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The Commonality of Plasticity Underlying Multipotent Tumor Cells and Embryonic Stem Cells

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Abstract Aggressive cancer cells and pluripotent stem cells converge in their capacity for self-renewal, proliferation and plasticity. Recent studies have capitalized on these similarities by demonstrating that tumors arise from specific cancer stem cell populations that, in a manner reminiscent of normal stem cells, are able to both self-renew and give rise to a heterogeneous tumor population. This stem cell like function of aggressive cancer cells is likely attributable to the ectopic expression of embryonic factors such as Nodal and Cancer Testis Specific Antigens (CTAs), which maintain a functional plasticity by promoting pluripotency and immortality. During development, the expression of these embryonic factors is tightly regulated by a dynamic array of mediators, including the spatial and temporal expression of inhibitors such as Lefty, and the epigenetic modulation of the genome. In aggressive cancer cells, particularly melanoma, this balance of regulatory mediators is disrupted, leading to the aberrant expression of pluripotency-associated genes. By exposing aggressive cancer cells to embryonic microenvironments, this balance of regulatory mediators is restored, thereby reprogramming tumor cells to a more benign phenotype. These stem cell-derived mediators, as well as the genes they regulate, provide therapeutic targets designed to specifically differentiate and eradicate aggressive cancers. J. Cell. Biochem. 101: 908–917, 2007.

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Aggressive cancer cells share numerous similarities with pluripotent stem cells. For example, like stem cells, tumor cells self-renew in a manner that confers near immortality, give rise to a phenotypically diverse progeny, and express several surface markers (including CD133, c-kit, and CD34) that traditionally demarcate stem cell populations [Hendrix et al., 2003b]. Moreover, the phenotype of both stem cells and cancer cells is profoundly influenced by bi-directional cellular communication with the microenvironment. During vertebrate development, multipotent precursor cells are gradually specified to particular fates through the autocrine or paracrine delivery of signaling molecules, and during cancer progression malignant cells similarly release and receive cues that promote tumor growth and metastasis. Of note, recent findings have illuminated a convergence between cancer and stem cells in the molecular messengers that they implement to regulate self-renewal, proliferation, and cell fate. These factors, classically associated with developmental processes, include members of the Wingless (Wnt), Notch, Cancer Testis Antigens (CTAs) and Transforming Growth Factor Beta (TGF-β) superfamilies [Bittner et al., 2000; Weeraratna et al., 2002; Hendrix et al., 2003b; Hoek et al., 2004; Balint et al., 2005; Topczewska et al., 2006].

Although this concept of relating cancer to stem cells has recently gained popularity

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(largely due to the identification of specific cancer stem cells) it is not a new one. Indeed, it was first conceived over 150 years ago by Rudolf Vichow who proposed that cancer arises from embryo-like cells, and Conheim and Durante who extended this idea to suggest that adult tissues contain embryonic remnants that normally lie dormant, but that can be activated to become cancer [Sell, 2004]. This concept, formally called the "embryonal rest" theory of cancer, is remarkably similar to the current cancer stem cell theory which maintains that cancer arises from a subpopulation of stem cells.

Recent technological advances have facilitated the development of the cancer stem cell theory by enabling scientists to specifically isolate and visualize cancer stem cells. These stem cells can be separated from the tumor bulk based on their expression of specific stem cell surface markers such as CD133, and then assayed for their tumorigenic potential. By definition the cancer stem cell is a single cancer cell that can form a tumor following transplantation. These cells can self-renew, deriving daughter cells and a phenotypically diverse population of less aggressive cancer cells that recapitulate the heterogeneity of the parental tumor [Reya et al., 2001]. The cancer stem cell theory was first brought to fruition for human leukemia when a subpopulation of leukemic cells was shown to be both required and sufficient to establish leukemia in mice [Lapidot et al., 1994; Reya et al., 2001]. Since this seminal discovery, cancer stem cells have been documented in malignancies as diverse as glioma, breast cancer and melanoma [Al-Hajj et al., 2003; Singh et al., 2004; Fang et al., 2005].

While evidence suggests that the majority of cancers depend on the presence of a cancer stem cell population for tumorigenicity, the origins of such neoplastic progenitors have not yet been unequivocally demonstrated. Cancer stem cells may initiate from mutations and/or epigenetic modifications in normal stem cells that reside in organ-specific stem cell niches such as the basal layer of the epidermis, the crypts of the intestine, the bone marrow, or the mammary gland terminal end buds [Reya et al., 2001; Toma et al., 2001; Marshman et al., 2002; Visvader and Lindeman, 2006]. Alternatively, as has been proposed for gastric cancer, mesenchymal stem cells recruited to the sites of inflammation or injury may transform into cancer stem cells [Houghton et al., 2004]. Finally, tumor cells may undergo genetic and/or epigenetic modifications that result in the manifestation of a plastic, pluripotent phenotype. In support of this latter concept, Krivtsov et al. [2006] recently demonstrated that a leukemia stem cell population can be derived from committed granulocyte macrophage progenitors.

Regardless of where they arise from or what cell surface markers characterize them; cancer stem cells have a profound, perhaps essential, role in tumor initiation. It is also likely that by facilitating cancer cell adaptability, they promote tumor progression and metastasis. In support of this concept, global gene analyses conducted in our laboratory and others suggest that aggressive cancer cell lines, which should inherently contain a high percentage of cancer stem cells, manifest a functional plasticity characterized by the simultaneous expression of genes from a variety of cell types concomitant with a reduction in the expression of genes specific to their parental cell lineage [Hendrix et al., 2003b]. For example, aggressive melanoma cells possess Keratins, intermediate filaments characterizing epithelial cells, and aberrantly express genes, including *Vascular Endothelial Cadherin* (*VE-Cadherin*), normally associated with endothelial cells [Hendrix et al., 1992, 2003a,b]. Furthermore, the expression of melanocyte-specific markers is dramatically reduced, and sometimes absent, in aggressive melanoma cells: Tyrosinase, which catalyzes the conversion of tyrosine to the pigment melanin [Hearing and Tsukamoto, 1991], is reduced by more than 35-fold in aggressive melanomas as compared to their poorly aggressive counterparts [Hendrix et al., 2003b]. Aggressive melanomas also have the ability to express developmental proteins like the Notch receptors and stem cell markers such as CD34 [Hendrix et al., 2003b; Hoek et al., 2004]. Collectively, this gene expression pattern confers upon aggressive melanoma cells a functional plasticity that enables them to thrive and metastasize. For example, in melanoma, the expression of Keratins is associated with enhanced invasion and metastasis [Hendrix et al., 1992], and VE-Cadherin is essential for the formation of tumor-derived vascular networks, a feature that provides the tumor with an auxiliary perfusion pathway [Hendrix et al., 2001]. In addition, reduced Tyrosinase levels are associated with a poor clinical outcome, likely because they enable melanoma cells to

evade immune surveillance [Takeuchi et al., 2003]. Hence, the plasticity conferred by the stem cell-like nature of aggressive cancer cells, particularly melanoma cells, greatly increases their metastatic capacity.

In a manner analogous to aggressive cancer cells, human embryonic stem cells (hESC) express genes that characterize differentiated tissues including brain, liver, and blood concurrent with self-renewal genes such as Oct-3/4 and Nanog [Golan-Mashiach et al., 2005; Lotem and Sachs, 2006]. This comparison evokes an intriguing question: Do cancer cells employ embryonic stem cell signaling pathways to sustain a niche that facilitates plasticity? In order to address this question we utilized the developing zebrafish as a biosensor for tumor-derived signals [Topczewska et al., 2006]. By injecting aggressive melanoma cells into zebrafish blastula, we investigated the possibility that aggressive cancer cells and embryonic stem cells can communicate bi-directionally using pathways characteristic of a microenvironmental stem cell niche. Morphological observations of the embryos that were engrafted with melanoma cells led us to the discovery that aggressive melanoma cells can indeed communicate with embryonic progenitors: The injected cancer cells orchestrated the formation of ectopic embryonic axes via the secretion of Nodal, a member of the TGF-β family. Nodal is a potent embryonic morphogen that initiates embryonic axis formation by acting as an organizing signal before gastrulation and determines left–right symmetry later in gestation [Schier, 2003]. Our studies determined that Nodal also promotes melanoma tumorigenicity and metastasis. We found that Nodal expression was absent in normal skin and was rare in poorly invasive radial growth phase melanomas. This is in contrast to invasive vertical growth phase melanomas and melanoma metastases where Nodal expression was frequently observed. Furthermore, inhibiting the expression of Nodal in aggressive melanoma cells resulted in decreased colony formation in soft agar concomitant with a marked abrogation of tumor formation in a mouse model [Topczewska et al., 2006].

Interestingly, Nodal has been shown to maintain the pluripotent status of embryonic stem cells [James et al., 2005; Vallier et al., 2005; Mesnard et al., 2006]. Indeed, inhibition of the Nodal signaling pathway results in hESC differentiation, and Nodal is one of the first genes to be downregulated as totipotent hESCs differentiate during embroid body formation [Vallier et al., 2004]. We determined that Nodal similarly maintains cancer cell plasticity. For example, depletion of Nodal in the aggressive melanoma cells lead to an upregulation of Tyrosinase, a reduction in VE-Cadherin, and a decrease in cellular invasion, thereby promoting the differentiated phenotype associated with melanocytes [Topczewska et al., 2006]. Of note, reminiscent of the staining patterns observed for cancer stem cell populations, we found that Nodal protein was expressed heterogeneously in both the clinical specimens and in the aggressive melanoma cell line employed in our studies. Hence, by virtue of its heterogeneous expression and requirement for tumor formation, Nodal may be an important marker of the melanoma stem cell phenotype. Unlike other cancer stem cell markers, which lack a defined function, we speculate that Nodal not only demarcates the cancer stem cell population but also facilitates tumor progression by sustaining a stem cell-like niche. The significance of this function is profound in that it suggests that aggressive cancer cells are able to actively maintain their own plasticity.

EMBRYONIC MICROENVIRONMENTS CAN REPROGRAM AGGRESSIVE CANCER CELLS: DIFFERENTIATION THERAPY

If hESCs and aggressive cancer cells share many similarities and converge at the signaling pathways which they implement to maintain plasticity, why is it that hESCs do not form tumors following blastocyst implantation? The simplest explanation is that hESCs differentiate in response to almost any microenvironmental alteration [Lotem and Sachs, 2006]. This is in contrast to aggressive melanoma cells, for example, whose plasticity is unabated by exposure to microenvironments such as extracellular matrices (ECM) conditioned by normal melanocytes or the ischemic limb of a mouse [Hendrix et al., 2002; Seftor et al., 2005]. Indeed, the only microenvironments that have been shown to effectively reprogram aggressive melanoma cells are those associated with embryological development.

Pierce and colleagues suggested that cancer is a problem of developmental biology and that an embryonic microenvironment capable

of differentiating a stem cell lineage should be able to reprogram cancers derived from that lineage [Gerschenson et al., 1986]. In support of this concept, embryonic microenvironments were shown to inhibit the tumorgenicity of a variety of cancer cell lines [Pierce et al., 1982; Podesta et al., 1984; Gerschenson et al., 1986]. For example, B16 murine melanoma cells were unable to form tumors and appeared to differentiate toward a neuronal phenotype following exposure to microenvironmental factors derived from the embryonic skin of a developing mouse [Gerschenson et al., 1986]. In another set of experiments, Bissell and colleagues documented that Rous sarcoma virus, which causes a rapidly growing tumor when injected into hatched chicks, is non-tumorigenic when injected into 4-day-old chick embryos, despite viral replication and v-src oncogene activation [Dolberg and Bissell, 1984].

More recently, we utilized the embryonic chick model to explore the possibility of reprogramming melanoma cells toward their neural crest-derived cell type of origin [Kulesa et al., 2006]. In this study, we utilized confocal imaging to track GFP-labeled metastatic melanoma cells following transplantation in ovo adjacent to host chick premigratory neural crest cells. The transplanted melanoma cells responded to host neural crest cues by migrating into the surrounding host tissue in a programmed manner. Furthermore, the melanoma cells displayed neural crest-like morphologies and were able to populate structures such as the brachial arches, sympathetic ganglia, and dorsal root, in a manner similar to neural crest cells. Interestingly, a subpopulation of melanoma cells that invaded the chick periphery was reprogrammed to express the melanocyte-associated protein Mart-1/Melan-A, thus confirming that melanoma cells can respond to developmental cues [Kulesa et al., 2006].

As a corollary to these findings, we employed an in vitro 3-D model to examine whether the microenvironment of hESCs could similarly reprogram the metastatic melanoma cell phenotype [Postovit et al., 2006]. Utilizing this approach we determined that, similar to Nodal inhibition, exposure of melanoma cells to hESC microenvironments resulted in the re-expression of melanocyte specific markers, as well as a reduction in invasive potential. In contrast to the study by Pierce and colleagues, the conditioned medium of hESCs did not alter the aggressive melanoma phenotype, suggesting that the hESC transdifferentiating factor(s) are preferentially deposited or stabilized in the matrix [Postovit et al., 2006]. Collectively, these findings suggest that embryonic microenvironments contain cues not associated with differentiated tissues, which may be harnessed to epigenetically reprogram metastatic melanoma cells.

EPIGENETIC MECHANISMS UNDERLYING THE REPROGRAMMING OF AGGRESSIVE CANCER CELLS

Tumor progression is accompanied by a complex array of genetic mutations and epigenetic alterations. 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 signaling and DNA modifications. We have determined that the ability of hESCs to reprogram aggressive melanoma cells is reversible over time (unpublished observation). As such, this phenomenon is likely due to epigenetic alterations.

As illustrated in Figure 1, we propose that hESCs reprogram melanoma cells at least in part by inhibiting the Nodal-signaling pathway. Nodal propagates its signal by binding to heterodimeric complexes between Type I (ALK 4/5/7) and Type II (ActRIIB) activin-like kinase receptors. Assembly of this complex results in phosphorylation and activation of ALK 4/5/7 by ActRIIB, followed by the ALK 4/5/7 mediated phosphorylation of Smad-2/3 [Schier, 2003]. This process is greatly enhanced by the epidermal growth factor type co-receptor (EGF-CFC), Cripto-1 [Schier, 2003]. Interestingly, Nodal is regulated via a positive feedback loop [Besser, 2004]. In order to control the levels of this potent morphogen hESCs also secrete Nodal inhibitors, most notably Lefty-A, Lefty-B [Chen and Shen, 2004], and Lefty-A/B, which are also upregulated in response to Nodal, specifically antagonize the Nodal-signaling pathway by interacting with Nodal and/or with Cripto-1 [Chen and Shen, 2004; Tabibzadeh and Hemmati-Brivanlou, 2006]. As summarized in Table I, and depicted in Figure 1, although aggressive melanoma cells express Nodal and the co-receptor Cripto-1, they do not express Lefty-A/B: As a consequence, Nodal is allowed to

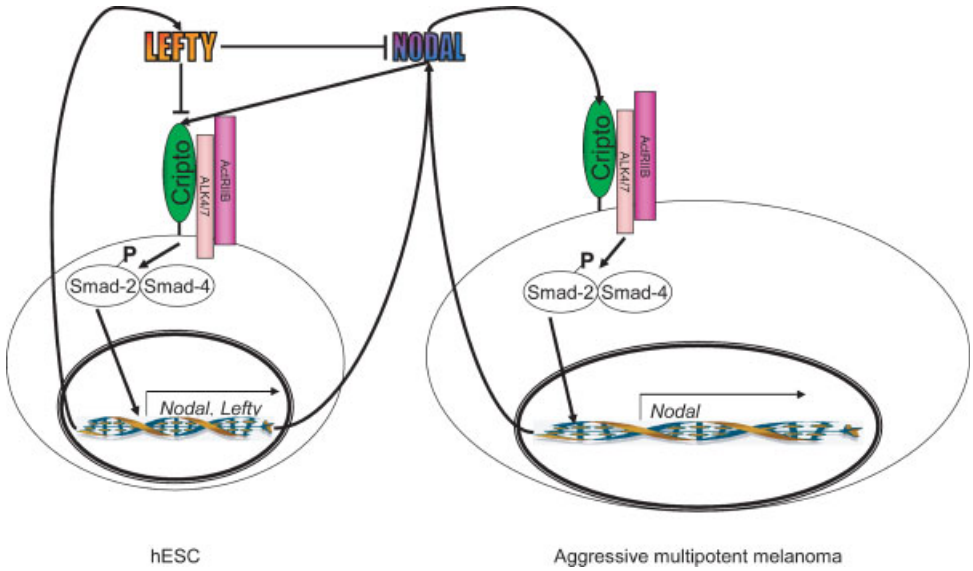

Fig. 1. Hypothetical model for the hESC-mediated reprogramming of aggressive melanoma cells through inhibition of Nodal signaling. Nodal signals by binding to heterodimeric complexes between Type I (ALK 4/7) and Type II (ActRIIB) activin-like kinase receptors. Assembly of this complex results in phosphorylation and activation of ALK 4/7 by ActRIIB, followed by the ALK 4/7 mediated phosphorylation of Smad-2/3. This process is enhanced by the EGF-CFC Cripto-1. Nodal is regulated via a positive feedback loop. In order to control the levels of this potent morphogen hESCs also secrete Nodal inhibitors, most notably Lefty. Lefty, which is upregulated in response to Nodal, specifically antagonizes the Nodal-signaling pathway by interacting with Nodal and/or with Cripto-1. Although aggressive melanoma cells express Nodal and the co-receptor Cripto-1, they do not express Lefty: As a consequence, Nodal is allowed to signal without control in melanoma cells. This is in contrast to hESCs which amply express all of the components of the Nodal-signaling pathway. HESC-derived Lefty may inhibit Nodal signaling in aggressive melanoma cells, thereby promoting their reprogramming toward a less aggressive, more differentiated phenotype.

signal without control in these melanoma cells. This is in contrast to hESCs which amply express all of the components of the Nodal signaling pathway, perhaps enabling them to differentiate in response to microenvironmental cues. Utilizing Dynabeads covalently coupled to anti-Lefty antibody, we have been able to successfully isolate Lefty from hESC-conditioned matrices (unpublished data). We propose that this hESC-derived Lefty is able to inhibit Nodal signaling in aggressive melanoma cells, consequently promoting their reversion toward a less aggressive, more differentiated phenotype.

It is also conceivable that embryonic microenvironments reprogram aggressive cancer cells via more conventional DNA-associated epigenetic alterations. For example, Jaenisch and colleagues elegantly demonstrated that nuclear transplantation of a RAS-inducible melanoma nucleus into an oocyte leads to the reprogramming of the melanoma genome, giving rise to ESCs with the capacity to differentiate into cell types such as melanocytes and fibroblasts. Because the melanoma nuclei became compatible with a broad developmental spectrum while predisposing mice to RAS-induced tumors later in life, this reprogram-

TABLE I. Summary of Nodal, Cripto, and Lefty Expression in Melanoma and Human Embryonic Stem Cell Lines

Cell type	Nodal	Cripto	Lefty	Reference
Aggressive melanoma cell line (C8161)	+	+*	–	Topczewska et al., 2006 and unpublished data
Human embryonic stem cell lines (H1, H9)	+	+	+	Besser, 2004 and unpublished data

+ Signifies that the gene is expressed.
+* Signifies expression in a subpopulation of cells.
– Signifies a lack of expression.

ming was likely attributable to epigenetic alterations [Hochedlinger et al., 2004]. When a nucleus is transferred into an oocyte it undergoes a dramatic hypomethylation that exposes its DNA promoters and enables transcription [Lotem and Sachs, 2006]. This hypomethylation confers the broad developmental spectrum observed when normal or neoplastic somatic nuclei are transplanted. As differentiation and cell fate specification ensue, there is a marked increase in DNA methylation leading to the downregulation of most genes and a consequential specialization in gene expression [Lotem and Sachs, 2006].

DNA methylation and histone modifications are the main epigenetic mechanisms used in eukaryotes to turn genes on or off in a cell type and tissue-specific manner [Fuks, 2005]. Of these, methylation of DNA cytosines that are followed by guanines (annotated as CpG dinucleotides) is the most investigated epigenetic modification in humans [Callinan and Feinberg, 2006]. Exemplifying its importance, DNA methylation is essential for normal embryonic development and is one of the main epigenetic changes that occur in cancer [Herman and Baylin, 2003; Bibikova et al., 2006]. Indeed, the cancer genome is characterized by the specific hypermethylation of promoter regions in tandem with a global hypomethylation [Das and Singal, 2004]. The genomic hypomethylation affects mainly intergenic and intronic regions of the DNA, in particular repeat sequences and transposable elements. Several studies have shown that hypomethylation of these repeats, especially satellite regions, are common in tumors [Costa et al., 2006] and can result in chromosomal instability and an increased mutation rate in some cases [Jones and Baylin, 2002]. In addition, by increasing the accessibility of DNA for transcriptional activators, hypomethylation can disturb imprinting and activate normally silent genes, such as those associated with other tissue types or those that promote tumor progression. For example, the CTA family member *MAGE-A1* is normally restricted to male germline cells but is overexpressed in melanoma due to promoter hypomethylation [De et al., 2004]. Hypermethylation of gene promoters that are important in normal cellular processes also characterizes the cancer genome [Costa et al., 2004]. This hypermethylation often increases tumor progression by blocking transcriptional activation and silencing tumor suppressor genes. For instance *Apaf-1*, a gene that mediates mitochondrial dependent apoptosis is silenced in malignant melanoma by

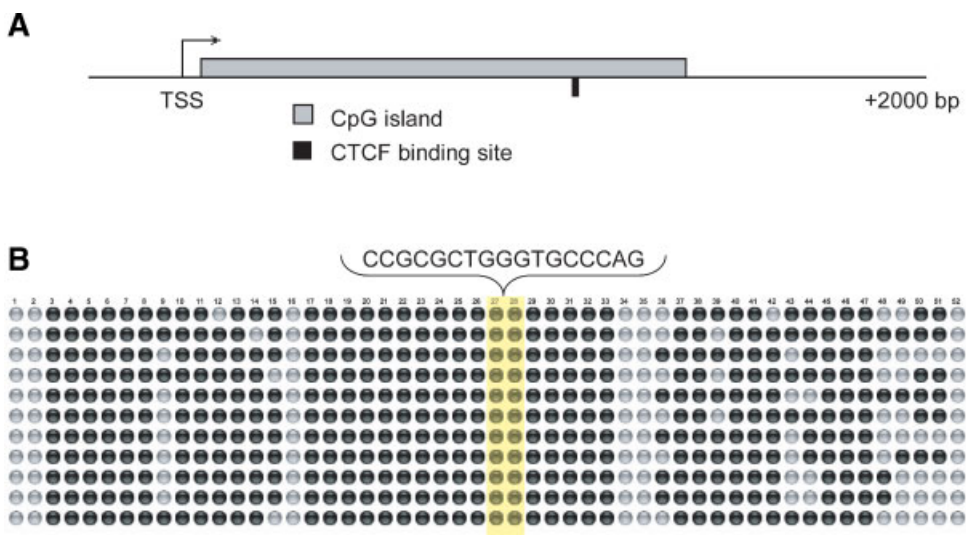

Fig. 2. A CpG island near the transcription start site (TSS) of the *Nodal* gene contains a putative CTCF binding site that is methylated in aggressive C8161 human cutaneous melanoma cells. The *Nodal* promoter was examined for the presence of CpG islands and CTCF binding sites. **A**: depicts a scaled map of the entire CpG island containing a putative CTCF binding site 55 bp downstream of the *Nodal* TSS. **B**: The methylation status of a portion of the Nodal CpG island containing the putative CTCF binding site (CCGCGCTGGGTGCCCAG; highlighted in yellow) in aggressive C8161 human cutaneous melanoma cells. Each circle represents a CpG dinucleotide in the CpG island: Black and gray circles symbolize methylated and unmethylated residues, respectively. Each row represents an individual clone or allele. The CTCF-binding region was methylated in 100% of the clones examined.

promoter methylation [Soengas et al., 2001]. Paradoxically, Feinberg and colleagues have illuminated the unique ability of methylation to activate genes, including the breast cancer stem cell marker *CD44* [Gius et al., 2004]. In this case, methylation may prevent the binding of the CTCF insulator protein, which has been shown to block the transcriptional activity of enhancer elements.

It is very possible that the mechanisms by which hESC microenvironments promote melanoma differentiation include modifications in DNA methylation. Given its importance in differentiation and melanoma tumorgenicity, as well as its normal silencing following embryogenesis, we decided to interrogate the possibility that Nodal expression is regulated in melanoma cells by changes in methylation. We first determined that there is a sizable CpG island (>1,300 base pairs) near the transcription start site (TSS) of the *Nodal* gene. Using microarray and bisufite-sequencing technologies, we determined that the second half of this CpG-rich region is heavily methylated in the aggressive melanoma cell line C8161 (Fig. 2). Given that C8161 cells express Nodal [Topczewska et al., 2006], this was an intriguing observation. Closer inspection and sequence alignment revealed that, in a manner similar to that previously described by Feinberg [Gius et al., 2004], the CpG island associated with Nodal contains a CTCF binding site. As depicted in Figure 2, this site is methylated in the aggressive melanoma cells. Hence, methylation may promote Nodal expression by preventing the binding of CTCF insulator proteins, and enabling the activity of the *Nodal* enhancer elements.

Interestingly, we also determined that exposure of aggressive melanoma cells to matrices

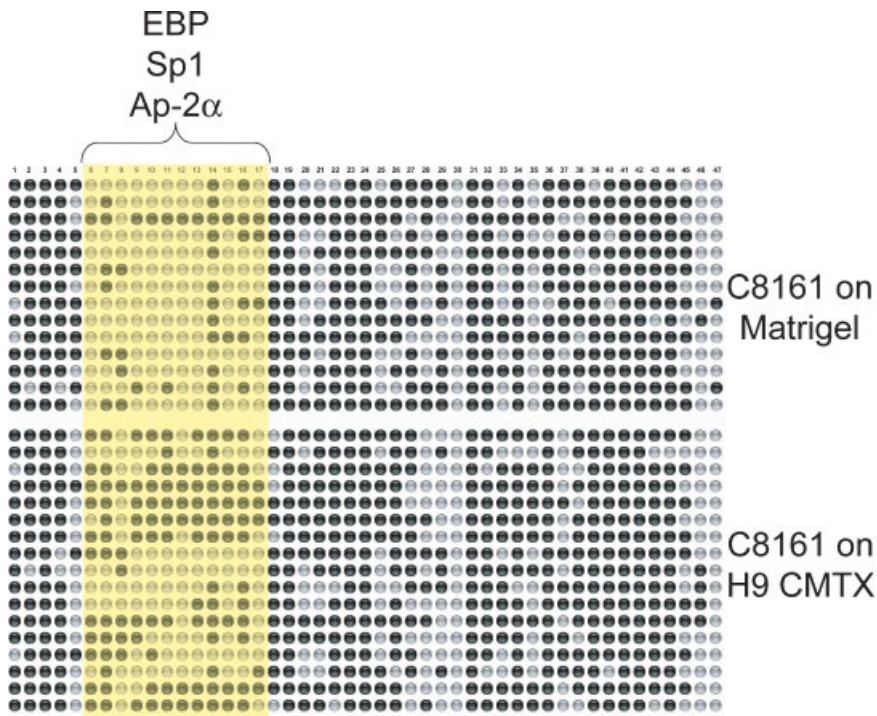

Fig. 3. Exposure of aggressive C8161 human melanoma cells to matrices conditioned by hESCs induces an increase in the methylation of specific cytosine residues (CpG residues 6–17) in the first half of the CpG island in the *Nodal* promoter. The graphs depict the methylation status of the first half of the *Nodal* CpG island in aggressive C8161 human cutaneous melanoma cells cultured either on Matrigel or on Matrigel conditioned by H9 hESCs. Each circle represents a CpG dinucleotide in the CpG island. Black and gray circles symbolize methylated and unmethylated residues, respectively. Each row represents an individual clone or allele. Although culture in the presence of a hESC microenvironment (H9 CMTX) globally increases methylation by only 6.8%, the area highlighted in yellow experienced a 32% increase in methylation when cells were cultured on H9 CMTX versus Matrigel alone. We have aligned this region with the DNA sequence and elucidated that the differentially methylated cytosines are associated with putative transcription factor binding sites. The highlighted area contains consensus sequences for EBP, Sp1, and AP-2α.

conditioned by hESCs resulted in a marked increase in site-specific methylation in the *Nodal* CpG island. Although hESC microenvironments did not drastically affect global methylation, and did not alter methylation at CTCF binding sites, Figure 3 illustrates that we observed specific areas, in the first half of the CpG island, where changes did occur. Sequence analyses determined that these areas contained putative consensus sequences for transcription factors including Sp1, Egr-1, and GATA-4. It is therefore plausible that hESC derived microenvironments can alter Nodal expression in melanoma cells by epigenetically methylating transcription factor binding sites. These modifications may canonically decrease the accessibility of the Nodal promoter for transcriptional activators, thereby decreasing Nodal expression commensurate with differentiating melanoma cells and abrogating their tumorgenicity. Collectively, our ongoing studies reveal that the epigenetic mechanisms by which embryonic microenvironments reprogram aggressive cancer cells is complex and multifaceted.

FUTURE DIRECTIONS AND SIGNIFICANCE

The microenvironment exerts control over the genome in both normal and cancer cells. Within this relationship lie the clues for new therapeutic strategies. Further investigation is warranted to elucidate the molecular basis underlying the epigenetic reprogramming of tumor cells, such as melanoma, by embryonic microenvironments. Future studies will follow up on the biological relevance of the newly discovered Nodal-signaling pathway in melanoma with respect to the control of cell fate determination and mechanisms underlying tumor cell plasticity. Based on our intriguing observations showing the reversion of metastatic melanoma cells to a melanocyte-like phenotype after exposure to embryonic microenvironments, further investigation will yield new insights into the molecular mechanisms involved in reprogramming multipotent tumor cells and possibly neutralizing the signals associated with their plastic phenotype.

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