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Reprogramming metastatic tumour cells with embryonic microenvironments

Mary J. C. Hendrix, Elisabeth A. Seftor*, Richard E. B. Seftor*, Jennifer Kasemeier-Kulesa‡, Paul M. Kulesa‡ and Lynne-Marie Postovit**

Abstract | Aggressive tumour cells share many characteristics with embryonic progenitors, contributing to the conundrum of tumour cell plasticity. Recent studies using embryonic models of human stem cells, the zebrafish and the chick have shown the reversion of the metastatic phenotype of aggressive melanoma cells, and revealed the convergence of embryonic and tumorigenic signalling pathways, which may help to identify new targets for therapeutic intervention. This Review will summarize the embryonic models used to reverse the metastatic melanoma phenotype, and highlight the prominent signalling pathways that have emerged as noteworthy targets for future consideration

Tumour cell plasticity
The ability of aggressive tumour cells to express multiple molecular phenotypes similar to pluripotent, embryonic-like stem cells.

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Plasticity underlies both multipotent tumour cells and embryonic stem cells, and requires special attention as we contemplate new therapeutic strategies to target the elusive metastatic phenotype. Furthermore, both normal stem cells and tumour cells are profoundly influenced by bi-directional communication with their respective microenvironments, which dictates cell fate determination and behaviour[1,2]. Although the concept of relating cancer to stem cells has recently gained popularity, primarily owing to the identification of specific tumour stem cells[3–13], there is a great deal to learn about stem cell regulation, which can result in normal or aberrant behaviour. It is interesting to note that over 150 years ago, Rudolf Virchow initially proposed that cancer arises from embryonic-like cells[14], an innovative concept that was further extended by Cohnheim and Durante[2], who suggested that adult tissues contain dormant embryonic remnants, but maintained the potential to become cancerous. This original concept was formerly referred to as the 'embryonal rest' theory of cancer, and is notably similar to the current cancer stem cell theory, which suggests that cancer arises from a subpopulation of tumour stem cells[15] (see BOX 1 for an overview of the evolution of the stem cell theory of cancer).

Evidence in support of these early concepts, which now appear to be remarkably insightful, came from several early studies in the 1970s and 1980s. Following the general theory that an embryonic microenvironment might have the capacity to reverse the metastatic phenotype of cancer cells, Mintz and Illmensee[16] showed that a mouse embryonic blastocyst microenvironment could suppress the tumorigenic phenotype of teratocarcinoma cells, and that the developmental plasticity of the tumour cells was manifested as they contributed to the formation of normal tissue. Later, Pierce and colleagues proposed that an embryonic microenvironment capable of differentiating a stem cell lineage should be able to reprogramme cancers derived from that lineage[17]. Support for this concept was derived from the testing of various cancer cell lines[17–19] that showed the tumour-inhibiting properties of embryonic microenvironments. One example from these studies showed the inability of B16 murine melanoma cells to form tumours following exposure to microenvironmental factors derived from the embryonic skin of a developing mouse[18]. Additional experiments (performed by Bissell and colleagues[20]) documented a similar tumour-suppressing microenvironment in a chick model, in which Rous sarcoma virus failed to induce sarcomas in the embryos.

Technological advances in microscopy and molecular biology have allowed scientists to extend observations made by the pioneers working at the intersection of developmental biology and cancer biology. Recent investigations have given unique insights into the molecular reprogramming of metastatic melanoma cells exposed to the embryonic microenvironments of human embryonic stem cells (hESCs), the zebrafish and the chick embryo. These observations have led to the discovery of a novel signalling pathway in melanoma that underlies stem cell plasticity and provides a new target for therapeutic intervention.

At a glance

- Aggressive tumour cells, such as melanoma, share many characteristics with embryonic progenitors, which contribute to the conundrum of tumour cell plasticity. The challenge is to better understand the aetiology of the plastic, multipotent phenotype and to develop strategies that might include their differentiation and subsequent targeting.
- A complex and still enigmatic relationship exists between stem cells and their microenvironment that has a crucial role in the determination of cell fate. Current studies identifying the molecular pathways that regulate stem cell plasticity are also examining the epigenetic role of the microenvironment.
- The microenvironment of human embryonic stem cells can epigenetically reprogramme multipotent metastatic melanoma cells to assume a melanocyte-like phenotype. In addition, the 'reverted' melanoma cells show significantly reduced invasive and tumorigenic ability.
- The embryonic neural crest microenvironment of the chick provides an attractive model system to explore melanoma tumour cell reprogramming. Human metastatic melanoma cells transplanted into the chick embryonic microenvironment did not form tumours, and a subset of these tumour cells were reprogrammed to a neural crest cell-like phenotype. The melanoma cells also followed neural crest migratory pathways and populated host peripheral structures in a programmed manner.
- Recent findings using the embryonic zebrafish have illuminated a convergence in the molecular messengers that metastatic tumour and normal stem cells implement during their respective bi-directional communication with the microenvironment, leading to the identification of Nodal.
- The inhibition of Nodal signalling reduces melanoma cell invasiveness, colony formation and tumorigenicity. Nodal inhibition also promotes the reversion of melanoma cells towards a melanocytic phenotype concomitant with loss of the plastic phenotype.
- Nodal may represent a new diagnostic marker for disease progression and a novel target for the treatment of aggressive cancers. Additional strategic targets contributing to the Nodal signalling pathway, including SMAD2 and SMAD3, cripto and the activin-like-kinase (ALK) receptor complex, are worth further consideration for inhibiting the plastic tumour cell phenotype.
- The discovery of key signalling pathways that underlie the commonality of plasticity of embryonic stem cells and multipotent tumour cells will probably result in new therapeutic strategies to suppress the metastatic phenotype.

Multipotent tumour cells

There is an impressive body of literature detailing the molecular signature of metastatic tumour cells, and more recently embryonic stem cells, that shows both cell types share an underlying plasticity[3–6,21–32]. For example, in the field of melanoma research comparative global gene analyses of aggressive and poorly aggressive human melanoma cell lines have revealed the unexpected finding that aggressive tumour cells express genes (and proteins) associated with various cell types (including progenitor cells), while concomitantly downregulating genes specific to their parental melanocytic lineage[21] (see BOX 2 for an overview of the key features of melanoma). These intriguing findings support the premise that aggressive melanoma cells acquire a multipotent, plastic phenotype, a concept that challenges our current thinking of how to target tumour cells with stem cell-like properties. Indeed, although previous therapeutic strategies have focused on eliminating a homogeneous tumour, new treatment modalities should attempt to target a heterogeneous population of cancer cells whose stem cell-like phenotype facilitates adaptation, and consequently survival. In the case of melanoma, the aggressive phenotype can engage in vasculogenic mimicry by expressing endothelial-associated genes, such as vascular endothelial cadherin (VE-Cadherin), which contribute to the formation of an extravascular fluid-conducting network in aggressive tumours and coincide with a poor clinical outcome[33–35]. Vasculogenic mimicry can occur simultaneously with angiogenesis, vessel co-option and intussusceptive microvascular growth[36]. Moreover, experimental evidence has shown the plastic functionality of human metastatic melanoma cells as they participate in the neovascularization of circulation-deficient muscle[37]. This ability of aggressive melanoma cells to manifest endothelial-like qualities allows them to adapt to the hypoxic microenvironment associated with rapidly growing tumours at the same time as enabling them to circumvent conventional anti-angiogenic therapies, which are directed at endothelial cell-specific neovascularization. Vasculogenic mimicry is one example of the plastic tumour cell phenotype, and has been demonstrated in several tumour types, including breast carcinoma, prostatic carcinoma, ovarian carcinoma, lung carcinoma, synoviosarcoma, rhabdomyosarcoma, phaeochromocytoma, Ewings sarcoma, and during embryogenesis as cytotrophoblasts form the placenta[38–42] (for review see REF. 43).

The real challenge is to better understand the aetiology of the plastic, multipotent phenotype expressed by the most aggressive tumour cells, and to develop strategies that might include their targeting and subsequent differentiation in cancer patients. The first step is to identify and characterize these illusive cell types. Toward this goal, new technologies have enabled scientists to specifically visualize, isolate and analyse multipotent cancer cells with stem cell properties, which have been discovered in several tumour types[7–13]. With respect to melanoma prognosis, the expression of melanocyte-specific markers is dramatically reduced, and not uncommonly absent, in aggressive melanoma tumour cells. For example, the expression of pigmentation pathway-related genes, such as melan-A (*MLANA*) and tyrosinase (which catalyses the conversion of tyrosine to the pigment melanin), are reduced by more than 20-fold and 35-fold, respectively, in aggressive melanomas compared with their poorly aggressive, isogenically matched counterparts[43]. MLANA and tyrosinase are crucial in the melanin biosynthesis pathway[44,45]. Furthermore, reduced tyrosinase levels have been correlated with a poor clinical outcome, and favourable disease outcome has been reported in melanoma patients in whom these differentiation melanocyte-associated antigen genes are expressed[46]. Therefore, it is tempting to speculate that efforts to re-express these genes in patients with metastatic disease could serve as a viable strategy for redifferentiating a highly aggressive, plastic melanoma tumour cell phenotype to a melanocytic-like cell. Indeed, the ability of highly aggressive tumour cells to express multiple cellular phenotypes and engage in functional plasticity, such as vasculogenic mimicry, defines their multipotent potential. As we gain new insights into the acquisition and maintenance of the multipotent tumour cell, we should be particularly vigilant about harnessing the potential of the microenvironment in reprogramming this deadly phenotype.

Neovascularization
A formation of functional microvascular networks with red blood cell perfusion that differs from angiogenesis, which is characterized by the protrusion and outgrowth of capillary buds and sprouts from pre-existing blood vessels.

Synoviosarcoma
A malignant neoplasm arising in the synovial membrane of the joints and in the synovial cells of tendons and bursae; also called malignant synovioma and synovial sarcoma.

Phaeochromocytoma
A tumour that forms in the centre of the adrenal gland that causes it to make too much adrenaline.

Box 1 | **Evolution of the stem cell theory of cancer**

Embryonal rest theory
In 1858 Rudolf Virchow proposed that cancer arises from embryo-like cells. This concept, based on the histological similarities of cancer and embryonic tissues, was extended by Cohnheim and Durante to suggest that adult tissues contain embryonic remnants that normally lie dormant, but can be activated to become cancerous.

Cancer stem cell defined
By definition, the cancer stem cell is a single cancer cell that can form a tumour following transplantation. Just like normal stem cells, cancer stem cells self-renew, deriving daughter cells and a phenotypically diverse population of cells that recapitulate the heterogeneity of the parental tissue: in the case of the cancer stem cell this tissue is a tumour. The first documentation of a cancer stem cell occurred in 1994 when a subpopulation of leukaemic cells was shown to be both required and sufficient to establish leukaemia in mice. Cancer stem cells have since been documented in malignancies as diverse as brain cancer, breast cancer, colon cancer and melanoma.

Tumour cell plasticity
Based on their expression of pluripotency-associated genes, as well as their 'lineage non-specific gene expression pattern' (that is, the loss of lineage-specific gene expression concomitant with the aberrant expression of genes normally restricted to other cell types), aggressive tumour cells are able to behave like stem cells. Therefore, aggressive cancer cells can mimic other cell types and can adapt to microenvironmental changes. Vasculogenic mimicry, in which tumour cells express genes associated with endothelial cells and consequently partake in neovascularization, is an example of this plasticity.

Differentiation therapy
Based on the similarities between cancer cells and stem cells, the concept of differentiation therapy infers that cancer is a problem of developmental biology and that an embryonic microenvironment capable of differentiating a stem cell lineage should be able to reprogramme cancers derived from that lineage. Studies beginning in the 1970s and continuing to present day have documented the ability of embryonic microenvironments to reprogramme aggressive cancer cells towards a less aggressive phenotype. At present, an *in vitro* hESC model, developed in our laboratory, provides a new system from which specific differentiation-inducing molecules can be isolated and studied.

Human embryonic stem cell microenvironment

A complex and enigmatic relationship exists between stem cells and their microenvironment that has a crucial role in determining cell fate. Within this relationship are clues for new therapeutic strategies. A large study aimed at identifying the molecular pathways that regulate stem cell plasticity is examining the potential epigenetic role of the microenvironment on the emergence of cell phenotype. Unlike genetic changes, epigenetic adjustments do not affect the primary DNA sequence. Rather, they involve interactions among cells and cell products, which lead to alterations in reversible phenomena such as cell signalling and DNA modifications. Because all cells in an organism share the same DNA code, differences in gene expression that occur during differentiation or in response to microenvironmental factors are due to epigenetic alterations.

Based on the similarities associated with the plastic phenotype of embryonic stem cells and multipotent melanoma tumour cells, together with the observation that human embryonic stem cells (hESCs) do not form tumours during blastocyst implantation, although they do form teratomas when transplanted into immunodeficient mice, one must assume that the microenvironment exerts dominant control over stem cell fate. Therefore, we proposed that the microenvironment of hESCs might possess the potential to reprogramme the metastatic melanoma phenotype. To explore this possibility, we developed a model[47], that allowed hESCs to condition a 3D matrix, which would subsequently receive multipotent metastatic melanoma cells (FIG. 1). In this model, the hESCs were removed before the addition of melanoma cells, so the melanoma cells were exposed only to the extracellular microenvironment of the hESCs, thereby removing the complexity of cell–cell interactions from the many mechanisms that could be working to epigenetically modulate cell behaviour. Although other groups have studied the ability of embryonic microenvironments to affect the phenotype of tumour cells (mentioned earlier[16–20]), this was the first study designed to specifically examine the effects of human-derived secreted embryonic factors on human cancer cells.

This study showed that the hESC microenvironment can dramatically alter the behaviour of metastatic melanoma cells. Specifically, the exposure of the tumour cells to the 3D matrices preconditioned by the hESCs induced melanoma cells to form spheroids (FIG. 1a) similar to the colonies formed by hESCs. Interestingly, the conditioned media from hESCs was not able to induce melanoma cell spheroid formation, indicating that hESCs can influence the tumour cell phenotype through the modification of the immediate microenvironment. Biochemical and molecular analyses of the melanoma cells (which were amelanotic) exposed to the hESC microenvironment showed the epigenetic induction of a melanocyte-specific phenotype marker, MLANA (FIG. 1b). By contrast, a normal melanocyte microenvironment does not share the ability of the hESC microenvironment to epigenetically reprogramme metastatic melanoma cells. Changes in the behaviour of melanoma cells exposed to the microenvironment of hESCs manifested as significant reductions in the invasive capacity of the tumour cells *in vitro* (FIG. 1c) and in tumorigenic potential *in vivo* (FIG. 1d), suggesting suppressive cues associated with the hESC microenvironment. However, it is noteworthy that exposure to the hESC microenvironment did not completely abrogate melanoma cell invasiveness or tumorigenicity or induce spheroid formation in all melanoma cells. This might be due to the heterogeneous nature of both the hESC-conditioned matrix and the melanoma cell line(s). For example, although the entire population of melanoma cells was tested in the invasion and tumorigenic assays, it seems that only the subpopulation of melanoma cells exposed to the areas of matrix that supported hESC colony clusters would be epigenetically altered. Indeed, this concept is supported by the morphological evidence indicating that melanoma spheroid formation specifically located to the areas of matrices previously occupied (and conditioned) by hESC colonies. Studies are underway that address the epigenetic changes induced by this stem cell microenvironment that are directing our attention towards the site-specific methylation of CpG islands of genes expressed by both hESCs and multipotent melanoma tumour cells, with particular focus on the tumour cell subpopulations that appear to be reprogrammed versus those that are not phenotypically altered.

Ewings sarcoma
A highly malignant, metastatic, small round-cell tumour of the bone that usually occurs in the diaphyses (shafts) of long bones, ribs and flat bones of children or adolescents.

Spheroid
A spherical aggregation of tumour cells, grown in tissue culture, that reflects many of the properties of solid tumours. Spheroids have been used for studying the penetration of anticancer drugs into tumour tissue.

Amelanotic
A complete lack of the pigment melanin in pigment-derived cells and tissues.

Box 2 | **Key features of melanoma**

Melanoma progresses from common nevi (moles) followed by dysplastic nevi with structural atypia. Dysplastic nevi progress to radial growth phase (RGP) melanoma, characterized by lateral growth that is largely confined to the epidermis. Vertical growth phase (VGP) melanoma ensues when expansive nodules of malignant cells penetrate the epidermal basement membrane. Unlike RGP melanomas, which cannot undergo anchorage-independent growth, VGP melanomas can undergo anchorage-independent growth and have acquired metastatic competency[45]. Metastatic melanoma has a mortality rate of more than 80% and a median survival of less than 7.5 months.

Melanoma develops from the transformation of melanocytes, which are skin pigment cells that arise from the neural crest, a highly migratory population that give rise to cells of multiple fates[67]. Pioneer transplantation and extirpation studies in amphibians and birds first elucidated the neural crest origin of melanocytes. Melanocytes in the retinal pigmented epithelium are not of neural crest origin, and derive from the neural epithelium of the optic cup. Trunk neural crest cells have a higher capacity than cranial neural crest cells to produce melanocytes, and follow a dorsolateral migratory pathway between the ectoderm and dermamyotome to the skin. Melanoblasts, which have the potential to produce melanin and are melanocyte progenitors, invade all skin areas and differentiate into melanocytes. How neural crest cells become committed to the melanocytic lineage and what factors control the survival, proliferation and differentiation of melanocyte precursors are still unclear[70,71].

hESCs are a promising source of new therapeutic approaches. There have been noteworthy advances since their original isolation[48] that have provided new techniques for their maintenance on feeder-free matrices, and elucidated signalling pathways that regulate their differentiation[49–55]. However, the microenvironment of hESCs remains an understudied area of research, especially its epigenetic reprogramming potential. The model presented in FIG. 1 permits a global assessment of the epigenetic effects of the hESC microenvironment on tumour cells, using human multipotent metastatic melanoma cells as a proof of principle. This model is easily adaptable for testing other cell types, and may be a viable tool for defining essential signalling pathways and molecular mechanisms that underlie the potent influence of the hESC microenvironment on reprogramming aberrant phenotypes involved in disease pathogenesis. Indeed, ongoing studies are demonstrating similar suppressive effects of the hESC microenvironment on metastatic breast and prostate cancer cells[56], which suggests a more universal use associated with the reprogramming potential of the hESC microenvironment, therefore warranting further characterization.

Embryonic zebrafish and metastatic melanoma

To further explore the microenvironmental factors that might underlie the similarity between stem cells and plastic melanoma cells, we used the embryonic zebrafish as an experimental model to investigate bi-directional cellular communication, a fundamental component of both embryological development and cancer progression. Recent findings have illuminated a convergence in the molecular messengers that metastatic tumour and stem cells implement during such bi-directional communication. These factors, including members of the wingless (Wnt), Notch and transforming growth factor β (TGFβ) superfamilies[1,21,23,57–59] are classically associated with developmental processes, and have emerged as integral modulators of tumour progression. This convergence of embryonic and cancer signalling pathways evokes an intriguing question: what embryonic stem cell signalling pathway(s) do cancer cells exploit to sustain plasticity? Our recent study uniquely addressed this question by using the developing zebrafish as a biosensor for tumour-derived signals[1]. As depicted in FIG. 2, fluorescently labelled melanoma cells were injected into blastula-stage zebrafish embryos in order to explore potential bi-directional interactions between cancer cells and stem cells. Morphological observations led to the discovery that multipotent metastatic melanoma cells can indeed communicate with embryonic progenitors; however, poorly aggressive melanoma cells could not. When the multipotent melanoma cells were injected towards the animal pole the embryos developed an abnormal anterior outgrowth, and when the cells were injected near the yolk margin a duplication of the body axis ensued. Interestingly, the melanoma cells did not directly form these structures, but rather recruited and instructed the host zebrafish cells. This phenomenon of ectopic outgrowth induction suggests that the ability of cancer cells to 'speak an embryonic language' is associated with the most aggressive and phenotypically plastic tumour cell subpopulations.

The inimitable ability of metastatic melanoma cells to organize embryonic development is reminiscent of the duplicated axis formation induced by the dorsal organizer in the classical dorsal lip transplantation studies of Spemann and Mangold[60]. Indeed, like the dorsal organizer, the metastatic melanoma cells organized the formation of an ectopic secondary axis complete with neuroectoderm, notochord and non-axial mesoderm when injected near the embryonic margin. The ectopic structures also maintained normal anterior and posterior patterning. Collectively, these observations suggested that the metastatic melanoma cells were secreting a potent embryonic morphogen that initiates embryonic axis formation and Nodal, a morphogen belonging to the TGFβ superfamily, was identified as the most likely candidate[61] (BOX 3). In support of this notion, the ectopic expression of Nodal homologues has been shown to induce structures in zebrafish similar to those induced by melanoma cells[62,63], and Nodal-responsive genes such as goosecoid were induced in zebrafish cells surrounding the melanoma cells in as little as one hour after engraftment[1]. The ability of the melanoma cells to induce ectopic outgrowths was also abrogated both directly, by inhibiting Nodal expression in the cancer cells, and indirectly, by overexpressing the endogenous Nodal inhibitor *lefty1* in the zebrafish embryo. Finally, Western blot analyses revealed that melanoma cells do indeed express and secrete Nodal protein, and in accordance with the zebrafish studies, Nodal expression is restricted to highly aggressive melanoma cell lines. This expression pattern was assessed for the first time in human melanoma tissues, in which Nodal protein was absent in normal skin and was rarely found in poorly invasive radial growth phase melanomas, but was highly expressed in invasive vertical growth phase melanomas and melanoma metastases[1]. These findings highlight

Feeder-free matrices
The growth of embryonic stem cells *in vitro* in the absence of an underlying layer of mouse-derived fibroblasts.

Animal pole
The point in the blastocyst that is farthest away from the yoke margin. In the zebrafish, mesoendoderm is not usually found here.

Dorsal organizer
A group of cells on the dorsal lip of the blastopore that induces the differentiation of cells in the embryo, controlling the growth and development of adjacent parts that eventually form the body axis.

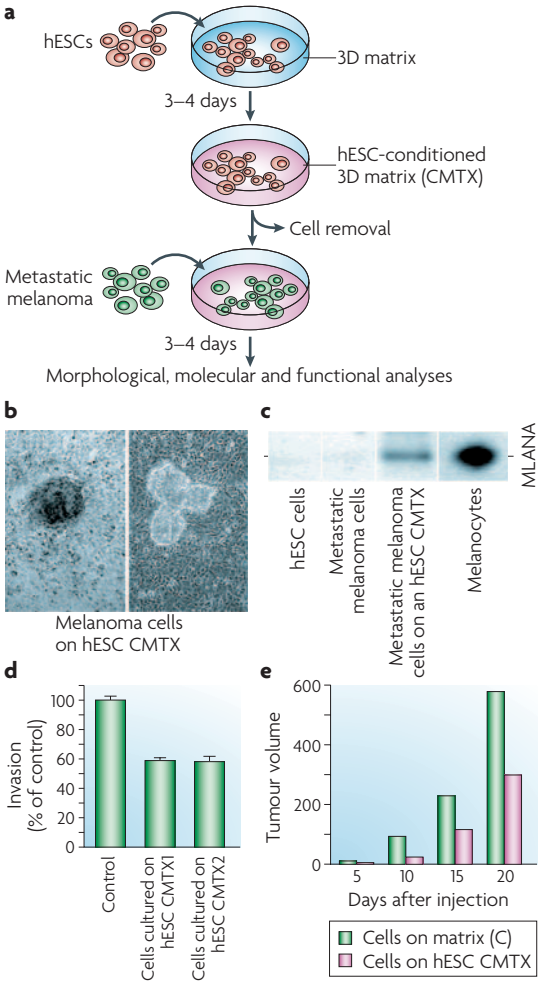

Figure 1 | **Human embryonic stem cell microenvironment model. a** | Compact colonies of human embryonic stem cells (hESCs) are seeded onto a 3D matrix for 3–4 days, then removed, resulting in a conditioned 3D matrix (CMTX) onto which human metastatic melanoma cells are then seeded and incubated for 3–4 days. Subsequently, changes in cell morphology, gene and protein expression as well as the behavioural function(s) of the tumour cells can be examined. **b** | Phase-contrast microscopy of metastatic melanoma cells induced to form spheroids (a marked morphological alteration reminiscent of how hESCs grow in culture) by the hESC microenvironment. **c** | Western blot analysis of whole-cell lysates for a melanocyte marker, MLANA, shows its induction in metastatic melanoma cells exposed to the hESC conditioned matrix, and its absence in hESCs and in melanoma cells grown on a control matrix. Protein analysis was performed on whole cell lysates including their underlying matrix. 40 μg of this total protein lysate was loaded per parameter. Melanocytes express MLANA as their phenotypic marker. **d** | Melanoma cells cultured on two different types of hESC microenvironments (CMTX 1 derived from the H1 hESC cell line, CMTX 2 derived from the hSF-6 hESC cell line) were 42% less invasive than melanoma cells cultured on a control matrix. **e** | *In vivo* tumour formation was reduced in nude mice that received orthotopic injection of metastatic melanoma cells cultured on an hESC CMTX compared with the injection of metastatic melanoma cells cultured on an unconditioned matrix (C). Portions of this figure have been modified with permission from REF. 47.

Neural tube
In the developing vertebrate nervous system, the neural tube is the precursor of the central nervous system, which comprises the brain and spinal cord.

the convergence of tumorigenic and embryonic signalling pathways, and implicate Nodal as a new diagnostic marker for disease progression and a novel target for the treatment of aggressive cancers such as melanomas.

Although the developing zebrafish has proven to be a powerful system for examining how cancer cells influence embryonic stem cells, it can also be used to study reciprocal interactions; that is, how embryonic microenvironments affect cancer cells. To this effect, extracts derived from zebrafish embryos have been shown to inhibit proliferation and induce apoptosis in several cancer cell types[64]. Furthermore, we have shown that the exposure of metastatic melanoma cells to an embryonic zebrafish microenvironment results in their reprogramming towards a non-tumorigenic phenotype. Indeed, although still present after a 3 month period of observation, melanoma cells implanted into zebrafish embryos lay dormant and were unable to form tumours[65] (FIG. 2). Interestingly, this phenomenon is unique to the embryonic zebrafish microenvironment, as human melanoma cells transplanted into zebrafish 2 days after fertilization (after morphogenesis and organogenesis are complete) form tumours and even induce angiogenesis[66]. The findings generated in the embryonic zebrafish model demonstrate the remarkable plasticity of multipotent metastatic melanoma cells and the utility of the developing zebrafish as a model for studying the epigenetic modulation of tumour cell phenotypes.

Neural crest microenvironment

The third model investigated for its potential to reprogramme the metastatic melanoma phenotype is the embryonic chick, specifically the neural crest regions. The neural crest is a multipotent cell population (sharing similar properties with aggressive melanoma) that emerges from the neural tube and invades the embryo in a programmed manner to form peripheral neurons and glia and the entire facial and visceral skeleton of the vertebrate head[67–69]. A subpopulation of neural crest cells distribute throughout the embryo and differentiate into pigment cells (from which melanocytes and melanoma derive) to protect the body from UV radiation[70,71]. In the embryo, the identity and fate of an individual migratory neural crest cell is thought to be regulated by an interplay between intrinsic signals in the neural tube and extrinsic cues mediated by the microenvironment through which they migrate[72–76]. The neural crest cell migratory pathways emerge as discrete segregated streams, separated by neural-crest-free exclusion zones that seem to prevent the intermixing of neural crest cell subpopulations and ensure their arrival at precise targets[77–81]. Based on the migratory and invasive properties shared by both neural crest cells and multipotent melanoma tumour cells, we used the embryonic chick model to specifically explore the possibility of reverting melanoma cells to their neural crest cellular derivatives (FIG. 3). When green fluorescent protein-labelled human metastatic melanoma cells were transplanted into the chick embryonic microenvironment, the melanoma cells invaded host neural crest targets, did not form tumours, and a subset of these tumour cells were reprogrammed to a neural

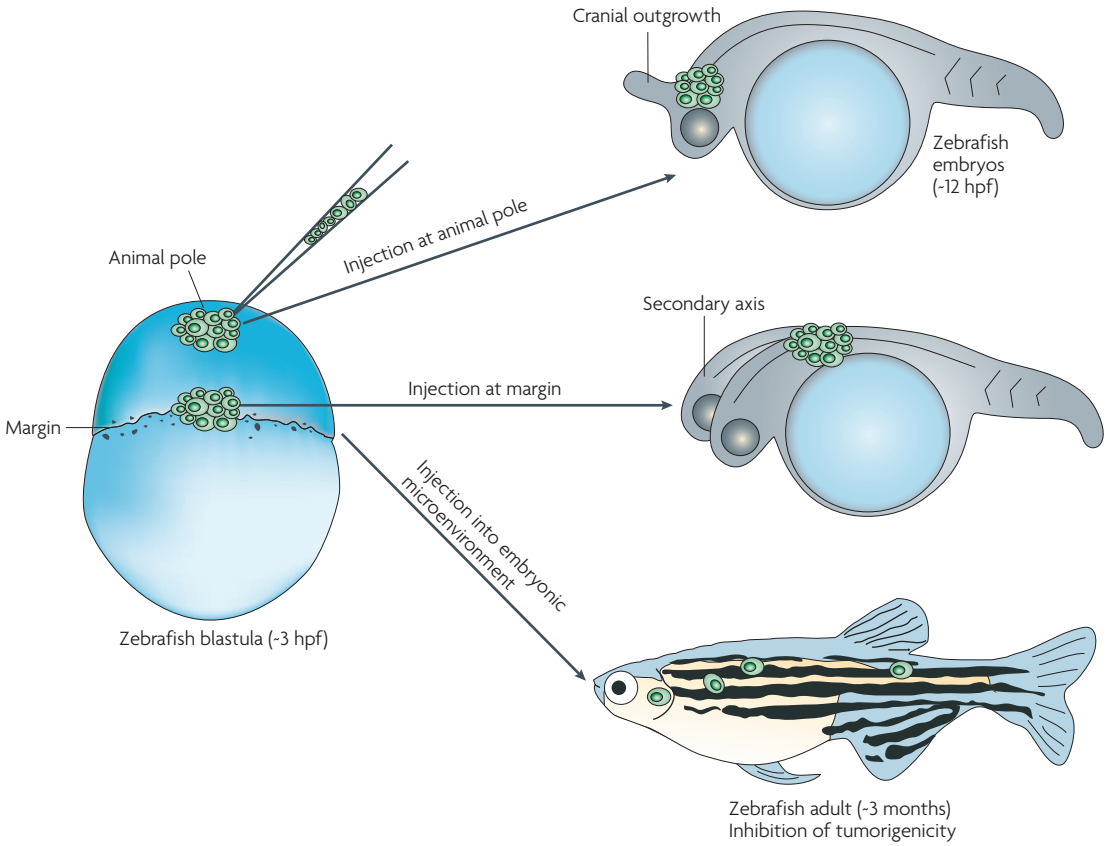

Figure 2 | **Transplantation of metastatic melanoma cells into the zebrafish embryo.** Green fluorescent protein-labelled aggressive human metastatic melanoma cells are injected into blastula-stage zebrafish embryos 3 hours post fertilization (hpf), either at the region(s) of the animal pole, resulting in an abnormal cranial outgrowth, or at the yolk margin, resulting in the formation of a secondary body axis (12 hpf). Therefore, the melanoma cells secrete factors that instruct the pluripotent zebrafish cells to form ectopic structures. Melanoma tumorigenicity is inhibited in the embryonic zebrafish microenvironment, for up to 3 months in the adult fish.

crest cell-like phenotype[82]. Specifically, the transplanted melanoma cells populated host peripheral structures in a programmed manner, including the branchial arches, dorsal root and sympathetic ganglia. A subpopulation of melanoma cells that invaded the chick periphery was reprogrammed to express the melanocyte-associated protein MLANA, and the neuronal marker TUJ1. These intriguing results demonstrate that metastatic melanoma cells can respond to developmental cues, and suggest that factors unique to the neural crest embryonic milieu may be implemented to reprogramme melanoma cells to a more benign melanocytic cell type. Therefore, improved insights into the mechanisms that govern tumour cell reprogramming, studied within the chick neural crest microenvironment, might be relevant for the identification of new cancer therapies.

The fate of an individual neural crest cell should be irreversible to ensure cell type-specific function; however, the mechanisms by which differentiated neural crest cells can change fate or reverse to precursor neural crest cells are poorly understood. Recent studies have shown that differentiated neural crest-derived quail pigment cells can be isolated from the skin and cultured *in vitro* to generate glia and myofibroblastic cells[83]. The phenotypic reprogramming involves the dedifferentiation of dividing pigment cells into cells that re-express neural crest early marker genes. Therefore, pigment cells seem to be phenotypically unstable, can revert to their neural crest cell-like ancestors and are endowed with self-renewal capacity when removed from their niche and introduced to appropriate culture conditions. The sources of the programming signals in the embryo are unclear, but may include neural crest cell–cell interactions and/or cell–environment interactions with the extracellular matrix and mesoderm along the migratory route and at target sites. These signals within the embryonic neural crest microenvironment might be specific to the neural crest and some other cell types. Fibroblasts grafted along chick embryonic neural crest cell pathways did not invade surrounding tissues and remained intact at the graft sites[84], whereas non-malignant B16 mouse melanoma cells injected into the chick trunk neural tube migrate into the neural crest microenvironment but undergo apoptosis[85]. The transplantation of very small numbers of neural crest cells has shown that the lack of neighbouring signals to reinforce their character may cause a considerable degree of neural crest cell plasticity[82]. Therefore, cell communication could have an important role in the identity and fate of a neural crest cell. In particular, migratory neural crest cells display

Embryonic axis
An imaginary line from the head end to the tail end of an embryo or, before that, the line of elongation of the primitive streak and groove.

Filopodial extensions
Filopodia are slender cytoplasmic projections that extend from the leading edge of migrating cells and contain actin filaments crosslinked into bundles by actin-binding proteins. They form focal adhesions that function to link the cell surface to the substratum and facilitate cell motility.

Box 3 | Nodal

- Nodal is an embryonic morphogen belonging to the TGFβ superfamily.
- Nodal is secreted into the microenvironment and has concentration-dependent effects on gene expression.
- In vertebrates, Nodal induces embryonic axis formation before gastrulation. It is an essential component of the dorsal organizing centre that instructs pluripotent stem cells to initiate notochord formation.
- In vertebrates, Nodal patterns the nervous system, induces the mesoderm and endoderm and determines left-right asymmetry.
- Nodal maintains the pluripotency of human embryonic stem cells (hESCs) and sustains pluripotent cells in the mouse epiblast before axial patterning.
- In humans, Nodal expression is largely restricted to embryonic tissues including the trophoblast, ESCs and the developing mammary gland.
- Nodal expression has been documented in human melanoma, breast cancer, and testicular cancer. So far the mechanisms by which cancer cells regain the expression of this embryonic morphogen remain elusive.
- Nodal sustains melanoma tumorigenicity and plasticity by promoting an interconverted phenotype as well as a non-specific gene-expression pattern.

extensive filopodial extensions and cell–cell contact during migration[86–88] that seem to have a role in cell guidance[86], and very similar morphologies are displayed by transplanted melanoma cells. Furthermore, the directionality of the tumour cells coincides with their migration toward the intended branchial arches. Whether neural crest or melanoma cell filopodial interactions are also involved in the translation of positional information between cells is unknown. Ongoing studies are using real-time monitoring of melanoma cell dynamics within the embryonic neural crest microenvironment combined with molecular analyses, and offer great potential to determine the signals that reprogramme metastatic melanoma cells.

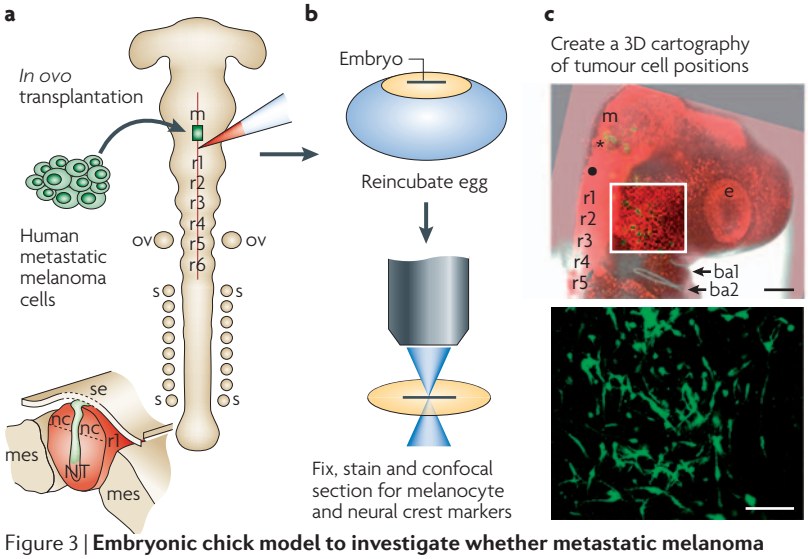

Figure 3 | **Embryonic chick model to investigate whether metastatic melanoma cells can be reverted back to their cellular derivatives by the neural crest microenvironment. a** | Green fluorescent protein (GFP)-labelled human metastatic melanoma cells are transplanted under the surface ectoderm (se) into the chick (6–8 somite (a paired, blocklike mass of mesoderm, arranged segmentally) stage) neural tube (NT) at various axial locations, including the midbrain (m) and hindbrain rhombomere segments (r1–r6). The DiI-label is a lipophilic tracer dye (shown in red within a glass needle and as a line along the vertebrate axis) that diffuses within the neural tube to label the host embryo premigratory neural crest (nc), but does not label the surrounding microenvironment (mes) into which the neural crest cells migrate. **b** | Following injection, the embryonic chick incubates *in ovo* for 48 hours, to allow the melanoma cells to invade the host. The embryos are subsequently harvested and analysed by 3D confocal sectioning to produce a cartography of tumour cell positions. **c** | Typical confocal sections through a chick embryo head reveal the invading melanoma (green) and host neural crest cells (red). The melanoma cells have emigrated from the transplant site (black circle), and are found within the unlabelled embryonic neural crest cell-rich microenvironment adjacent to the neural tube and towards some of the neural crest target destinations, including the first branchial arch (ba1), face (white box) and eye (e). The bottom image (magnification of the box area) reveals the long projections (asterisk) and cell morphologies of individual invading tumour cells. The scale bars are 200 μm. ov, otic vescile; s, somite.

Targeting Nodal signalling

The discovery of Nodal as a crucial component underlying the bi-directional communication between human metastatic melanoma cells and zebrafish stem cells prompted further investigation into the molecular mechanisms that underlie the effect of Nodal. Essentially, Nodal propagates its signal by mediating the phosphorylation of SMAD2 and possibly SMAD3 (REF. 61) (FIG. 4). Phosphorylated SMAD2 and SMAD3 associates with SMAD4 and translocates to the nucleus, where it regulates gene expression through an association with transcription factors including forkhead box H1 (FOXH1) (REF. 61). The epidermal growth factor type co-receptor (EGF-CFC), cripto , enhances this process[61]. Extracellular Nodal inhibitors, most notably LEFTY1 and LEFTY2, control this system by spatially and temporally restricting Nodal signalling levels[89]. Indeed, LEFTY1 and LEFTY2, which are also upregulated in response to Nodal, specifically antagonize the Nodal signalling pathway by interacting with Nodal and/or with cripto, thereby providing a negative-feedback mechanism[61]. The complexity of the Nodal signalling pathway provides several plausible therapeutic targets and strategies (highlighted in FIG. 4).

Unlike hESCs, which express both the activating and inhibitory (lefty) components of the Nodal signalling pathway, metastatic melanoma cells express only Nodal and no lefty, whereas subpopulations express the co-receptor cripto (REF. 90). Therefore, Nodal signals without regulatory control in metastatic melanoma cells. Thus, we used two potential strategies for blocking Nodal signalling: small-molecule inhibitors designed to block activin-like-kinase (ALK) activity, and Nodal-specific morpholinos (MO)[1]. Using an (ALK 4/5/7) small-molecule inhibitor

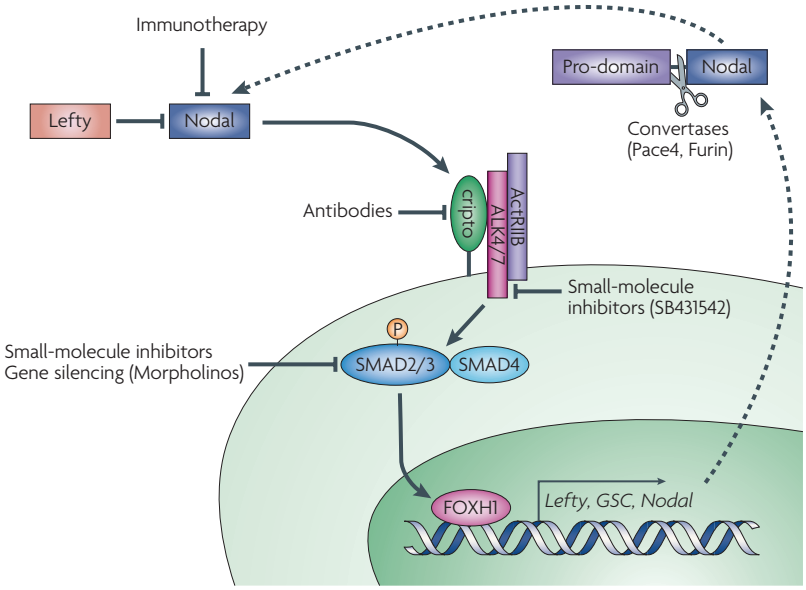

Figure 4 | **The Nodal signalling pathway.** Extracellularly, the Nodal precursor is cleaved by the subtilisin-like proprotein convertases (SPCs), Pace4 and furin. Nodal propagates its signal by binding to heterodimeric complexes between type I (activin-like-kinases (ALKs) 4 and 7) and type II ALK receptors (ACTRIIB), resulting in the phosphorylation and activation of ALK 4/7 by ACTRIIB, followed by the ALK 4 and 7-mediated phosphorylation of SMAD2 and possibly SMAD3. Phosphorylated SMAD2 and SMAD3 associates with SMAD4 and translocates to the nucleus, where it regulates gene expression through its association with transcription factors such as forkhead box H1 (FOXH1). The Nodal signalling cascade is promoted by the epidermal growth factor-co-receptor (EGF–CFC) cripto, which, like Nodal, is associated with tumorigenesis. Nodal signalling promotes the transcription of genes including *LEFTY1*, *LEFTY2* (*Lefty*) and goosecoid (*GSC*), and also upregulates its own expression through a positive-feedback loop. Extracellular Nodal inhibitors, most notably LEFTY1 and LEFTY2, spatially and temporally restrict Nodal signalling levels through their antagonism of Nodal and/or cripto, thereby creating a negative-feedback mechanism. The Nodal signalling pathway provides several potential therapeutic targets for the treatment of metastatic melanoma. Small molecules such as SB431542 could be used to specifically block the activation of the ALK 4 and 7 receptors. Alternatively, downstream cytoplasmic effectors including SMAD2 could be inhibited by gene silencing with agents including Morpholinos, or by specific small-molecule inhibitors. Cripto-specific neutralizing antibodies could be a viable treatment for metastatic melanoma. Extracellular Nodal inhibitors, most notably the Lefty proteins, might also be used. Due to the function of Nodal as a promoter of melanoma tumorigenicity as well as its restricted expression pattern, Nodal-specific immunotherapy could be used. The rationale behind this approach would be to target the host immune system against aggressive Nodal-producing tumour cells at the same time as neutralizing Nodal signalling by selectively blocking its interactions with its receptors.

Morpholinos
Morpholino oligos are short chains of about 25 Morpholino subunits comprised of a nucleic acid base, a morpholine ring and a non-ionic phosphorodiamidate intersubunit linkage. Their high mRNA-binding affinity and specificity permits them to sterically block translation initiation in the cytosol, modify pre-mRNA splicing in the nucleus or directly block miRNA activity to effectively knock down the expression of targeted genes.

(SB431542) in metastatic melanoma cells abrogated Nodal signalling, reducing SMAD2 phosphorylation and decreasing Nodal expression[1]. These data suggest that Nodal is regulated in metastatic melanoma cells, at least in part, by a SMAD2-dependent positive-feedback loop. Additional studies with SB431542 promoted the reversion of metastatic melanoma cells towards a less plastic, melanocyte-like phenotype, demonstrated by reduced invasive capacity *in vitro*, coincident with the abrogation of vasculogenic mimicry, and re-expression of tyrosinase (a melanocytic marker)[1]. The treatment of metastatic melanoma cells with Nodal MO significantly diminished their anchorage-independent growth, which could be rescued by the inclusion of recombinant Nodal. Using a nude mouse model and the orthotopic transplantation of metastatic melanoma cells treated with Nodal MO resulted in a 30% decrease in tumour incidence and a decrease in tumour growth, measured at 17 days after inoculation; however, immunohistochemistry revealed that the tumours that formed regained Nodal expression by day 17 (REF. 1). Therefore, new strategies to diminish Nodal expression for longer duration are currently under consideration. In a manner similar to Nodal inhibition, knocking down SMAD2 expression resulted in decreased melanoma invasiveness and anchorage-independent growth[1]. However, the inclusion of recombinant Nodal did not rescue the ability of the aggressive melanoma cells to undergo anchorage independent growth when SMAD2 was knocked down, suggesting that Nodal-dependent tumorigenicity is mediated through a SMAD2 dependent mechanism[1]. Therefore, inhibition of SMAD2 might present an attractive therapeutic target designed to inhibit melanoma tumour formation.

Further testing of the Nodal–Lefty pathway in the chick embryo and hESC microenvironment models revealed two significant observations that further support the rationale for exploiting this signalling pathway as a viable therapeutic target. When Nodal-deficient metastatic melanoma cells are transplanted into the chick embryonic neural crest microenvironment, the tumour cells fail to migrate and populate neural crest-cell migratory pathways and peripheral targets (P.M.K., J.K.-K. and L.M.D., unpublished observations). In the hESC model, we have determined that Lefty is deposited into the microenvironment by hESCs, resulting in the downregulation of nodal expression in metastatic melanoma cells (L.M.P., R.E.B.S., E.A.S. and M.J.C.H., unpublished observations). In current studies, Lefty is being isolated from hESCs using an anti-Lefty antibody bound to magnetic beads (dynabeads), to determine its potential efficacy as a therapeutic agent, as physiological levels of recombinant Lefty are ineffective in altering Nodal signalling in melanoma cells (L.M.P., R.E.B.S., E.A.S. and M.J.C.H., unpublished observations).

Nodal expression is restricted to embryonic tissues such as hESCs and to cancers, making it an ideal target for immunological therapies[91,92]. One approach could employ specific antibodies to identify and neutralize Nodal protein, which would target the host immune system against aggressive Nodal-producing tumour cells at the same time as neutralizing Nodal signalling by selectively blocking its interactions with its receptors. This type of targeted immunotherapy has proven to be efficacious in the treatment of melanoma[93]. For example, greater than 30% of patients treated with antibodies directed to the cancer-testes specific antigen, melanoma antigen family A3 (MAGE3), have demonstrated disease regression[93]. Targeted immunotherapy against Nodal could be accomplished with either conventional antibodies or with emerging nanotechnology[94], ultimately preventing tumour cell plasticity while concomitantly providing a mechanism for targeted tumour destruction.

The direct inhibition of cripto, Nodal's co-receptor, might provide a more selective therapeutic target. Like Nodal, cripto is highly expressed in cancer but is rarely detected in normal adult tissues[95]. Furthermore, cripto is

a stem cell marker that is thought to maintain the pluripotency of hESCs, perhaps through a Nodal-dependent pathway[96,97]. In addition, cripto expression promotes de-differentiation and invasion in breast carcinoma cells[98,99]. These functions of cripto in epithelial cancers mirror those of Nodal in melanoma, implying a convergence of their tumour-promoting functions. Although the presence of cripto has not yet been documented in metastatic melanoma, a subpopulation of melanoma cells express this co-receptor on their cell surface. Because cripto is a glycosylphosphatidylinositol-linked protein, it may also be secreted[100]. In this manner, it could bind to extracellular Nodal, thereby facilitating the activation of ALK 4/7 receptor complexes[101]. Therefore, antibodies designed to block the function of cripto or its association with Nodal may be of therapeutic relevance. Indeed, cripto-neutralizing antibodies have been shown to inhibit the growth of breast, colon and testicular cancers, both *in vitro* and *in vivo*, and anti-cripto therapeutics are currently under development, as is the application of cripto as a serological marker for breast and colon cancer[97,102,103].

Future perspectives

There is an immediate need to elucidate the intricacies of the Nodal signalling pathway in melanoma and other tumours, which holds great promise for yielding new therapeutic targets. It is equally important to appreciate the complexity of the Nodal signalling pathway and the possible intersection with other signalling pathways that underlie stem cell fate. Our preliminary data would suggest that the notch pathway, also crucial in stem cell fate determination, is a probable candidate to pursue based on the potential for molecular cross-talk with Nodal signalling. Furthermore, as Nodal is a potent embryonic morphogen from the TGFβ family, the possibility also exists that other members of this superfamily might have an impact on Nodal-associated signalling. Complementary studies are underway to better understand the epigenetic role of the microenvironment in reprogramming multipotent, metastatic tumour cells, particularly with respect to downregulating and silencing the aberrant expression of the Nodal plasticity gene.

In summary, we have gained new insights into the potential of three embryonic microenvironments to reprogramme phenotypically plastic metastatic tumour cells towards a more benign cell type. However, it is important to recognize that these unique models do not entirely mimic real-life human disease. Nonetheless, the discovery of key signalling pathways that underlie the commonality of plasticity of embryonic stem cells and multipotent tumour cells might yield new therapeutic strategies to suppress the metastatic phenotype.

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Competing interests statement
The authors declare no competing financial interests.

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