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Running title: EMT features feed back heterogeneity in cell response.

## Abstract

The continuing efforts to exploit the death receptor agonists, such as the TNF-related apoptosis-inducing ligand (TRAIL), for cancer therapy have largely been impaired by the anti-apoptotic and pro-survival signaling pathways leading to drug resistance. Cell migration, invasion, differentiation, immune evasion and anoikis resistance are plastic processes sharing features of the epithelial-mesenchymal transition (EMT), that have been shown to give cancer cells the ability to escape cell death upon cytotoxic treatments. EMT have recently been suggested to drive a heterogeneous cellular environment that seemed favorable for tumor progression. Recent studies pointed out a link between EMT and cell sensitivity to TRAIL, whereas others highlighted their effects on the induction of EMT. This review aims to explore the molecular mechanisms by which death signals can elicit an increase in response heterogeneity in the metastasis context, and to evaluate the impact of these processes on cell responses to cancer therapeutics.

## The epithelial-mesenchymal transition (EMT) features

EMT is a physiological process that occurs during embryogenesis (type 1 EMT), wound healing phases (type 2 EMT) and metastasis (type 3 EMT). In these distinct situations where it undertakes development (1), cellular homeostasis and repair (2), EMT is characterized by the loss of some epithelial features and in parallel, by the gain of new mesenchymal properties such as acquisition invasive capacities and resistance to apoptosis. The newly acquired phenotype owns stem cells capacities that confer to cells, pluripotency and plasticity but also a different sensitiveness to both endogenous and environmental signals (3). It is a critical process for tumor initiation and progression (4).

During EMT, E-cadherin, the main cadherin responsible of the epithelial cells adherent junctions but also a regulator of actin cytoskeleton homeostasis and organization (5,6), is down-regulated primarily via the TGF-β/SMAD signaling leading to loss of cell-cell adhesion (7). To date, cancer cells with low level of E-cadherin are rather considered invasive (or aggressive) while those with a high level are associated with stress-resistance and survival (8). However, E-cadherin expression was recently shown to be crucial for metastasis by preventing ROS-dependent cell death and by allowing cancer cell dissemination (9), a report in accordance with the clinical evidences showing metastatic E-cad+ tumors (10,11).

Other pathways than TGF-β/SMAD also play a central role in relaying EMT signal. This is the case of the tyrosin kinases receptors (RTKs), Notch, Hedgehog, the canonical and the non-

canonical Wnt pathways (12). They all activate the EMT-inducing transcription factors (EMT-TFs). Among them, Snail family (Snail and Slug), ZEB1/2, TWIST1/2, TCF3, FOXC2, PRRX1, YAP/TAZ and SOX4/9 are targeting E-cadherin repression or cooperating with core EMT-TFs (13). Because EMT-TFs are differentially expressed depending on the cancer stage, as observed for example in endometrioid endometrial carcinoma (14), the spatiotemporal expression of the EMT-TF TWIST1 can be a mechanism for the hierarchical role of EMT-TFs observed during cancer progression (15), while the miR-34/SNAIL and the miR-200/ZEB axis not only regulate E-cadherin expression but also the hybrid phenotype with a double-negative feedback (16–19).

Evidences concerning the acquisition of stem cell characteristics associated with induction of partial EMT, also known as a hybrid E/M state, were described and associated with an increase in tumor-propagating cells (TPCs) frequency (20). By showing that the earliest EMT state can exhibit a high TPC frequency, the authors demonstrated that intermediate states can also provide stem cell properties leading to drug resistance and cancer progression, and this mechanism does not require the establishment of a full EMT as it has been assumed before. Indeed, a report that EMT was not required for lung metastasis, was based on the following observation that metastasis in secondary sites mostly exhibit epithelial phenotype (21). However, the actual tools and methods used to claim such a controversial conclusion were questioned and found insufficient to rule out the EMT process during cancer progression (22). Therefore, the hybrid E/M system is still the proposed mechanism by which EMT drives metastasis dissemination (23–25).

Cell death resistance has been shown in a partial EMT states (26), and the hybrid E/M phenotype was described in tumor as a source of cancer cell response heterogeneity –differences in sensitivity to apoptotic stimulus such as anoikis and anticancer drugs (27,28). The main signaling pathways involved in development and regenerating processes are also involved in this mechanism in neoplastic tissue. Among them, the Notch-Jagged pathway is stabilizing the hybrid E/M phenotype and is necessary to expand the fraction of CSCs. This was shown in a triple negative breast cancer model under the influence of IL-6, a pro-inflammatory cytokine able to activate the EMT program (29). Still in the mammary gland, the EMT program is also increasing stem-like features through the control of the Hedgehog signaling pathway (30) whereas Wnt pathways regulate the stem cell program of the hybrid E/M phenotypes that counts for the increasing drug resistance (31–33).

CSCs have tumorigenic potentials which depends on the EMT state (33). EMT also confers stem cell properties to cancer cells by inducing non-genetic and heritable epigenetic changes (34). But these newly acquired properties are known to be reversible through the induction of a mesenchymal to epithelial program called MET (35), with the EMT/MET switch contributing to

cell phenotypic plasticity (36,37). Because a large number of phenotypic states exist between partial-EMT and the well differentiated states of EMT, this phenotypic diversity is then increasing intra-tumoral heterogeneity (38) and is a potential source of drug resistance observed in patients (39).

***EMT and cell survival***

Evidence that EMT not only provides the mechanistic basis of metastasis but also of resistance to apoptosis, has been demonstrated in different model tissues, such as breast (40), lung (21), prostate (41) or pancreas (42) with a process potentially dependent of miR-200 (43,44), TWIST and Snail1 expression (45,46). The decreased expression of key proteins such as cadherins and the integrins is accompanied by the loss of cell adhesion with extracellular matrix and with the neighboring cells during the EMT process. These leads to the activation of intracellular pro-survival signals known as "non-canonical pathways" and mainly mediated by the PI3K/Akt pathway.

The PI3K/Akt signaling pathway plays a pivotal role in controlling cancer cell survival. More specifically, it allows the activation of the MAPK pathway responsible for the activation of downstream P90RSK thus inhibiting of the pro-apoptotic Bad protein Bad (47). In prostate cancer, the PI3K/Akt signaling is activated downstream by the involvement of the Notch and the Hedgehog pathways activation. While Hedgehog is increasing the expression of the anti-apoptotic Bcl-2, Notch mediates pro-survival mechanisms under the control of Akt thus leading to Docetaxol resistance (48). Akt activation also drives NF-kB activation which in turn controls the expression of the anti-apoptotic proteins FLIP and XIAP (49).

The main EMT-TFs responsible for migration, invasion or dedifferentiation also play a role in cell survival by modulating the expression of pro- and anti-apoptotic proteins. For example, Twist increases Bcl-2 leading to apoptosis resistance (50) whereas SNAI1 interact with PARP1 (51,52). Other apoptotic regulators are involved in the EMT-dependent survival mechanisms. Among them, TGF-β, a tumor repressor with a dual role in cancer that depends on the environmental conditions such as matrix rigidity (53) or cancer progression stage (54), was shown to induce apoptosis and to interplay with the PI3K/Akt pathway (55).

In conclusion, many environmental factors derived from extracellular matrix, cancer-associated fibroblasts (CAFs), immune cells and vessels are responsible for the increase of EMT-TFs listed above and involved in a multidrug resistance (MDR) phenomena. They not only regulate the expression of pro- or anti-apoptotic proteins but also those of ABC transporter genes (56). Moreover, signals that induce EMT such as TGF-β could modulate the cancer cells response to

anticancer drugs (as with endogenous anti-mitotic signals) by cytokinesis failure, a heritable mechanism that leads to genomic instability (57). The dual role of TGF-β is applied in this context and can lead to opposite effects, depending on the cancer mutations and the model studied. For example, in Ras-mutant pancreatic cancer cells, sensitivity to apoptosis is then controlled by the TGF-β induced EMT. In other words, TGF-β induces EMT and subsequent apoptosis is conferring a tumor suppressive property to the EMT program (58).

## Death receptor-mediated features of EMT

Activation of transmembrane receptors of the tumor necrosis factor superfamily (TNF-SF), such as Fas/CD95, TNF-R1/R2 and TNF-related apoptosis-inducing ligand (TRAIL) receptors 1 and 2 (TRAIL-R1/2, DR4/DR5), by their respective ligands Fas-L, TNF-α and APO-2L/TRAIL (Table 1), can lead to the induction of cell death (59,60). The binding of the ligand to its receptor allows the formation of a Death-Inducing Signaling Complex –DISC, including caspase-8 (61), which can transduce a pro-apoptotic signal via caspase cascade leading to cell death (62).

In addition to apoptosis induce a range of cell responses upon binding to death receptors. Among them, differentiation was shown regulated by TRAIL-induced caspases activation in intestinal cells (63), osteoclasts (64) and keratinocytes (65). A close relationship exists between the differentiation steps during early stage of development and cancer progression leading to metastasis, involving common molecular factors and pathways (66), such as death receptor activation or dysregulation. Indeed, metastasis and invasion are processes associated with TRAIL treatment and shown to be dependent of the NF-κB pathway (67). In human cholangiocarcinoma cell lines, TRAIL promotes cell migration and invasion under the control of the NF-κB-dependent pathway (68). As cancer progression can be initiated through the induction of EMT, involving different steps of cell differentiation, it becomes important to better understand the molecular mechanisms leading to acquirement of heterogenous EMT features upon death receptor engagement, further impacting cell response (Figure 1).

### *Death receptor cooperates with cell membrane components: TRAIL and loss of cell adhesion.*

TRAIL has been reported to induce loss of adhesion followed by drug-resistance in several cellular contexts. First through the apoptotic pathways, TRAIL activates cleavage of substrates involved in cell differentiation and remodeling (69). Then, by interacting with cadherins at the plasma membrane (Table 1), DR4/5 stabilization and activation can be impaired leading to changes in TRAIL-sensitivity, as discussed below.

Occludin and claudin, two transmembrane proteins forming tight junctions (TJs), play a

barrier role in controlling fluid transport but also in proliferation and differentiation of the epithelium. As an epithelial marker, occludin is downregulated during EMT (70). Epigenetic silencing of occludin is leading to metastasis of cancer cells via modulation of unique sets of apoptosis-associated genes (71). Moreover, its knockdown decreases TRAIL-induced cell death thus conferring an important role of occludin in apoptosis (72). Indeed, occludin (and to a lesser extent claudin) is interacting physically with FADD and the DISC when tight junctions are disrupted, a phenomenon allowing cell defense (an antibacterial strategy), by activating the extrinsic cell death signal (73,74).

Studies reported that distribution of TRAIL receptors in lipid rafts can be linked to cell sensitivity to TRAIL ligand (75–78). But how they interact with membrane components remains poorly understood. First, DR4/5 can couple with E-cadherin (79). Secondly, E-cadherin/α-catenin linkage with dynamic cytoskeleton is essential for efficient assembly of the active death-receptor complexes (80). Consequently, this receptor clustering allows formation of active TRAIL signaling complexes and sensitizes some cancer cells to death induced by TRAIL. Because EMT leads to the dysregulation and disassembly of this E-cadherin/TRAIL complex, cancer cells with a mesenchymal phenotype are increasing their protection against TRAIL-induced apoptosis (81). However, in patients with early-detected colorectal cancer, DR4 and DR5 can be expressed in parallel of E-cadherin but their co-localization at the membrane is not systematic (82). Moreover, the potential interactions between the death-receptors (including the decoy receptors known as death-receptor competitors which are lacking the intracellular death domain responsible for the propagation of TRAIL-induced apoptotic signal) and the cadherins remained unanswered despite the potential mechanistic impact these cell processes hold (83).

A study focused on the natural anti-metastatic agent antrocin, shown that it could act as an EMT inhibitor, restoring the E-cadherin protein level in parallel of the DR5 increase expression (84) whereas another study shows that DR5 knockdown could increase E-cadherin expression and diminish migration in breast cancer, which further suggest a specific regulatory step (85). But how DR4/DR5 and E-cadherin expressions are simultaneously regulated is still not well understood. However, the Hedgehog pathway and the modulation of some miRNAs may be involved in this regulatory process. Indeed, TRAIL-induced apoptosis resistance in chronic condition was shown to be under the control of miR-21, miR-30c and miR-100 in lung cancer (86). The overexpression of these miRNAs inhibits the expression of caspases 3/8 and the EMT marker E-cadherin. They also activate the NF-kB pathway which regulates in a feedback loop the expression of the miRNAs involved. Among them, miR-21 seems to play a pivotal role in modulating expression of both E-cadherin and DR4/DR5 as observed in a glioblastoma model (87). In this study, the Hedgehog

inhibitor NPV-LDE-225 blocks the EMT process and allows the increase of TRAIL-induced apoptosis efficiency by increasing DR4/DR5 and E-cadherin expressions. It finally diminishes those of miR-21 but also stem cells markers such as NANOG, OCT4, SOX2 and c-Myc making both miR-21 and the hedgehog signaling pathway the possible master regulators of this mechanism. In addition, the natural Hedgehog inhibitor cordycepin can also induce apoptosis in breast cancer models, with the upregulation of DR4/DR5 and E-cadherin (88).

More recently, the cadherin/DR cooperation was studied in head and neck cancers (HNSCCs): N-cadherin, the major mesenchymal marker, was shown to enhance cell growth by inhibiting apoptosis (89). N-cadherin overexpression was associated with increase in DcR2 but also with decrease of DR5 whereas its knockdown led to the opposite results suggesting the existence of a signaling network between cadherins and death receptors. Moreover, N-cadherin was observed to interact with DcR2 in these same models, a process allowing cell survival via cleavage of caspases by activating MAPK/ERK pathway. Because E-cadherin/N-Cadherin switch is the hallmark of EMT (90,91) and because cell sensitivity to TRAIL is changing with the cell status (from epithelial-mesenchymal), it is now reasonable to hypothesize that a similar switch may exist between DR5 and DcR2 and could represent in turn a TRAIL-sensitivity marker. Further investigations are needed to clarify this possible regulation even if limited data exist in the literature supporting this regulatory process. Indeed, we know that both DR5 and DcR2 expressions are under the control of P53 and a negative feedback loop was proposed between DcR2 and P53 (92). (Overexpression of P53 can lead to the increase of DcR2 which in turn can attenuate DR5-mediated apoptosis (93).)

While E-cadherin cooperates with DR5 at the membrane level of epithelial cancer cells and N-cadherin with DcR2 in the mesenchymal ones, another member of the cadherin family was described to physically interacts with TRAIL receptors, FAT1. FAT1 is a cadherin like-protein with tumor suppressor functions which is playing a central role in developmental processes and cell communication (94,95). This adhesive molecule is highly expressed in several fetal epithelia, but its mutation is leading to an aberrant activation of the WNT signaling pathway leading to tumorigenesis (96). In glioblastoma cells, FAT1 acts as a negative regulator of DR4/5. After knockdown of FAT1, cancer cells became more sensitive to TRAIL-induced apoptosis, a process very closed to those aforementioned: by interacting with FAT1, DRs finally prevent the DISC activation (97).

Death receptor cooperates physically with other membrane proteins not specially involved in EMT but also involved in cancer progression leading to metastasis. For example, carcinoembryonic antigen (CEA, CD66e), mainly found in colorectal cancer, is a cell surface glycoprotein which is increased along with DR5 when cells are in suspension. Interestingly, it binds

and inhibits DR5 leading to the decrease activity of caspase-8. Increase of cell survival (in vitro) and colonization of secondary tissues (in vivo) were also observed. Together, these events stimulate cancer progression and metastasis (98).

***EMT regulates TRAIL-sensitivity***

Targeting mesenchymal cancer cells displaying stem cells characteristics with TRAIL, has been proposed to reduce resistance in different cancers such as squamous and adenocarcinoma lung cancer (99). This association is emerging in other pathologies such as biliary atresia, a common viral dependent cholangiopathy where EMT was shown to block biliary innate immune response via TRAIL-mediated apoptosis (100,101) or in HBV infection, where hepatitis B virus may activate in certain condition an EMT-like state which is ER-stress dependent (102).

Interestingly, some homologies have been observed between differential sensitivity to TRAIL and the EMT process. TRAIL resistance of non-genetic origins -from variable activations and expression levels of pro- and anti-apoptotic proteins (103–105), was shown to be transient and sustainable (106). Similar observations were made in the EMT context. Indeed, during cell division, variable partitioning of macromolecules in daughter cells was proposed to increase EMT heterogeneity (107) illustrating that non-genetic mechanisms play an important role in cellular heterogeneity and plasticity leading to different cell states. Because cancer cells can switch from an epithelial state to a mesenchymal one in order to adapt to the tumor microenvironment and to progress to metastasis, the intermediate states known as hybrid epithelial / mesenchymal linked to differences in sensitivity to chemotherapeutic agents (108) are now emerging as promising targets against cancer progression (109).

One of the first observations was related to nitric oxide (NO) donors such as DETANONOate. This chemical can sensitize cancer cells to TRAIL-induced apoptosis through different mechanisms. First, it contributes to increase the expression of RKIP, a metastatic tumor suppressor. Then, it inhibits both the NF-kB pathway responsible of cell resistance to chemotherapies and the YY1 transcription factor which is in turn responsible for the regulation of Fas and DR5 (the main receptor for TRAIL). Finally, NO donors contribute to the inhibition of the Snail transcription factor, an E-cadherin repressor, thus repressing the EMT process. In brief, by dysregulating the NF-κB/Snail/YY1/RKIP/PTEN axis, NO donors prevent metastatic potential and resistance to apoptosis (110,111). Similar observations were done in urothelial cancer cell lines where mesenchymal cells showed higher resistance to TRAIL treatments than epithelial cells. Indeed, the latter present a lower level of XIAP and Bcl-2 proteins which account in part for the anti-apoptotic effects. These data appear to be an additional point in favor of the importance of

targeting EMT markers and/or processes as a strategy against cancer progression (112).

A compelling observation suggesting a link between EMT and resistance to TRAIL-induced apoptosis is the deregulation of transcription factors such as Snail and Slug (45,113). Both are not only involved in the downregulation of adherent proteins known as epithelial markers such as E-cadherin, claudins or occludins but also in the inhibition of pro-apoptotic proteins such as Bcl-2, Bid, Puma and Caspase-9. Moreover, the upregulation of Snail and Slug leads to the increase of P53 protein level that mediates resistance through anoikis (114). Thus, reverting EMT appears to be a strategy to sensitize cancer cells to TRAIL therapy. Srivastava *et al.* used a benzamide histone deacetylase inhibitor (MS-275 also called Entinostat) to target HDAC1/3 leading to the increase of apoptosis-inducing potential of TRAIL in different cancer cell lines in vitro (113). This treatment enabled to sensitize TRAIL-resistant cancer cells, a phenomenon also observed in vivo (breast cancer xenografts in nude mice) where MS-275 inhibits EMT, decreases NF-κB pathway activation and finally increased DR4/DR5 receptors and pro-apoptotic protein expressions. In pancreatic cancer stem cells, the same team demonstrated that the GLI transcription factor inhibitor (GANT-61) which targets the Hedgehog pathway allowed EMT inhibition with in parallel the increase of the DR4 and DR5 expressions (115).

Another mechanism proposed earlier is the dysregulation of miRNAs, specially Mir-9, which has been found downregulated in many cancers (116). This microRNA can modulate the expression of IFN-induced genes and MHC class I molecules. Among these IFN-induced genes, TRAIL was shown to be one of them. Indeed, increase of Mir-9 is associated with overexpression of TRAIL (117). TRAIL overexpression was also found in MCF-7 cancer cells who have acquired resistance to Metformin treatment. By inducing autophagy in certain cancer cells, TRAIL can protect them by blunting the treatment cytotoxicity thus contributing to TRAIL resistance (118). Mir-9 is also known to interact with TGF-beta signaling pathway during EMT (119) however facts are still lacking about TRAIL sensitivity. It has only been reported that TGFβ-induced EMT plays a critical role during irradiation of the breast cancer cell line HMLE leading to radioresistance of stem-like breast cancer cells generated. Indeed, in this study, mesenchymal $CD24^{-/low}/CD44^{+}$ CSCs were shown to exert apoptosis resistance through differential activation of death receptors such as TRAIL and in parallel via the increase expression of the anti-apoptotic marker survivin (120). The changes observed in TRAIL gene expressions are likely to be associated with an EMT signature in such cases. Then another miRNA candidate was proposed to play such an important role in TRAIL-induced apoptosis resistance. For example, by downregulating the PI3K/AKT regulator PTEN, miR221 induces EMT and invasiveness of breast cancer cells (121).

Lu et al., proposed a mechanism of EMT-dependent apoptosis inhibition where loss of E-

cadherin (which bounds selectively to DR4 and DR5 but not to Fas owning the DISC formation and caspase-8 activation) drives cancer cell resistance to TRAIL treatment (79). Another study reported that EMT reversal by ML327, an isoxazole-based small molecule probe that represses E-cadherin level and partially reverses the EMT phenotype, is accompanied by an enhanced response to TRAIL in carcinoma cells and this, in an E-cadherin-independent manner (122).

Involvement of the mitochondrial pathway in models such as melanoma is also critical in TRAIL sensitivity (123) but its relationship with EMT remained less described. In lung cancer, when the EMT marker MUC1 (responsible for pro-oncogenic signal transduction) is silenced, TRAIL treatment becomes more efficient. This increased sensitivity is possible due to the MUC1-BAX association leading to the prevention of the mitochondrial permeabilization in response to apoptotic stimuli (124).

Depending on the EMT status and on the expression levels of pro-and anti-apoptotic proteins under the control of the EMT-TFs, cancer cells will respond to anticancer therapies differently, with greater sensitivity in epithelial cells (125).

***TRAIL and resistance to anoikis in the metastasis context***

The term «anoikis», from the Greek anoikos “without a home”, was proposed in the 90's by Frisch and Francis (1994) to describe an apoptosis phenomenon following loss of cell-to-ECM interactions. The authors explained that anoikis occurs to abrogate an escape mechanism, meaning the possibility for a cell to reattach in an inappropriate tissue. This mechanism allows the limitation of oncogenic transformations without disrupting plasticity and cell migration necessary during development, repair and cell tissue homeostasis. Anoikis and its resistance also increase the diversity of phenotypes (127). Thus, resistance to anoikis became a hallmark of malignant cells with their ability to grow under anchorage-independent conditions (128).

In epithelial cells, anchorage to ECM represents an environmental signal which is mediated by integrins. Indeed, integrins β1- and β3-subunits, when in contact with ECM components such as collagens, phosphorylate FAK which in turn phosphorylates Akt leading to the inhibition of pro-apoptotic proteins such as Bad. Consequently, lack of ligation of integrins β1- and β3-subunits induces decrease of both FAK protein and its activity but also those of proto-oncogene tyrosine-protein kinase Src or Integrin-Linked Kinase (ILK) leading to the inhibition of the pro-survival pathway PKB/Akt (128,129).

Evidences for a function of death receptors in anoikis has been previously described (130). When MDCK and HaCat cells are losing their interactions with ECM, a caspase-8-dependent

apoptotic cascade is triggered. This increasing caspase-8 activity after cell detachment occurs through FADD recruitment without DR4/DR5 activation, a process observed independently of the death ligands fixation (131). Authors also noticed that Bcl-2 and Bcl-XL inhibit caspase-8 induced-anoikis probably via a mitochondrial positive feed-back by caspase-3. These data were further supported by another study showing that extrinsic apoptosis leading to anoikis was also triggered by caspase-8 in keratinocytes (129). This work revealed the positive feed-back described above as a complementary interaction between the two apoptosis pathways. A negative post-transcriptional regulation of DR5 via miR126-3p was also proposed to explain the decrease in extrinsic apoptosis pathway signaling without affecting death receptor mRNA level (132), but how TRAIL is associated with anoikis resistance during cancer progression was left unanswered.

Although DRs drive anoikis in normal cells, they fail to induce such a process in malignant ones, probably via a FLIP -dependent process (133). In breast cancer, cell anchorage suppresses TRAIL gene expression whereas detached cells increase its level. The autocrine role of endogenous TRAIL was then suspected to be associated with anoikis through activation of the DR5 (and to a less extent DR4). Because the detached cells were found more sensitive to TRAIL, circulating tumor cells were considered as a potential target for TRAIL therapy (134). In fact, DR4/DR5 signaling allows caspases activation leading to cleavage of Akt/PKB proteins and to its decreased expression levels. Because the Akt pathway plays a central role in mediating survival signal, cell detachment via loss of integrin interactions with ECM is the first step to the inhibition of this anti-apoptotic signaling (135). In colorectal cancer cells (CRC), the DR5 is increasing in cell suspension. The use of antagonists or DR5 knockdown is sufficient to inhibit anoikis whereas no effects were observed concerning DR4. Exogenous TRAIL failed to increase anoikis as noticed earlier in breast cancer model and finally the proposed mechanism hypothesizes that DR5 is activated by cross-linked soluble and membrane-bound TRAIL ligand (128).

Mechanisms of anoikis resistance are numerous and they depend on the mutation status of the cancer cell model studied. Although one can suspect that a constitutive activation of pro-survival pathway could inhibit the apoptotic processes engaged after loss of anchorage, thanks to acquired mutations, non-genetic heterogeneity associated with differences in protein expression levels can also largely impact cell fate decision. In the specific case of TRAIL-induced anoikis resistance, several mechanisms were reported in the last two decades. For example, decrease in DR4/DR5 expressions was described to explain such a resistance. In hepatoma cells, low level of DR4/DR5 expression was associated with resistance of TRAIL-induced apoptosis cascade even if upregulation of TRAIL mRNA was noticed (136). Yet, no modulation in DR4/DR5 expressions was observed between attached and detached human colon epithelial cells where TRAIL resistance was shown. Only increases in FAK and ILK activities and, in a second time, in the downstream Akt

pathway activation, protect colon cells from TRAIL-induced apoptosis (137). Similar conclusions were reported in an ovarian model (138) and in HL-60 cells (139). Interestingly, FAK not only stimulates the Akt pathway activation but also interacts with caspase-8 in an adhesion-dependent manner thus blocking the apoptotic extrinsic pathway in this condition (140,141). But how TRAIL interacts with the Integrin/FAK/Akt pathway remains unclear. More recently, TRAIL was described as a mediator of FAK signaling in regulation of entosis (an invasion process involving two cells, one is merging via the cytoplasm with the other) and necrosis, in primary human mammary epithelial cells (127). Indeed, during detachment induced cell death, even if TRAIL is rapidly increasing and this for a long time (from 3h to 72h), FAK successfully inhibits TRAIL and protects cells during all the experience.

Generally, mechanisms of anoikis resistance linked to TRAIL treatment are shared with common apoptosis resistance mechanisms, especially those which interact with the extrinsic pathway. Indeed, decrease in caspase-8 expression and its activity is associated with TRAIL resistance (142). Modulation of c-FLIP protein level, the main endogenous pro-caspase-8 inhibitor (143), but also increase in IAPs proteins family (144) are other targets and regulators of this TRAIL-dependent resistance.

***TRAIL regulates the PD-L1-dependent immunogenic response***

In lung cancer or melanoma, PD-1/PD-L1 expression and activation is an indicator of poor prognosis for patients (145,146) but their inhibition has become a strategy to stimulate immune response and increase cell death (147,148). There is a growing body of evidence suggesting intricate regulation processes between TRAIL and PD-L1 expressions. In 2010, Tu et al., analyzed the effect of the hepatitis C virus core protein (HCVc) on human liver and specially on innate immune Kupffer cells (KC). They found that it was able to induce up-regulation of PD-L1 under interleukins (IL-1β-10) and TNF-α secretion, along with the inhibition of the cell surface expression of the cytotoxic molecule TRAIL, a process dependent on the PI3K/Akt pathway activation (149). Moreover, in Chronic Lymphocytic Leukemia (CLL), the therapeutic agent trabectedin induces apoptosis of both human primary leukemic cells, selected myeloid and lymphoid immunosuppressive cells mainly through the TRAIL/TNF pathway. In parallel, trabectedin also blocks the PD-1/PD-L1 axis by targeting PD-L1$^+$ CLL cells, PD-L1$^+$ monocytes/macrophages, and PD-1$^+$ T cells (150). Complementary data were reported in murine melanoma (151) and in hepatocellular carcinoma cells (152). Even if this association is not completely understood, we now know that IFN-γ, a cytokine responsible for the increase expression of PD-L1, can also sensitize cancer cells to TRAIL-mediated apoptosis through downregulation of c-FLIP (153,154). Based on the relationship between immune cells of the tumor microenvironment and cancer cells, a very

attractive approach has been proposed using a bifunctional fusion protein, designated anti-PD-L1:TRAIL (see Bremer, 2013) that targets with success both immune cells (myeloid effector cells and T cells activity) and cancer cells sensitized by this method (156).

EMT plays a central role in the immunogenicity. It was shown to promote metastasis via immunosuppression (157,158) but evidences that PD-L1 overexpression was correlated with induction of EMT was demonstrated in NSCLC and more recently in breast cancer via a ZEB-1/miR-200 mechanism (159,160). Upstream of this signaling cascade, GSK-3β/β-catenin is controlling the ZEB-1/miR-200 axis and allows β-catenin nuclear translocation under the negative control of SDH5, a succinate dehydrogenase component of the tricarboxylic acid cycle (161,162). In NSCLCs, EMT specifically regulates PD-L1 expression with the need of epigenetic reprogramming thus leading to immune escape (163). This mechanism requires both demethylation of PD-L1 promoter thanks to TGF-β action and activation of NF-κB via TNF-α stimulation but is not accompanied by the increase in DR4/DR5 or TRAIL expression (164), suggesting that an inversely proportional relationship between the expression of PD-L1 and the increase in resistance to TRAIL dependently on the decrease in DRs expression, would occurs under the control of EMT. This proposed mechanism has also been observed in glioblastoma (GBM) where cannabidiol (CBD) upregulated gene and protein expression of DR5/TRAIL-R2 and sensitize GBM cells to TRAIL-induced apoptosis. The authors noticed that, as expected, CBD notably decreased in GBM surface levels of PD-L1 (165).

Different regulation pathways were proposed to explain the simultaneous expressions of TRAIL receptors and PD-L1. In tumor IFN-driven resistance, stimulation of cancer cells by IFN-γ leads to the nuclear translocation of STAT1. The activation of the IFN-γ/STAT1 axis is then responsible for the increase of PD-L1 and in parallel the decrease of TRAIL-R2 (166,167). Blockade of IFN-γ receptor in this same resistant model leads to the increase of TRAIL-R2 and allows the natural killer (NK) to stimulate the extrinsic apoptosis in the cancer cells. Another regulation mentioning the role of miRNA-429 in PD-L1 expression and TRAIL sensitivity was recently described. Indeed, miR-429 is a member of the miR-200 family than can inhibit ZEB1/2 or PTEN/Akt upregulation making this miRNA an EMT regulator (168). In gastric cancer, PD-L1 is positively correlated with TRAIL resistance where miR-429 is downregulated (169). The authors observed that miR-429 targets the 3'UTR of PD-L1. They proposed a mechanism where PD-L1 interacts with p-EGFR leading to the activation of the pro-survival Akt pathway thus blocking the TRAIL-dependent apoptosis process.

Finally, in KRAS-mutated cancer cells, oncogenic RAS allows the stabilization of PD-L1 mRNA leading to its increase and escape of immunosurveillance. This phenomenon partly accounts

for the chemotherapeutic resistance observed. Interestingly in pancreatic ductal adenocarcinoma (PDCA), cancer cells also express endogenous TRAIL with autocrine function. Via DR5 activation, TRAIL stimulates the migration and invasion of KRAS-mutated cancer cells in a Rac1 dependent manner. Knowing that Rac1 is usually inhibited via ROCK under the control of KRAS in normal condition, authors proposed a new strategy to target both KRAS and TRAIL to stimulate the immunogenic response and increase patient survival (170).

***Death receptors expression in circulating tumor cells (CTCs/CTMs)***

CTCs are considered as putative precursors that might contribute, alone or in cluster, to cancer cell dissemination in the body leading to metastasis (171,172). This cancer progression step is often called "the leukemic phase" of solid tumor as suggested by Mocellin et al., (2006). In patients' blood, not only CTCs are collected but also apoptotic CTCs and CTCs clusters described as circulating tumor microemboli (CTM) with higher metastatic potential. Together, they represent poor prognostic and pharmacodynamic biomarkers of solid tumors (174–177). Remarkably, only a small proportion of CTCs can give rise to metastasis (178,179).

Anoikis resistance appears to be critical for the etiology of CTC (180–182). CTCs from prostatic cancer cells lose their adhesive capacity through down-regulation of E-cadherin, γ-catenin and β4-integrin with in parallel the gain of anti-apoptotic mechanisms increasing their resistance to cytotoxic stresses induced by immune cells (183). Among them, authors observed the decrease of HSP90B1, a chaperone protein that enable escape from immune surveillance but also the increase of Bcl-2 under the control of the Akt pathway signaling activation. In another model namely the pancreatic cancer cells, Wnt2 was proposed as a candidate CTC gene. It was shown to be responsible for the anoikis resistance through the activation of the non-canonical WNT/TAK1/FN1 signaling pathway (184). Such examples are emerging in the literature, but all have in common a double signature: the decrease of epithelial markers and the gain of anti-apoptotic capacities as observed during EMT.

In CTCs from breast cancer, the molecular features of EMT were found inversely correlated with TRAIL plasma cytokine expression (185). Unfortunately, DRs expression levels were not reported in this study. However, it seems that soluble TRAIL could have only weak apoptotic effects on CTCs independently of the DRs concentrations as observed in a computational model (186). Different regulatory processes were proposed to understand DRs modulations in CTCs, such as the c-Jun N-terminal kinases (JNK) pathway. In pancreatic cancer stem cells, JNK inhibition allows the decrease of DcR1 via an IL-8 dependent autocrine process while DR4/5 expressions are increased thereby sensitizing cells to TRAIL treatment (187). Consequently, the authors observed

diminished tumor burden and number of CTCs. Autophagic processes have also been shown to regulate CTCs sensitivity to TRAIL (188), and to protect invasive cancer cells from anoikis (189,190). In a breast cancer cell line, DR4/5 expression is decreased in cell suspension in contrast to adherent cells thus increasing the TRAIL resistance. Mechanistically, DR4/5 undergo a rapid endocytosis, a sequestration in nucleus and a degradation in autophagosome (Di et al., 2013).

Given that EMT provides mesenchymal cells with the ability to resist to apoptosis, anoikis and some stem cells characteristics (regulated by different factors such as TGF-β, Wnt or Notch (192)), more evidence would be needed to evaluate whether death receptor agonists could favor the emergence of CTCs through EMT mechanisms and further assess CTCs-sensitivity to these drugs.

## Conclusions and Perspectives

Death receptor activation allows pleiotropic effects whether related to cell death (apoptosis, necrosis, necroptosis, pyroptosis ...) or to survival (differentiation, division, migration, entosis, EMT, ...). But cell fate will ultimately depend on a wide range of environmental and cell contexts with both genetic and non-genetic variations. This response heterogeneity is at the origin of cell resistance, an adaptive mechanism which impairs cancer drug development and therapeutic strategies (193). In this review, we examined how EMT participates to increase this response heterogeneity which in turn, enhance cancer cell survival. There are other possible mechanisms by which EMT could increase response heterogeneity, through interactions with the tumor microenvironment. First, cancer cells growth is usually accompanied with a decrease in the availability of oxygen and other necessary elements within the tumor. This transient ischemia stimulates the expression of the hypoxia-inducible factors family (HIF-1) that mediates the angiogenic response and control different EMT-TFs (TCF3, ZEB1/2 and TWIST1) responsible for E-cadherin downregulation (194,195). Secondly, carcinoma-associated fibroblasts (CAFs) are stroma cells which secrete soluble TGF-β, MMPs, HGF and uPA. These CAFs are also recruited and activated from resident fibroblasts via equivalent secretion of factors produced by cancer cells in EMT (196). Finally, inflammation stimulates and maintains EMT through production of cytokines (TGF-β, TNF-α, IL-1β, IL-6, IL-8, CXCL1 and CCL18) by infiltrating immune cells including tumor-associated macrophages (TAMs) and lymphocytes (197,198). Because the EMT program is regulated temporally and spatially (activation at the invasive front of the tumor), the differential communication between cancer cells and the microenvironment can further contributes to increase response heterogeneity to drug treatments (29,199).

## Figure Legend

**Figure 1. Effects of death receptor agonists on EMT-mediated cancer cell heterogeneity.** Binding of death ligands can activate pathways including caspase-8-dependent apoptosis and survival. Cancer cells that survive to treatment can give rise to different response such as proliferation, senescence or differentiation. Epithelial-mesenchymal transition (EMT) is one of the cell biological process that contributes to cellular plasticity, allowing cancer cells to switch from an epithelial state to a mesenchymal one. Cells lose their adhesion capacities, acquire stem cells characteristics (CSCs) and can migrate until invading secondary sites via the lymphatic system and the blood circulation. EMT also provides resistance to anoikis, an apoptotic process following loss of cell contacts with extracellular matrix and decreases the immunogenic response. Together, these events can participate to the increased resistance of circulating tumor cells alone (CTCs) or in clusters (CTMs), allowing cancer progression and metastasis. Finally, EMT increases response heterogeneity by enhancing cell diversity within the tumor, which can further increase the clonal heterogeneity and cancer cell resistance to chemotherapies.

**Table 1. EMT-associated molecular features of TRAIL receptors.**

Claudin, Occludin and E-Cadherin are described as DR4/5 positive regulators whereas FAT1 is considered a negative regulator. N-cadherin is a positive DcR2 regulator.

## Authors' contributions

Writing – Original Draft, L.P., M.M., and J.R.; Writing – Review & Editing, L.P. and J.R.; Resources, P.H. and J.R.; Funding Acquisition, J.R.

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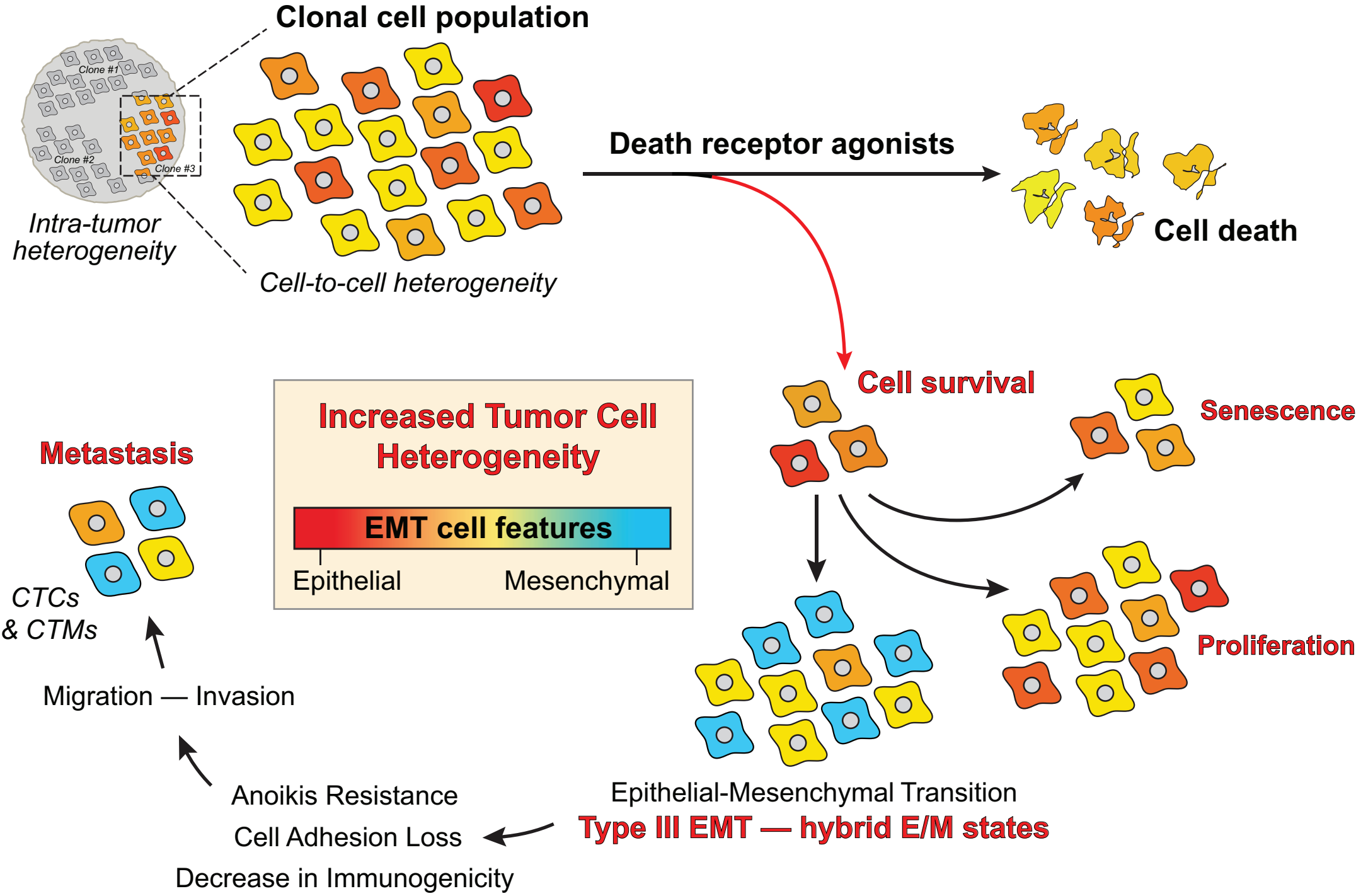
Clonal cell population
Clone #1
Clone #2
Clone #3
Intra-tumor heterogeneity
Cell-to-cell heterogeneity
Death receptor agonists
Cell death
Cell survival
Senescence
Proliferation
Increased Tumor Cell Heterogeneity
EMT cell features
Epithelial
Mesenchymal
Metastasis
CTCs & CTMs
Migration — Invasion
Anoikis Resistance
Cell Adhesion Loss
Decrease in Immunogenicity
Epithelial-Mesenchymal Transition
Type III EMT — hybrid E/M states

| Death Receptor | Alternative names | Functions | Pathways involved | EMT-associated molecular features | References |
|---|---|---|---|---|---|
| DR4 | TRAIL-R1, Apo2, *TNFRSF10A* | Pro-apoptotic, Pro-survival | TAK1, MAPKs, NF-kB, Caspases-8/10 | Claudin & Occludin (associated with FADD at the DISC), E-cadherin and FAT1 (protein interactions) | 72,73,74,79,81, 82,97 |
| DR5 | TRAIL-R2, *TNFRSF10B* | | | | |
| DcR1 | TRAIL-R3, *TNFRSF10C* | Anti-apoptotic | – | – | – |
| DcR2 | TRAIL-R4, *TNFRSF10D* | Anti-apoptotic | _ | Associated with N-Cadherin overexpression | 89 |
| DcR3 | TR6, M68, *TNFRSF6B* | Anti-apoptotic | – | – | – |
| OPG | OCIF, PDB5, *TNFRSF11B* | Anti-apoptotic | – | – | – |

Table 1