (A): Hierarchical clustering of the peaks data generated by deepCAGE sequencing of HG-SOC-MSCs and N-MSCs for the differentially expressed genes (single peak analysis). Red and green bars represent, respectively, upregulated or downregulated genes with respect to the average of all samples. (B): qRT-PCR validation of selected genes differentially expressed between HG-SOC-MSCs and N-MSCs. (C): HeatMap of qRT-PCR confirmed genes from TPM matrix. (D): Heat-map showing the results of the annotation of functional analysis. Starting from the genes upregulated in HG-SOC-MSCs versus N-MSCs, and in HG-SOC-MSCs or N-MSCs versus 100 random-selected samples datasets, the biological functions and associated transcriptional regulators were obtained using the Ingenuity Pathway Analysis (IPA) tool and then clustered. Left panel: each block represents a single functional theme. Colored vertical bars represent the functional themes that are overrepresented in both the N-MSCs and HG-SOC-MSCs with respect to the random gene lists, but that are also globally overrepresented in the HG-SOC-MSCs with respect to the N-MSCs. Among them, a vast group is composed of terms that are predominantly cancer-related (red bar). Another group of functional terms is represented by themes related to cellular, tissue, and organismal development (blue bar). Right panel: the same analysis was performed for the IPA-associated transcriptional regulators. Each block represents the single transcription factor activation status, predicted by the differential expression levels of its known targets. The bar intensity reflects the statistical significance (corrected for multiple testing) of the analysis and the actual value is also plotted in trace line. Abbreviations: HG-SOC, high-grade serous ovarian cancers; MSC, mesenchymal stem/stromal cell.
aberrations were clearly found between matched PBMC and HG-SOC genomes ($p < .03$, paired t test).

In order to test for tumorigenicity, cultured HG-SOC-MSCs were injected into NOD-SCID mice in parallel to SKOV3 cells chosen as a tumorigenic ovarian cancer-derived cell line. $1 \times 10^6$ HG-SOC-MSCs (at passage P3) from three separate patients were injected subcutaneously in duplicate into mice and followed for 12 weeks. These cells were incapable of forming tumors. In contrast, $1 \times 10^6$ SKOV3 cells (in five replicas) were capable of forming subcutaneous tumors in 2 weeks (Supporting Information Table S3).

HG-SOC-MSCs and N-MSCs Differ in the Relative Levels of Activation of Biological Functions and Transcriptional Regulators

The transcriptional identity of HG-SOC-MSCs was analyzed by comparing their profiles to the large collection of samples in the FANTOM5 Project [36]. Ten normal tissue-derived MSC populations (N-MSCs) obtained from ten different patients (three from adipose tissue, three from bone-marrow, and four from heart tissue) and nine HG-SOC-MSCs (obtained from four different patients) (Supporting Information Table S1B) were profiled by deep-CAGE technology [37] as part of the
FANTOM5 project. In order to analyze the promoter activity peaks in terms of deep-CAGE-derived expression profiles, we performed a differential analysis using a read count-based statistics approach [38] considering each peak as representative of the expression of the related gene (hereafter referred to as differential expression analysis). This enabled us to identify a list of differentially expressed genes associated to the peak activity: 625 genes that were more highly expressed in HG-SOC-MSCs and 450 more highly expressed in N-MSCs were identified ($p$-value < $10^{-5}$, false discovery rate (FDR) corrected) as shown in Figure 3A. Some differentially expressed genes were experimentally validated by qRT-PCR analysis, in N-MSCs with respect to HG-SOC-MSCs (Fig. 3B). The resulting HeatMap in Figure 3C shows the agreement between the expression analysis based on CAGE-seq peak activity and the qRT-PCR results (for the qRT-PCR results at single sample level refer Supporting Information Fig. S4A). These results allowed us to compare the HG-SOC-MSCs and N-MSCs deep-CAGE profiles with the FANTOM5 phase1 dataset which includes 889 distinct samples (see Materials and Methods) and the obtained results were subjected to the AFA. Figure 3D (left panel for functions, right panel for transcriptional regulators) clearly shows that N-MSCs and HG-SOC-MSCs share the activation of common core biological functions and transcriptional

![Figure 5](image.png)

**Figure 5.** Response of HG-SOC-MSCs and N-MSCs to EGF deprivation. *(A):* Representative immunofluorescence images of HG-SOC-MSCs and N-MSCs after 5 days of culture in the presence (10 ng/ml) or absence of EGF, showing the content of keratin-7 (KRT7) and vimentin (VIM) (upper panels). Scale bar = 100 μm. Quantification of the percentage of cells positive for KRT7 and VIM (lower panels), showing that EGF deprivation leads to a significant increase in KRT7 of the HG-SOC-MSCs, but not N-MSCs. 100% of cells (both HG-SOC-MSCs and N-MSCs) were positive for the VIM staining irrespective of the presence or absence of EGF. *(B):* qRT-PCR results of KRT7 and VIM genes in HG-SOC-MSCs (from three different HG-SOC patients) and N-MSCs (from normal tissue sources of three different patients: two from adipose tissue and one from bone marrow). Each bar represents the gene expression ratio of the gene for MSCs cultured for 5 days in the absence or in the presence of 10 ng/ml hEGF ($-\text{EGF}/+\text{EGF}$ ratio). Abbreviations: EGF, epidermal growth factor; HG-SOC, high-grade serous ovarian cancers; MSC, mesenchymal stem/stromal cell.
regulators, the differences residing in the different level of their activation. Importantly, among the most upregulated biological themes in HG-SOC-MSCs versus N-MSCs, the following emerged as significant: cancer-related and developmental functional terms (Fig. 3D, red bar and blue bar, respectively), extracellular matrix, cell adhesion, and cell motility (Supporting Information Table S4). The transcriptional regulators analysis (including the activation of TGFB, VEGF, and HGF) led to the same conclusion, suggesting that globally the differences between HG-SOC-MSCs and N-MSCs are quantitative, with similar core functions and transcriptional regulators activated, however at different levels. Similar results were obtained using DAVID/EASE [39] (Supporting Information Table S5). The transcriptional identity of HG-SOC-MSCs was also investigated with respect to the expression of genes previously reported to be associated with ovarian cancer-derived MSCs [16–18], showing an overall gene expression concordance of 75% (Supporting Information Fig. S5). Specifically, a most significant correlation was found for BMP2,4 [18] and secreted factors involved in fibrovascular organization [16], thus enforcing their critical functions in the homeostasis of the HG-SOC microenvironment.

HG-SOC-MSCs Are Close Relatives of Mesothelial Cells
The FANTOM5 project dataset represents to date the broadest transcription start site-based atlas obtained from the majority of the mammalian cells and tissues [36]. Using this comprehensive dataset we addressed the degree of relationship between the HG-SOC-derived MSCs and the full set of primary cells, cell lines, and tissues (n = 889), in search of the closest cell/tissue relatives. Starting from the RLE-TPM matrix (see Materials and Methods), and excluding genes with low variance across samples (normalized SD > 0.5), the resulting hierarchical cluster analysis is shown in Figure 4A. When first examined, all the samples aggregated into four major groups: cluster 1 mainly composed of blood and immune-related primary cells; cluster 2 composed of adult tissues, especially of neuronal origin; cluster 3 mainly composed of mesenchymal tissue/primary cells; cluster 4 mainly composed of cancer tissues/cell lines. HG-SOC-MSCs and N-MSCs are both included in cluster 3. Interestingly, when we directly compared the different MSCs populations, we noticed that the MSCs derived from heart were the most similar to HG-SOC-MSCs (Fig. 4B, and for hierarchical clustering analysis at single sample level refer Supporting Information Fig. S6). To find the closest transcriptional relatives of HG-SOC-MSCs we used a hierarchical sample ontology (modified version of the Freeman ontology, hereafter referred to as sample ontology, see Materials and Methods) to divide the FANTOM5 samples into biological-related groups (n = 220). We then performed a pairwise differential expression analysis between the HG-SOC-MSCs group and all the other biological groups. The analysis showed that, in terms of differentially expressed genes, HG-SOC-MSCs are one of the most similar to primary mesothelial cells and several cell types (smooth muscle cells and fibroblasts) (Supporting Information Table S6A) hypothesized to derive from mesothelial precursors [40].

HG-SOC-MSCs But Not N-MSCs Show Epidermal Growth Factor-Dependent Alteration of Cell Morphology and KRT7 Expression
It is known that, in vivo, mesothelial cells are keratin-rich but its content falls when they begin dividing rapidly in vitro [41]. In culture, mesothelial cells are able to switch between fibroblastoid and epithelioid morphologies depending on their state of growth and the presence of epidermal growth factor.
Given the expansion medium in which both the HG-SOC-MSCs and N-MSCs were grown, containing the growth factors hEGF and hPDGF-BB, and given the identified lineage relationship with the mesothelial cells, we decided to culture both the HG-SOC-MSCs and the N-MSCs, in the reported expansion medium, in the presence or in the absence of hEGF (10 ng/ml final concentration). As shown in Figure 5A and in Supporting Information Figure S7, after 5 days of culture without hEGF (−EGF), a significant fraction of HG-SOC-MSCs has undergone a morphological switch revealing a more epithelioid morphology, compared to the fibroblastoid appearance of the control HG-SOC-MSCs (+EGF). Such morphological

Figure 7. Survival analysis. (A): qRT-PCR analysis of HG-SOC-MSCs mesothelial marker genes and selected mesothelial-related genes. Relative expression levels in HG-SOC-MSCs with respect to N-MSCs. All the genes are significantly over-expressed (at least p < .05) in HG-SOC-MSCs with respect to N-MSCs. (B): Survival curves are shown for serous ovarian cancer patients with high (red) and low (black) expression of the selected gene signature. Progression-free survival (PFS) time (PFS < 96 months) has been selected as clinical outcome. Hazard ratio and significance are also reported. SOC patients expressing higher levels of the selected genes in tumors displayed shorter PFS time (p = 2.5^-05). (C): Survival curves are shown for breast cancer patients with high (red) and low (black) expression of the selected genes. PFS time (PFS < 96 months) has been selected as clinical outcome. Hazard ratio and significance are also shown. Patients expressing higher levels of the selected genes in tumors displayed no significant change in first progression survival time (p = .06). (D): Survival curves are shown for lung cancer patients with high (red) and low (black) expression of the selected genes. FP time (FP < 96 months) has been selected as clinical outcome. Hazard ratio and significance are also shown. Patients expressing higher levels of the selected genes in tumors displayed no significant change in first progression survival time (p = .06). Abbreviations: HG-SOC, high-grade serous ovarian cancers; MSC, mesenchymal stem/stromal cell.
switch was accompanied with a strong increase in the positivity for the epithelial cell marker keratin 7 (KRT7), as observed and quantified by immunofluorescence imaging (Fig. 5A, \( p\)-value < \(10^{-14}\)). Conversely, considerably lower levels of KRT7 were observed in N-MSCs, both in the presence and in the absence of EGF, with no significant differences between the two conditions. In parallel, the expression of vimentin (VIM) was also evaluated in HG-SOC-MSCs and N-MSCs. As shown in Figure 5A, 100% positive staining was observed for the two cell types both in the presence or absence of EGF. To complete these observations, the relative expression of KRT7 and VIM genes was evaluated by qRT-PCR. As shown in Figure 5B, the expression of KRT7 markedly increased (\( p\)-value < .035) after 5 days of EGF deprivation in HG-SOC-MSCs as compared to N-MSCs, while the expression of the mesenchymal marker VIM remained stable (for the qRT-PCR results at single sample level refer Supporting Information Fig. S4B). It is worth noting that KRT7 gene, in the reported expansion medium (+EGF), is expressed at very high levels in HG-SOC-MSCs compared to N-MSCs (Fig. 7A).

Differential Gene Peaks Usage Analysis Supports the Relationship Between HG-SOC-MSC and Mesothelial Lineage

To explore the full potential of FANTOM5 CAGE-seq data, we investigated the distribution of the peaks for all genes across their entire length, including the promoter region, highlighting that, on average, nine peaks are associated to each gene and in 15% of the cases more than one peak could be considered active (TPM > 15). The presence of multiple active peaks could be an indication of open chromatin status leading to multiple transcription start sites, that could be related to epigenetic-based mechanisms of transcriptional regulation [42]. In particular, we investigated the differences of multiple active peaks between N-MSCs and HG-SOC-MSCs. As shown for two representative genes (Fig. 6A, 6B) in spite of the presence of a dominant peak, there were significant differences when considering the presence of multiple peaks along the entire gene length. We analytically identified 203 genes with differential number of active peaks between N-MSCs and HG-SOC-MSCs (Supporting Information Tables S7A and S7B). Only a fraction of the resulting genes (22%) were highlighted in the previous differential expression analysis, unveiling differences not otherwise detected. Finally differential gene peaks usage data were used to search the HG-SOC-MSCs closest cell/tissue relatives (see Materials and Methods): as shown in Supporting Information Table S6B, this analysis further confirmed the evidence that HG-SOC-MSCs are close relatives of mesothelial cells.

A Specific HG-SOC-MSCs Mesothelial-Related Gene Signature Correlating With Serous Ovarian Cancer Prognosis

Starting from the lists of genes found to be differentially expressed between HG-SOC-MSCs and N-MSCs (derived from the differential gene expression analysis or from the differential gene peaks usage analysis), we performed an exhaustive literature search using automated tools (Ingenuity Pathway Analysis tool or molecular signatures database [43]) or manual tools (PubMed, Protein Atlas [44]) to search for mesothelial-related genes. Among them, we selected six genes previously reported as markers of the mesothelial lineage (CALB2, SPP1, KRT7, MSLN, CNN1, and LRRN4) and four mesothelial/mesothelioma-related genes (TLR2, FN1, MCAM/CD146, and KRT8) [41, 45–54]. Their differential expression in HG-SOC-MSCs with respect to N-MSCs was confirmed by qRT-PCR analysis (Fig. 7A).

In order to study the specificity of the identified transcriptional signature with respect to other types of cancers, starting from the genes previously confirmed by qRT-PCR analysis we generated a HSM-GS composed of nine genes (CALB2, SPP1, KRT7, MSLN, CNN1, FN1, MCAM/CD146, TLR2, and KRT8). To this purpose, we analyzed high-throughput gene expression datasets (GSE23066 for non-small cell lung cancer, GSE29270 for breast cancer, and GSE36474 for myeloma) comparing MSCs derived either from cancerous tissue or from the healthy tissue counterpart. As shown in Supporting Information Table S8, the HSM-GS signature is unique to MSCs derived from HG-SOC and not other cancers.

We were then interested to see if the mesothelial-related gene signature of HG-SOC-MSCs might correlate with ovarian cancer prognosis. We analyzed several microarray datasets of serous ovarian cancer, to our knowledge encompassing all publicly available SOC profiles based on the Affymetrix platform, collectively consisting of more than 900 patients [31]. Kaplan-Meier survival analysis of the combined datasets showed that SOC patients with higher levels of the selected genes displayed shorter PFS time (\( p = 2.5^{-05}\)), as shown in Figure 7B. To evaluate the HG-SOC-MSCs gene signature specificity we performed the same analysis on several breast cancer microarray datasets comprising more than 2,500 patients [32] and several lung cancer microarray datasets comprising more than 700 patients [33]. Interestingly we observed that breast cancer patients expressing higher levels of the HG-SOC-MSCs gene signature displayed longer PFS time (\( p = 7.1^{-05}\) in Fig. 7C), while lung cancer patients did not show significant correlation between the HSM-GS gene signature and FP survival time (\( p = .06\) in Fig. 7D). Different performances of the HG-SOC-MSCs signature in different kind of tumors suggest that we actually found a specific clinical correlation with serous ovarian cancer bad prognosis, possibly implying their tumor growth-promoting activity.

**DISCUSSION**

In this work, we analyzed the identity of HG-SOC-MSCs. First, we demonstrated their ability to differentiate in vitro into various cell types including cells of the mesodermal lineages such as osteoblasts, chondrocytes, and adipocytes, previously reported to discriminate MSC from terminally differentiated fibroblasts [55, 56]. Importantly, similarly to other studies, we demonstrated that, when compared with the matched tumor tissue, HG-SOC-MSCs do not possess gross chromosomal aberrations and are not tumorigenic in vivo. When HG-SOC-MSCs were compared to various normal adipose-, bone marrow-, and heart-derived MSCs (collectively named N-MSCs) isolated using the same protocol, we were able to observe significant biological differences in terms of immunophenotype, proliferation rate and ability to increase population density. In particular, among the cell surface markers, CD105, a component of the TGF-beta receptor complex, shows significantly lower...
expression in HG-SOC-MSCs than in N-MSCs, in this case lacking a correlation with limited differentiation capability. We can speculate that this could point to a context-specific subpopulation of MSCs, as proposed in this study. In line with this, it was recently shown that in adipose tissue-derived murine MSCs [57], CD105+ cells represent a distinct multipotent MSC subpopulation with greater capacity to differentiate into adipocytes and osteocytes and more efficient at inhibiting T-cell proliferation in vitro. It is also worth noting that the cell adhesion molecule CD146, encoded by the MCAM gene, shows significantly higher expression in HG-SOC-MSCs than in N-MSCs. Recent evidence indicates that CD146 is actively involved in various processes, such as development, signaling transduction, cell migration, angiogenesis, immune response and, as such, it has been identified as a cancer biomarker [58, 60]. Interestingly, it was previously shown that MSCs with greater differentiation potential express higher levels of CD146 on the cell surface, and that within a bone marrow MSC population, specific subpopulations highly expressing CD146 are committed toward a vascular smooth muscle cell lineage [61]. It was also shown that CD146 expression on MSCs is associated with their perivascular topography [62] and that it differentiates between perivascular (normoxic) and endosteal (hypoxic) localization of nonhematopoietic bone marrow MSC populations [63]. These observations prompted us to embed these cells into the FANTOM5 Project with the aim of investigating their transcriptional identity. Taking advantage of the deep-CAGE-seq data we were able to show that HG-SOC-MSCs possess distinct and specific CAGE-peak promoter activities with respect to N-MSCs as assessed by differential expression analysis. When comparing the expression of a compendium of genes, highlighted as relevant in previous ovarian cancer-derived MSCs, to the expression of the transcriptional elements upregulated in our HG-SOC-MSCs, it was possible to show an overall gene expression concordance of 75%, with the strongest correlation found for previously identified BMP2,4 [18] and genes involved in fibrovascular control [16]. Moreover, when looking at the distribution of all CAGE-peaks across the entire gene length (gene peaks usage analysis), the derived differential gene list suggested the possible existence of a specific epigenetic-based mechanism explaining such global CAGE-peak patterns. The existence of specific identities in MSCs has been recently described in murine MSC-like cells derived from different tissues (bone-marrow, heart, and kidney) [64], and individual Hox codes or Hox expression fingerprints have been associated to human MSC populations of different anatomical origins [65]. Importantly, such specific heterogeneity seems to be critical at the functional level since bone marrow-derived MSC-like cells cannot replace MSC-like cells of proepicardial origin in myocardial infarction [66], and MSCs originating from different sources show a different healing performance in cardiac regeneration [67]. Altogether the highlighted shared functional programs between HG-SOC-MSCs and N-MSCs suggest that the global differences are based on quantitative levels of transcriptional output rather than on qualitative differences in the expressed genes. Among all the FANTOM5 samples, both gene expression and gene peak usage analysis results suggested that HG-SOC-MSCs were close relatives of mesothelial cells, smooth muscle cells, and fibroblasts and that they are related to their district of origin; we may speculate that we would have obtained similar findings when using N-MSCs derived from normal ovarian tissue. In line with these results, and according to previous studies [41], we decided to test the hypothesis that HG-SOC-MSCs, similar to cultured mesothelial cells, could be able to switch between a fibroblastoid and an epithelioid state in an EGF-dependent manner. Interestingly, HG-SOC-MSCs but not N-MSCs showed such ability highlighting changes in morphology and in the expression of specific markers (i.e., KRT7), once again marking a possible relationship with the mesothelial lineage. Noticeably we were able to trace CAGE-peak differences also within the various normal tissue-derived MSCs: most specifically and relevantly, heart-derived MSCs showed a similar overall mesothelial signature as found in HG-SOC-MSCs with respect to adipose and bone marrow-derived N-MSCs. Based on the identified relationship between the HG-SOC-MSCs and the mesothelial lineage, and in order to address a potential role of HG-SOC-MSCs in serous ovarian cancer, we derived a gene signature composed of nine genes. These genes consist of five established mesothelial markers and four mesothelial-related genes. When this signature was used to interrogate a large SOC meta-dataset, a statistically significant correlation with bad prognosis was found in patients expressing higher levels of the signature, possibly implying an HG-SOC-MSCs mediated tumor growth-promoting activity. Such correlation was rather specific for HG-SOC given that its performance was either statistically nonsignificant in the case of lung cancer or correlated with good prognosis in the case of breast cancer. It is interesting to note that, comparing this specific signature to the transcriptional networks or genes previously reported to be associated with ovarian cancer-derived MSCs [16–18], several interesting connections can be found. Among them, several reports are pointing to the relationship between the expression of BMP2 and the upregulation of SP1 and FN1, while TLR2 and SPP1 are reported to mediate the upregulation of MMP2 and secreted growth factors involved in the fibrovascular network formation [68–72]. This would suggest a potential relationship between the HG-SOC-MSCs signature and the transcriptional networks highlighted as relevant in other ovarian cancer-derived MSCs studies. With respect to the close relationship of HG-SOC-MSCs with mesothelial lineage, it has been recently shown [40] that the mesothelium derived from various adult mouse organs contains the precursors of fibroblasts and smooth muscle cells. We therefore hypothesize that the HG-SOC-MSCs could derive from the ovarian mesothelium or other local mesothelia. Similarly, all the accumulated evidence [73–77] support the contiguous mesothelium of ovarian surface epithelium and fallopian tube fimbriae as the site of origin of the cancer cell compartment of high-grade serous ovarian, peritoneal, and fallopian tube cancers (collectively referred to as “pelvic serous carcinomas”). As an alternative to the above-proposed hypothesis, the HG-SOC microenvironment as orchestrated by the dominant cancer cell compartment could instruct, via epigenetic mechanisms, the conditioning of recruited bone marrow MSCs to acquire the overall mesothelial signatures common to their specific origin. This hypothesis has been recently proposed for HG-SOC as a mechanism to explain the HoxA9 cancer cell-mediated activation of MSCs and peritoneal cells to acquire CAFs features [78]. Future transcriptomic analysis of
mesothelial-derived cells obtained from different tissue districts will uncover their complex biology and heterogeneity, shedding light on the potential role they might play in the microenvironment of HG-SOC and other aggressive tumors.

**CONCLUSIONS**

Altogether, the FANTOM5 deep-CAGE sequencing data allowed us to assign a specific identity for MSCs derived from HG-SOC. We demonstrate a cell-type-specific transcriptional activity associated with HG-SOC-MSCs, which identifies them with respect to N-MSCs from other tissue districts and positions them close to primary mesothelial and mesothelial-derived cells within the 889-sample FANTOM5 dataset. This unveils either their possible local mesothelial origin or their specific conditioning by the dominant mesothelial-derived HG-SOC cancer cell compartment.

**ACKNOWLEDGMENTS**

C.S. is supported by Italian Association for Cancer Research (AIRC), AIRC Special Program Molecular Clinical Oncology “5 per mille,” FIRB “ONCODIET” project, and National Technology Cluster CTNO1_00177_817708 project, both from the Ministry for Education, University and Research (M.I.U.R). Y.C. is supported by a Ph.D. Fellowship from the School of Molecular Biomedicine University of Trieste. S.P. is supported by the FIRB “ONCODIET” project from the Ministry for Education, University and Research (M.I.U.R). C.A.B. is supported by FIRB project RBAP11Z4Z9, 2012–2014, and AIRC “5 per mille” Special Program 2011, Pr. 12214. A.P.B. is supported by the FIRB project RBAP11ETKA_007. D.C. is supported by the ERC-7FP SP 2 IDEAS QUIPQROQUO G.A. n. 269051. M.M. is supported by the FIRB RBAP11Z4Z9 project from the Ministry for Education, University and Research (M.I.U.R). Y.C. is supported by Italian Association for Cancer Research (AIRC), AIRC Special Program Molecular Clinical Oncology “5 per mille,” FIRB “ONCODIET” project, and National Technology Cluster CTN01_00177_817708 project, both from the Ministry for Education, University and Research (M.I.U.R). Y.C. is supported by Italian Association for Cancer Research (AIRC). The authors indicate no potential conflicts of interest.

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