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# REPROGRAMMING METASTATIC TUMOR CELLS WITH THE EMBRYONIC MICROENVIRONMENT

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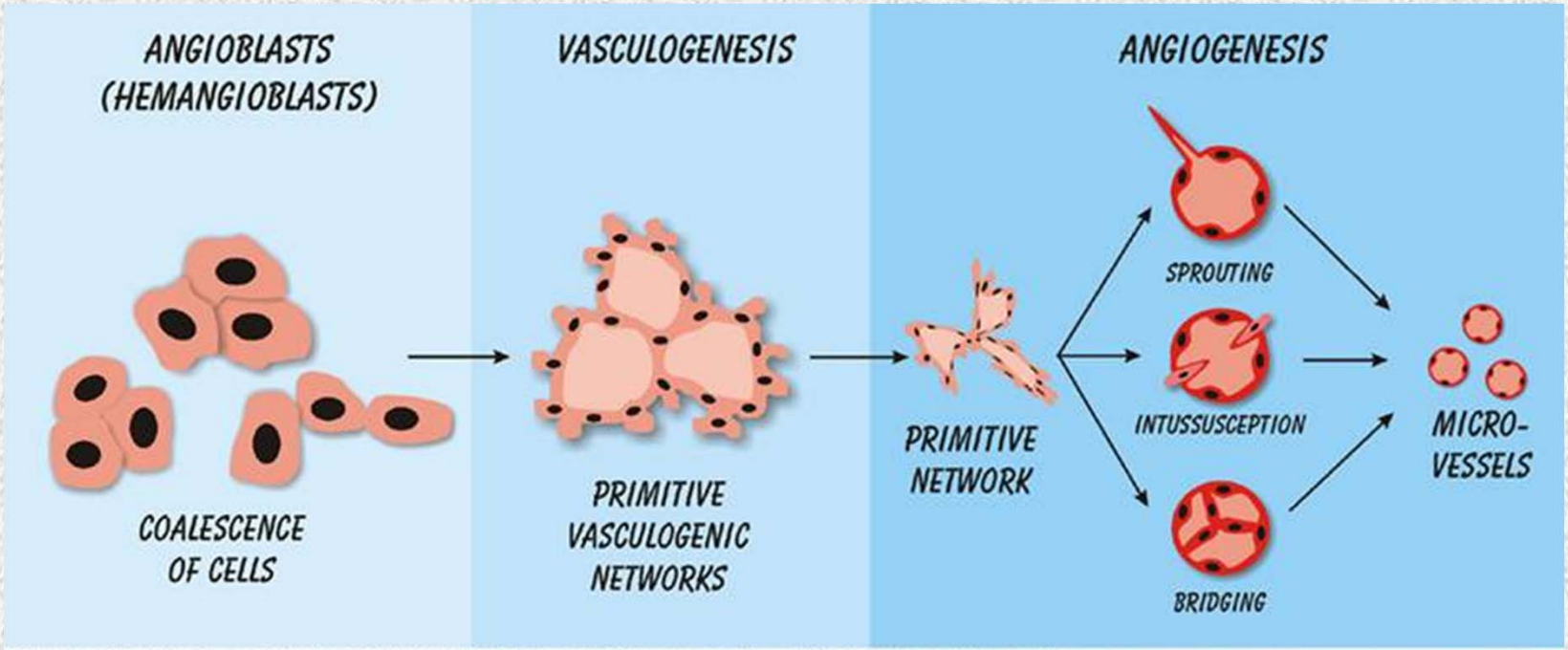
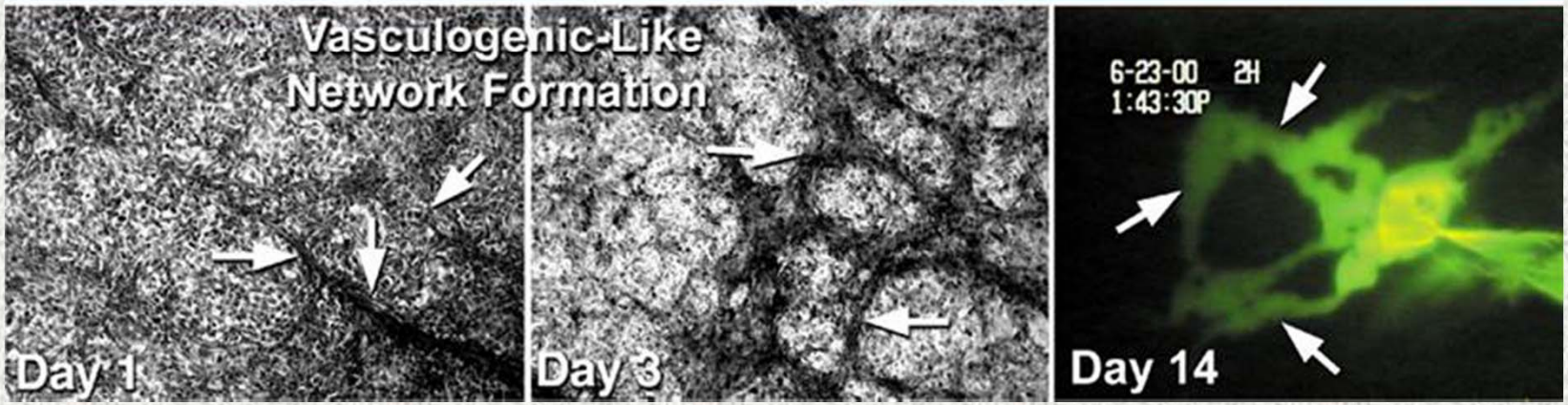
**Chicago, Illinois**

# Classifications of Melanoma

- Multiple categories of melanoma are distinguished in the current WHO classification of skin tumors.
- The classification has its basis in the histogenetic categories proposed by Wallace Clark and colleagues in the 1960's.
- However, recent molecular studies have revealed subclassifications of melanoma based on oncogenic mutations and differentiation status.
- In particular, the identification of plastic, multipotent stem cell subpopulations within melanoma tumors further confounds the classification and targeting of the most aggressive tumor cells.

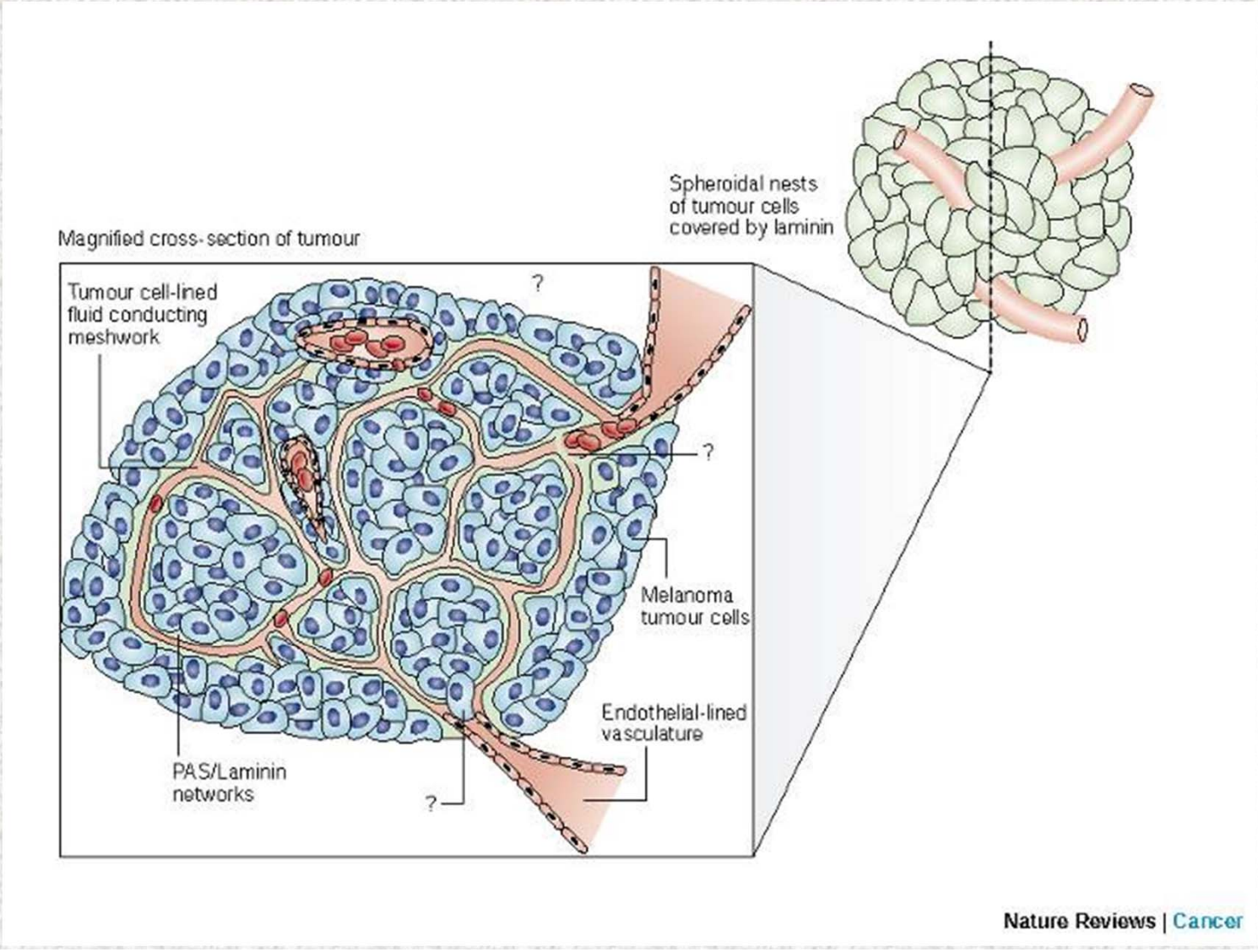
# VASCULOGENESIS AND ANGIOGENESIS




"Vasculogenic Mimicry" AJP 155:739-752, 1999

# Vasculogenic Mimicry Contributes to an Extravascular Perfusion Pathway:Collaboration with Angiogenesis



**Questions:**

- **What are the cellular and molecular determinants underlying tumor cell plasticity?**
- **What is the role of the microenvironment in contributing to this phenotype?**
- **What are the clinical implications of targeting melanoma tumor cell plasticity?**

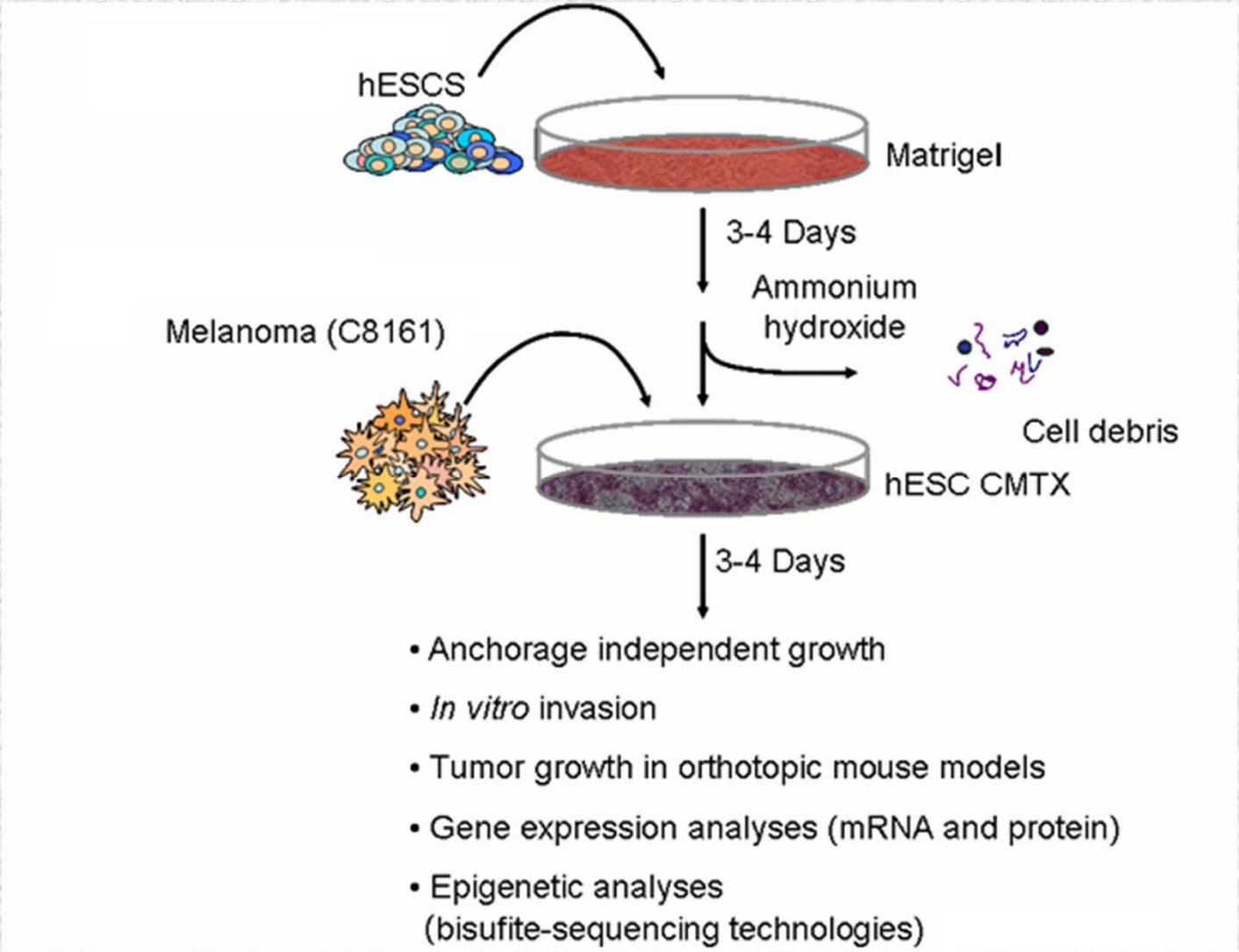
# Molecular Profile of Aggressive Melanoma Cells Expressing Multiple Cellular Phenotypes

| Gene | Function | Ratio |
|---|---|---|
| ESM-1 | Endothelial surface molecule | 44 |
| **VE-cadherin** | **Endothelial adhesion molecule** | **10.7** |
| **Laminin** | **Extracellular matrix** | **50** |
| **MMPs** | **Matrix metalloproteinases** | **2-36** |
| TIE-1 | Endothelial protein receptor | >100 |
| **EphA2 (Eck)** | **Epithelial cell kinase** | **77** |
| **Keratins 7, 8, 18** | **Epithelial Intermediate Filaments** | **20-80** |
| **MART-1/Melan A** | **Melanocyte antigen** | **5.1↓** |
| LSP1 | Lymphocyte specific protein | 4.8 |
| HCLS1 | Hematopoietic-lineage protein | 19 |
| KIT | Stem cell factor receptor | 2.5 |
| **Nodal** | **Embryonic stem cell marker** | **20** |
| **Notch** | **Stem cell marker** | **5.3** |
| VEGF-C | Ligand for Flt-4 | 30 |
| Fibronectin | Extracellular matrix | 27 |

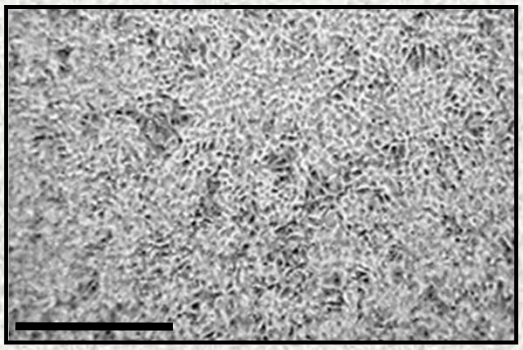
**Based on the multipotent potential of aggressive melanoma cells, could exposure to embryonic microenvironment(s) reverse the metastatic phenotype?**

- hESC (Stem Cells 24:501-505, 2006)
- Zebrafish embryo (Nature Medicine 12:925-932, 2006)
- Chick embryo (PNAS 103:3752-3757, 2006)
- Nature Reviews Cancer 7:246-255, 2007

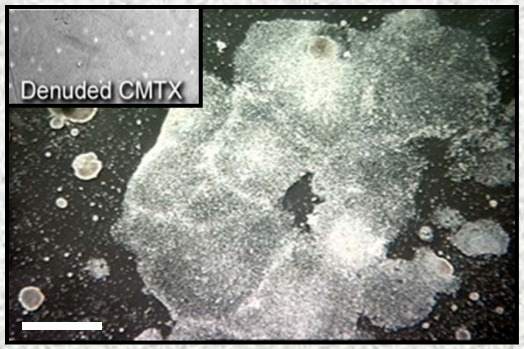
# Development of a three-dimensional model to study the epigenetic effects induced by the microenvironment of human embryonic stem cells



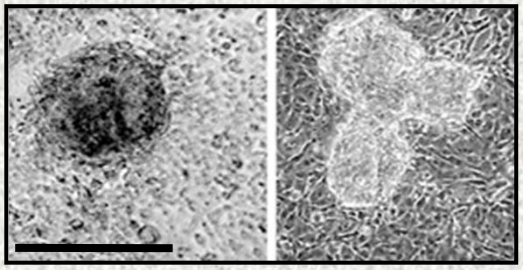
# The microenvironment of human embryonic stem cells induces melanoma spheroid formation



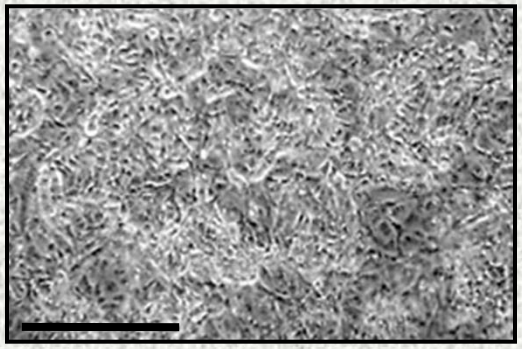
*C8161 Cells on Matrigel*




*hESCs on Matrigel*



*C8161 Cells on Matrigel Conditioned by hESCs*

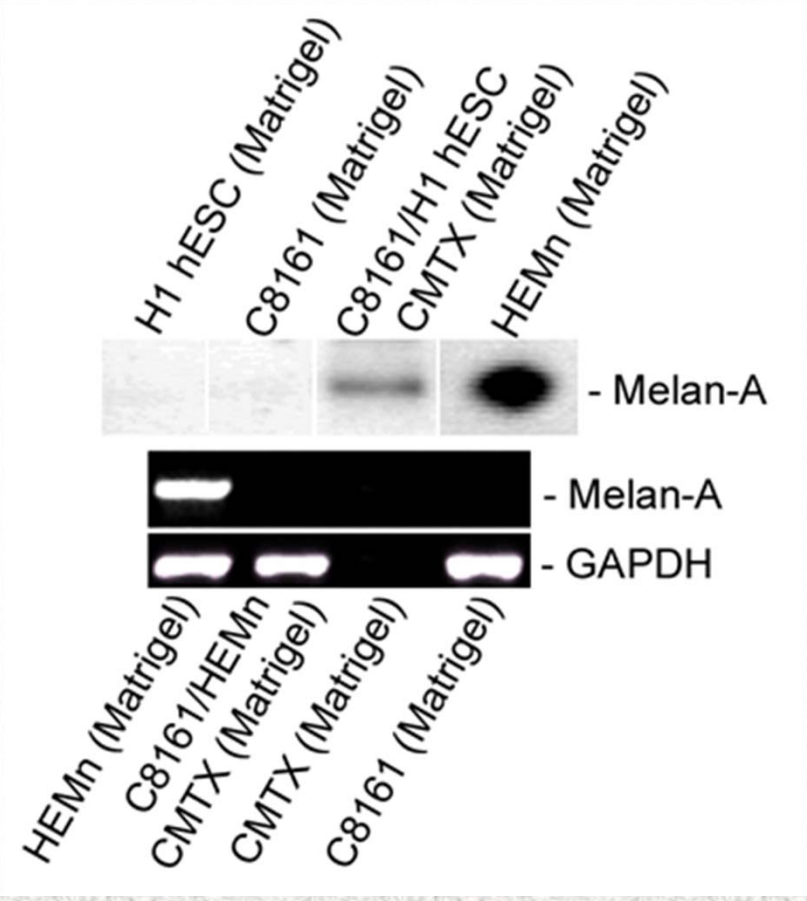


*C8161 Cells on Matrigel Cultured with Medium Conditioned by hESCs*

**The microenvironment of hESCs induces melanoma spheroid formation.** Phase contrast microscopy showing the confluent growth of C8161 amelanotic, human metastatic cutaneous melanoma cells on 3-D Matrigel matrix, compared with the formation of colonies by H9 hESCs on 3-D Matrigel matrix; following removal of the hESCs from their 3-D matrix (leaving a denuded preconditioned matrix, CMTX, shown in inset), the C8161 tumor cells seeded onto the hESC preconditioned matrices now form spheroids similar to hESC colonies. (Bar equals 500 μm). In contrast, C8161 cells exposed to medium conditioned by hESCs cells are unable to form spheroids.

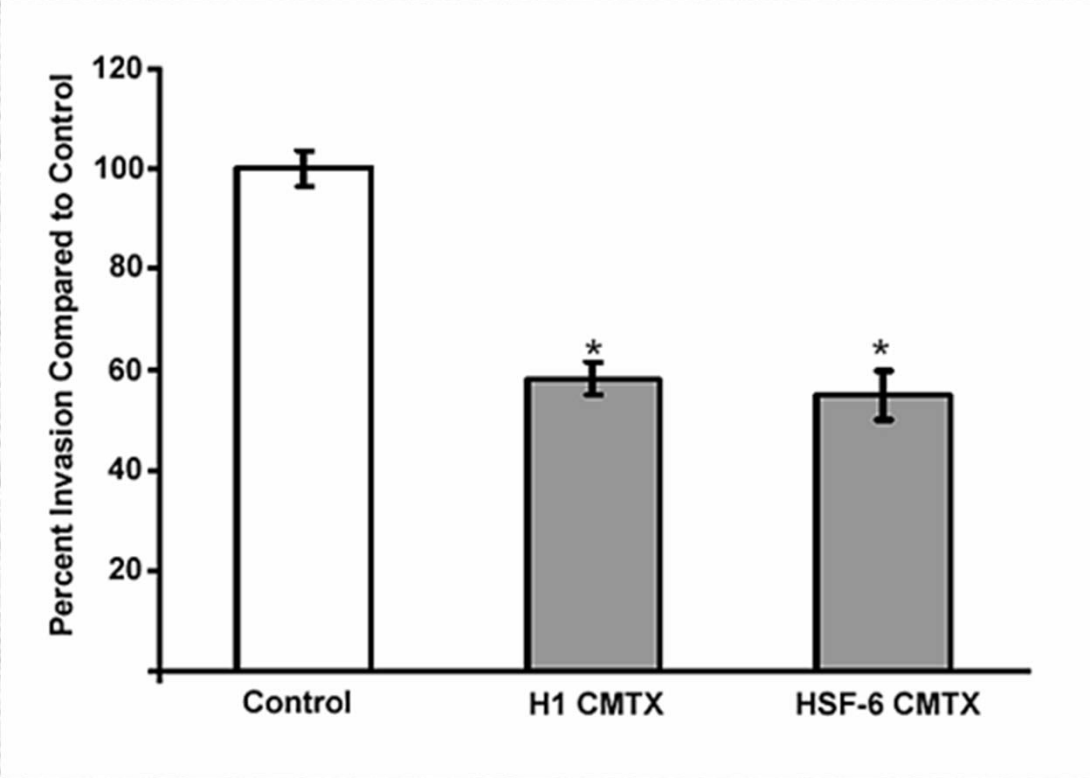
*Federal Registry hESCs

# 3D matrices preconditioned by human embryonic stem cells promote epigenetic changes in aggressive amelanotic melanoma cells



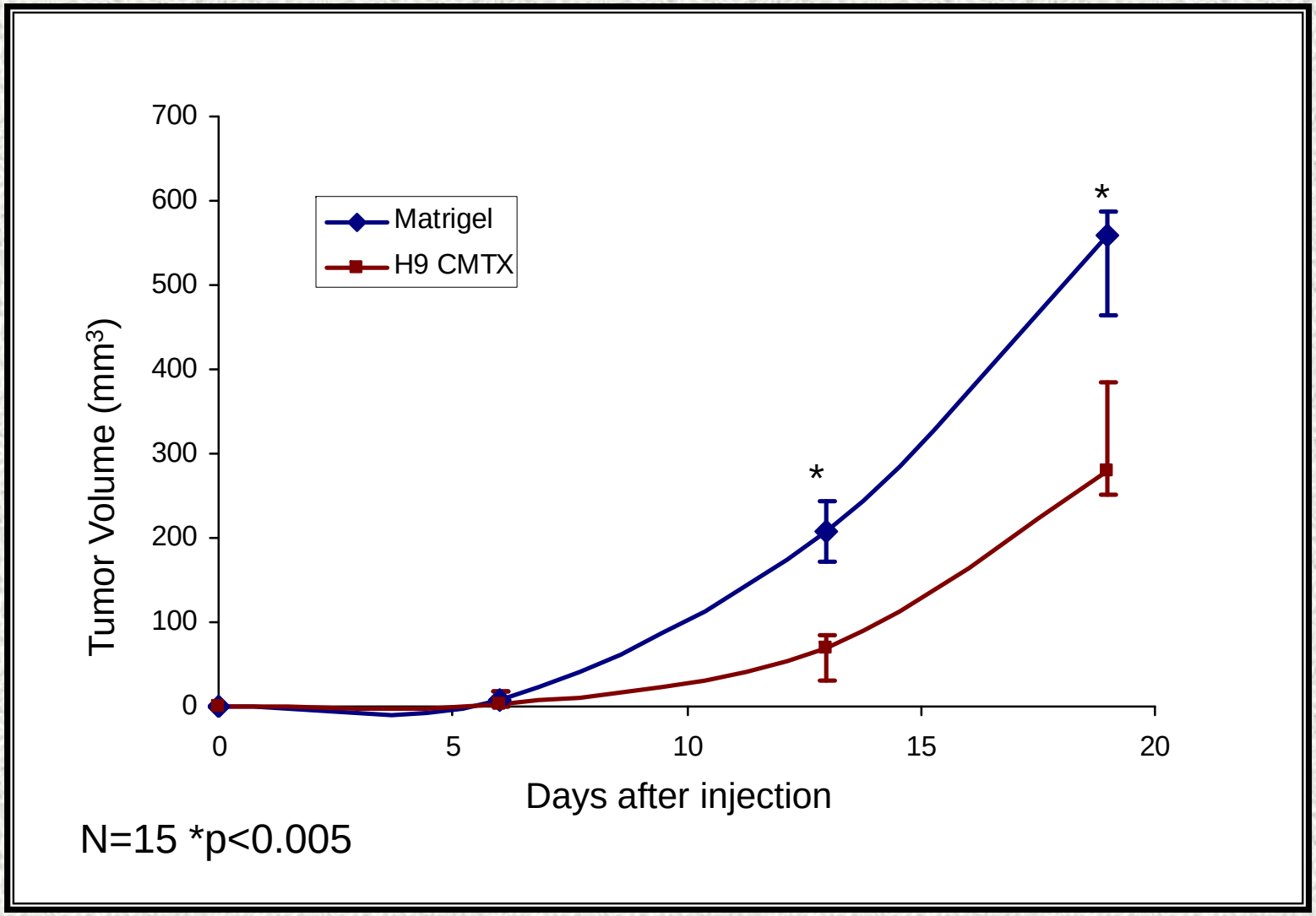

**Western blot analysis of whole cell lysates (with an equal amount of protein loaded per sample), for a melanocyte marker, Melan-A, shows its absence in H1 hESCs on Matrigel and C8161 tumor cells on Matrigel; and the induction of Melan-A in C8161 cells exposed to the H1 hESCs preconditioned matrix, CMTX (Matrigel), compared with Melan-A in control human epidermal melanocytes (HEMn) on Matrigel (upper panel). Semi-quantitative RT-PCR analysis of Melan-A gene expression in HEMn cultured on Matrigel compared to C8161 cells exposed to a HEMN preconditioned matrix, CMTX (Matrigel), compared with C8161 cells on Matrigel. The CMTX lane serves as a control demonstrating the complete removal of the HEMn cells from the preconditioned matrix prior to seeding the C8161 melanoma cells. GAPDH was used as a loading control for RNA (lower panel).**

# The microenvironment of human embryonic stem cells decreases metastatic melanoma cell invasion



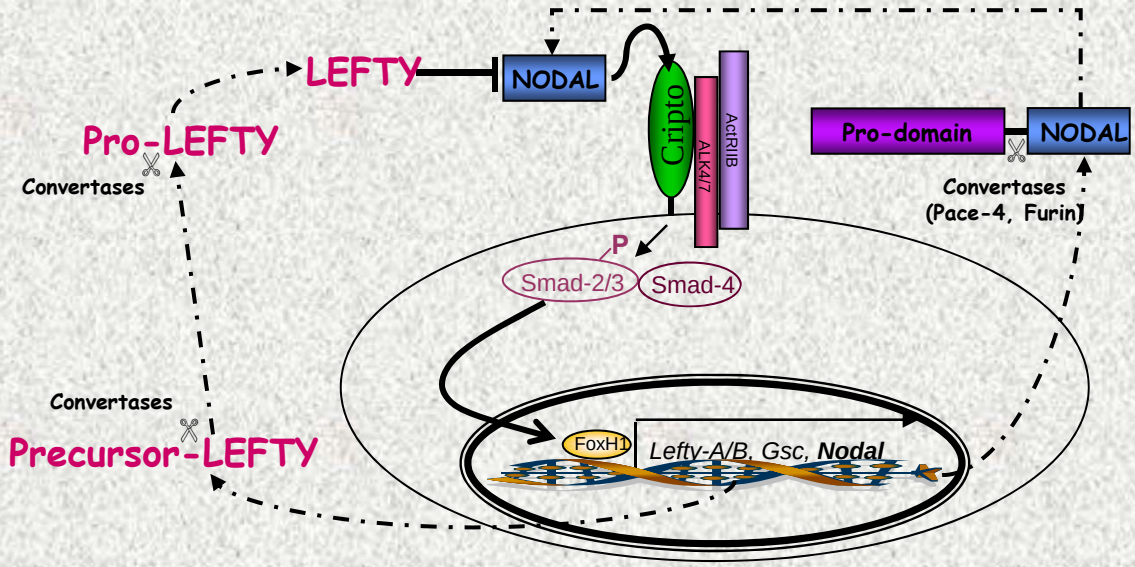

**Invasion of C8161 cells following culture on unconditioned Matrigel (Control) or Matrigel preconditioned by either H1 or HSF-6 hESCs was calculated as a percentage of cells able to invade through a defined matrix (collagen IV, laminin, and gelatin)-coated membrane during a 24 hour period using the MICS assay. Bars represent the mean, normalized, invasion indices ± standard deviations. The values indicated by an asterisk (*) are significantly different from the invasion index of control cells.**

# The microenvironment of human embryonic stem cells decreases tumor formation by metastatic melanoma cells

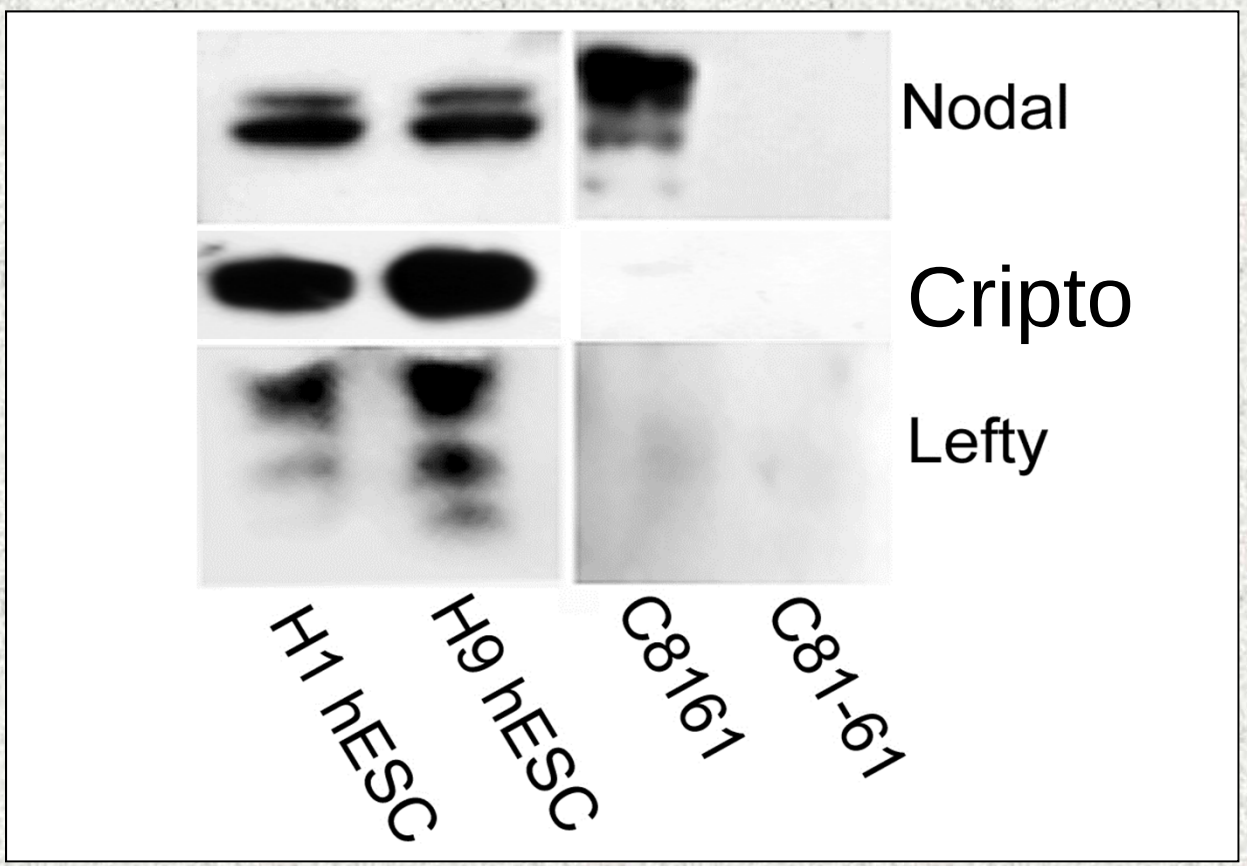


# What factor(s) might be responsible for the reversion of the metastatic phenotype?

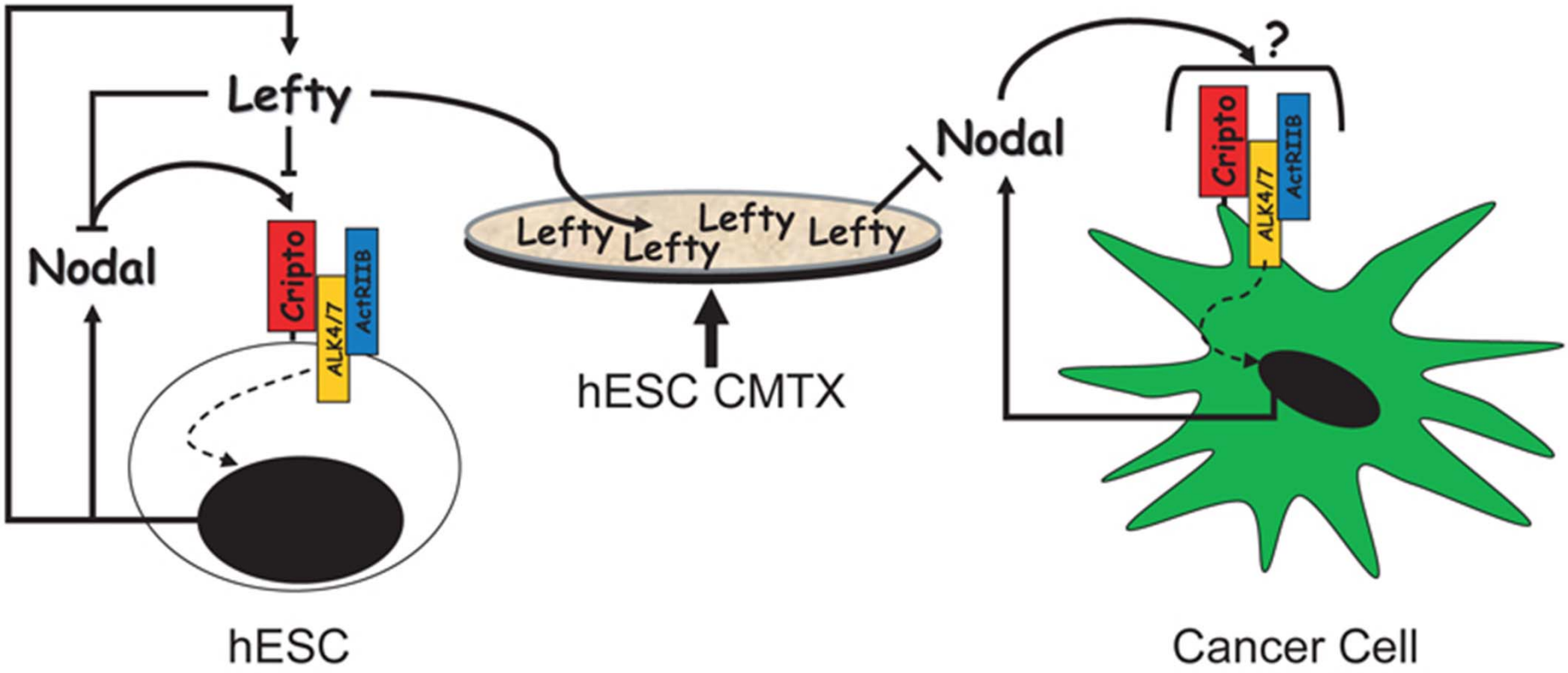
- Microarray analysis revealed that Nodal, a stem cell marker, is over-expressed by 20-fold in aggressive melanoma cells compared with poorly aggressive melanoma.
- Nodal is an embryonic morphogen belonging to the TGFβ superfamily and maintains hESC pluripotency.
- In humans, Nodal expression is largely restricted to embryonic tissues including the trophoblast, hESCs and the developing mammary gland -- but is lost in normal adult tissues.
- Lefty A (2) and Lefty B (1) are extracellular Nodal inhibitors, also members of the TGFβ superfamily -- critical in cell-fate differentiation events.



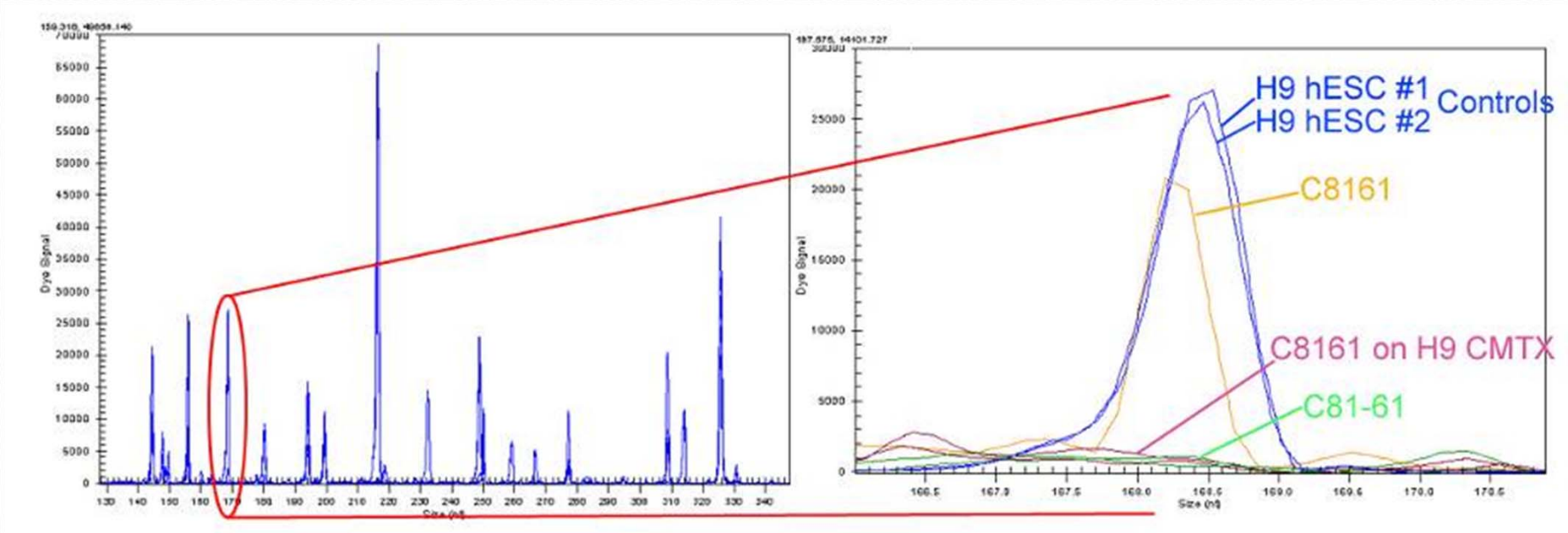
**Nodal protein is expressed by H1 and H9 hESCs as well as the metastatic melanoma cell line C8161, while Lefty (Nodal's inhibitor) and Cripto (Nodal's co-receptor) proteins are only expressed by the hESCs**



# Hypothetical Model: Lefty secreted by hESCs into the CMTX reprograms metastatic melanoma cells by inhibiting Nodal signaling



# The plasticity gene Nodal is knocked-down by 87% in metastatic melanoma cells exposed to the hESC (H9) CMTX (multiplex PCR analysis)

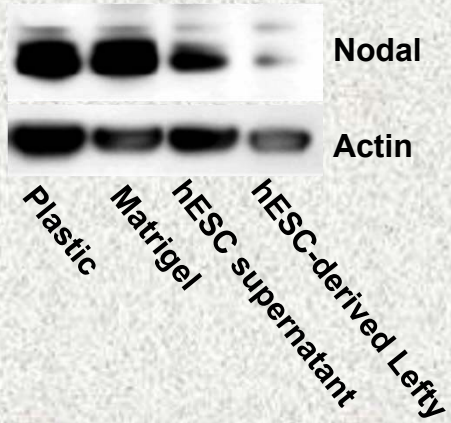



**C8161 on a control matrix**

**C8161 on the H9 CMTX**

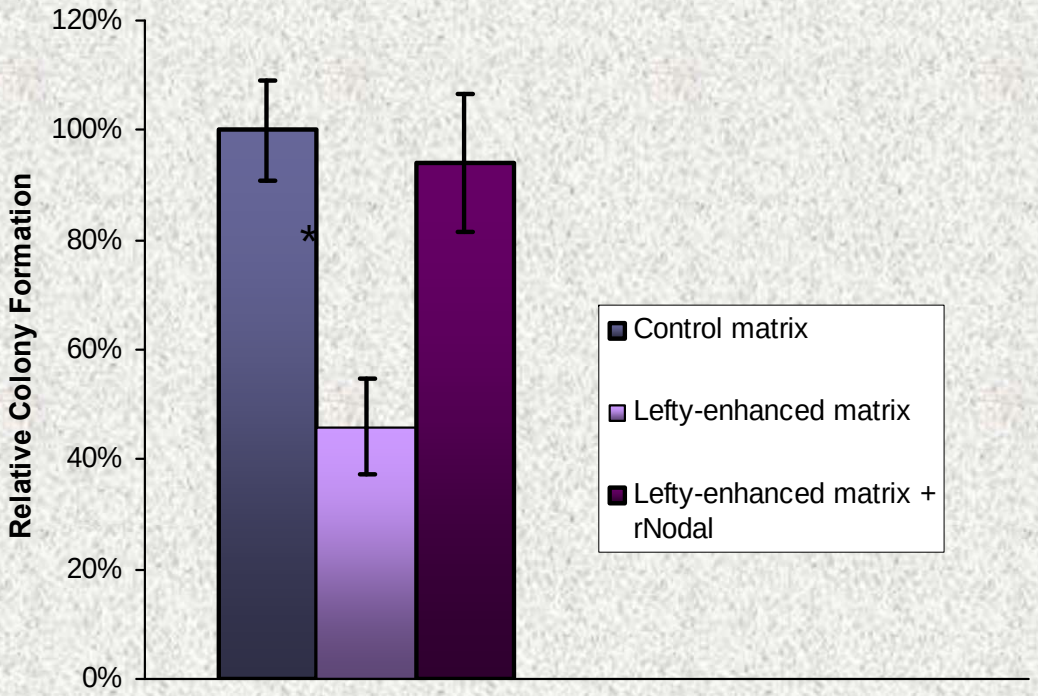
# Nodal protein expression is diminished and clonogenicity reduced in metastatic melanoma cells exposed to hESC-derived Lefty

**Western Blot Analysis of Nodal Protein in C8161 Cells**

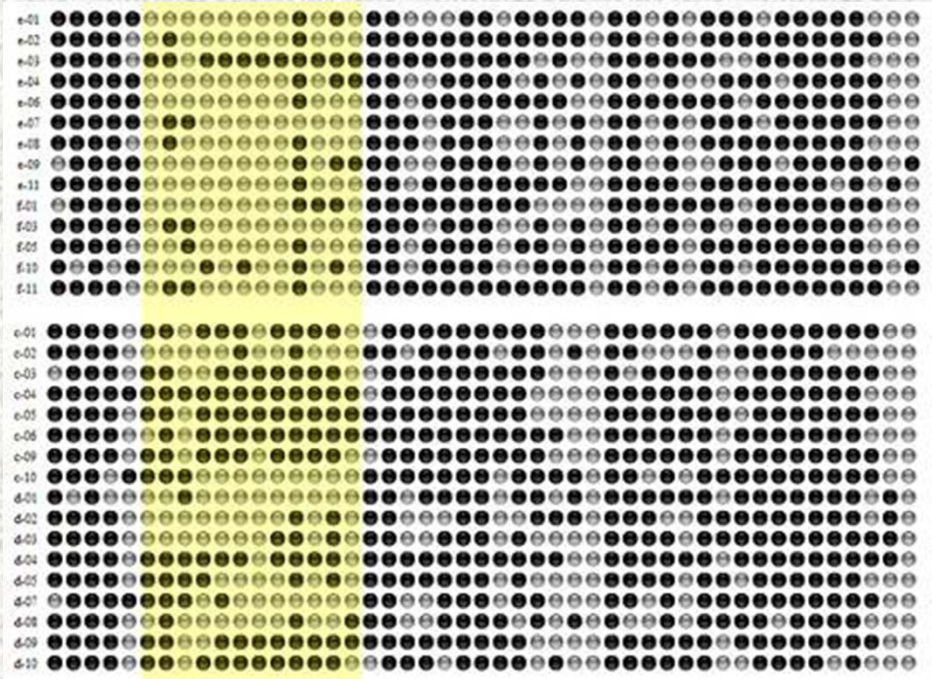



C8161 cells were cultured for 3 days on plastic, Matrigel, or Matrigel supplemented with hESC supernatant or with Lefty protein purified from hESCs. Western blot analyses were conducted with lysates of C8161 including their underlying matrix.

**Anchorage-Independent Growth (C8161 cells)**



# Possible mechanisms underlying the epigenetic reprogramming of plastic, metastatic melanoma cells



**C8161 on Matrigel (Control)**

**C8161 on H9 CMTX**

**↑32% methylation of Nodal**
**Nodal maintains the pluripotency of hESC; recently discovered to contribute to melanoma plasticity**

**Sequencing based methylation analyses.** Each circle in the left panel represents a CpG dinucleotide in the CpG island of Nodal: Black and grey circles symbolize methylated and unmethylated residues respectively. This sequence-based methodology will allow us to focus in on each individual CpG dinucleotide in order to observe subtle differences. For example, although culture of the C8161 cells in the presence of a hESC microenvironment (H9 CMTX) globally increases methylation by only 6.8%, the region highlighted in yellow has a 32% increase in methylation when cells are cultured on H9 CMTX versus Matrigel alone. We will align such regions with the DNA sequence in order to elucidate whether these differentially methylated cytosines are associated with elements including transcription factor binding sites.

**Based on the multipotent potential of aggressive melanoma cells and their ability to express the embryonic morphogen Nodal, could these tumor cells communicate with embryonic microenvironment(s) *in vivo*?**

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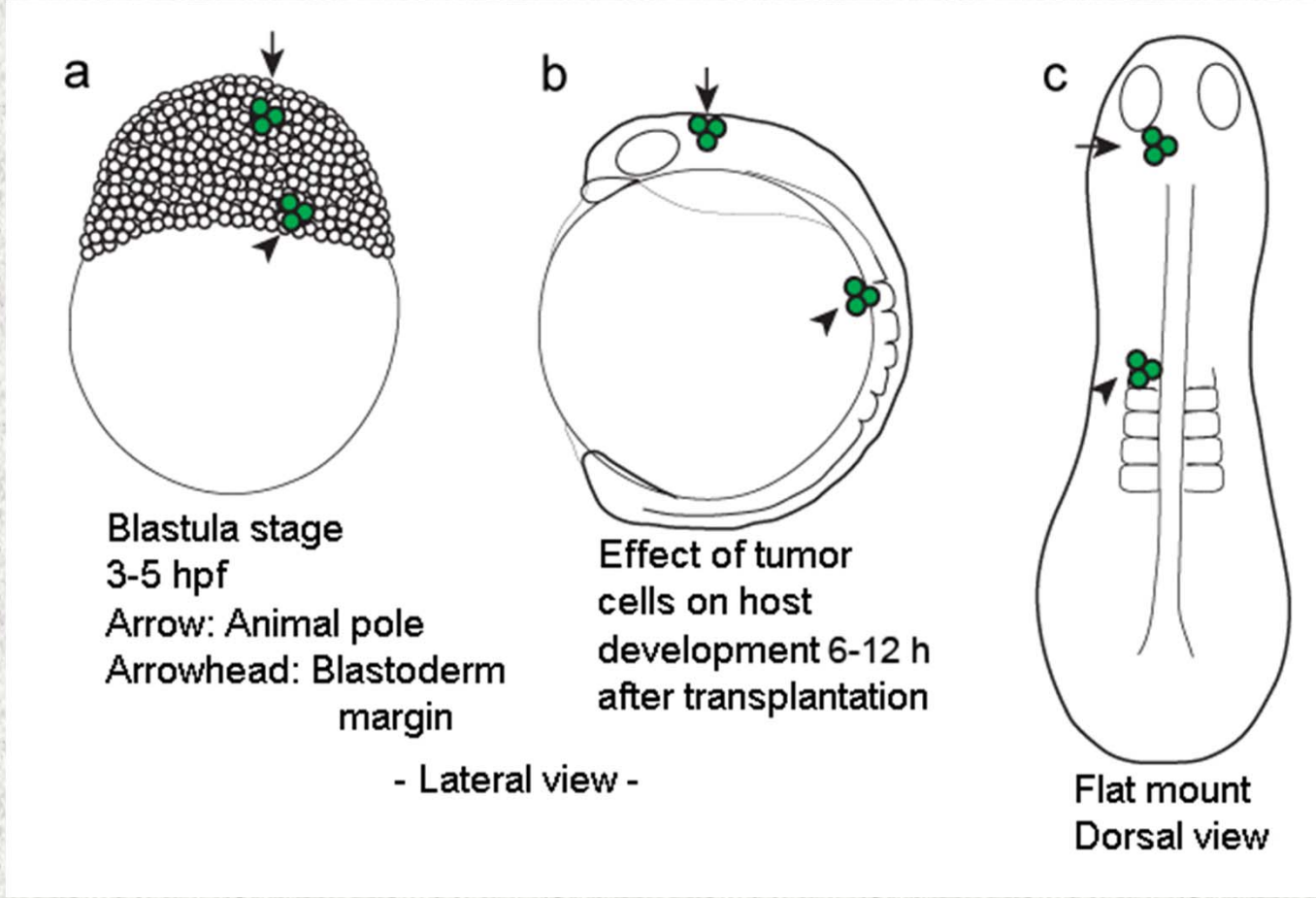
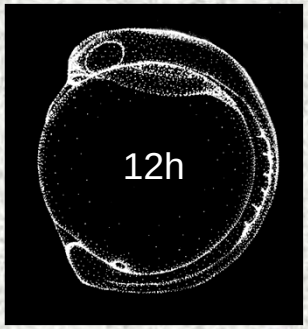
- ✓ **hESC (Stem Cells 24:501-505, 2006)**
- ⊙ **Zebrafish embryo (Nature Medicine 12:925-932, 2006)**
- **Chick embryo (PNAS 103:3752-3757, 2006)**
- **Nature Reviews Cancer (7:246-255, 2007)**

# Transplantation of melanoma cells into zebrafish embryos: Can they communicate *via* Nodal signaling?

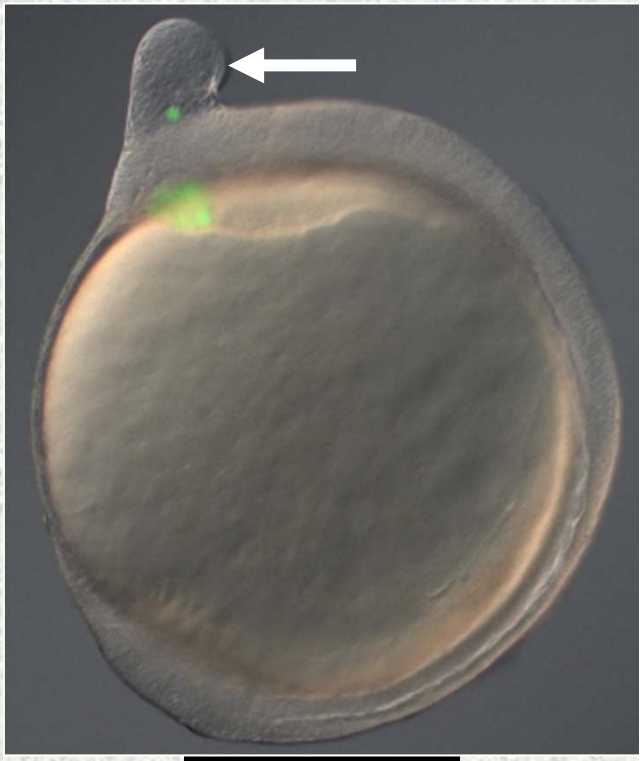


Embryos injected (animal pole) with aggressive melanoma cells form ectopic outgrowth(s) on the head



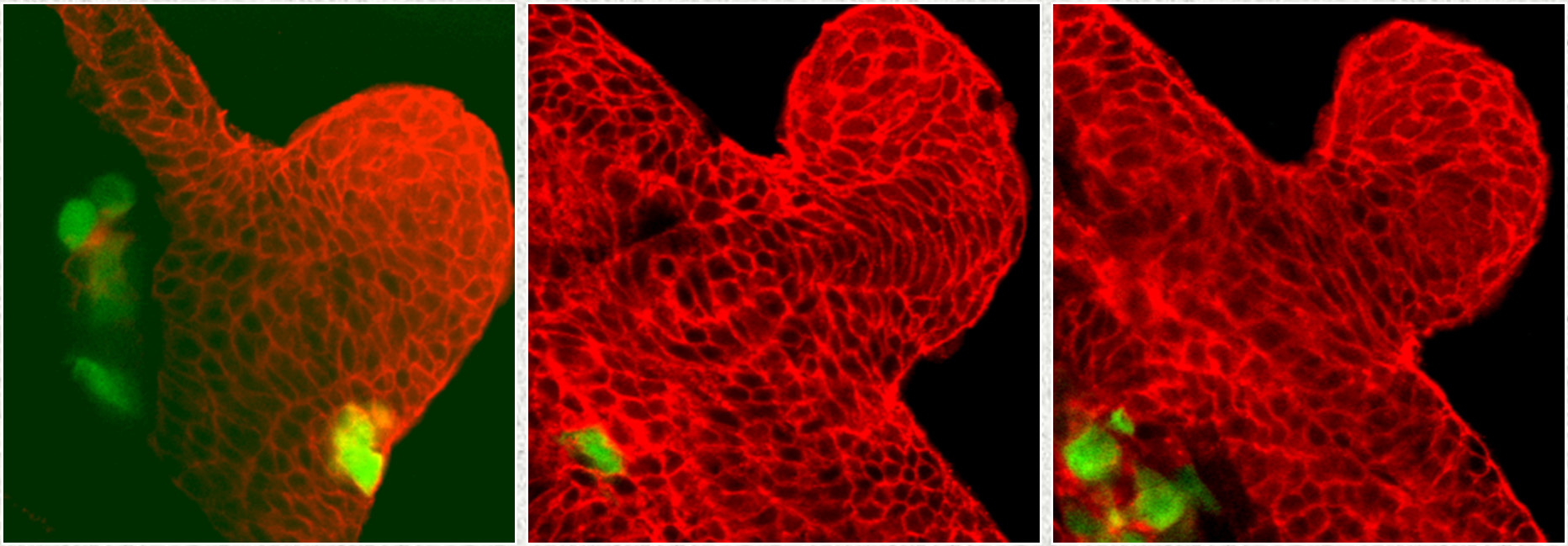

C81-61-GFP

*Vehicle*

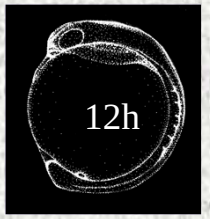


C8161-GFP

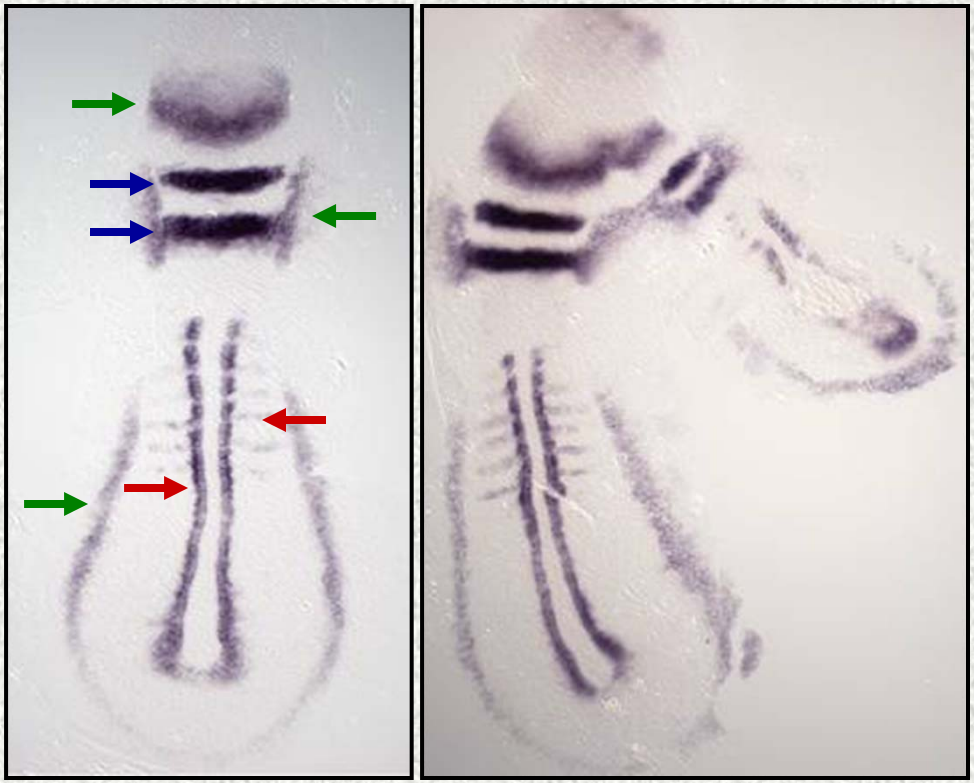
# Aggressive Melanoma Cells Direct Zebrafish Cells to Form Ectopic Outgrowth(s)

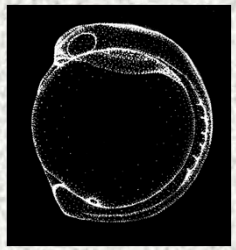


Whole mount immunohistochemistry with antibody against **β-catenin**
after transplantation of **C8161-GFP cells** at animal pole



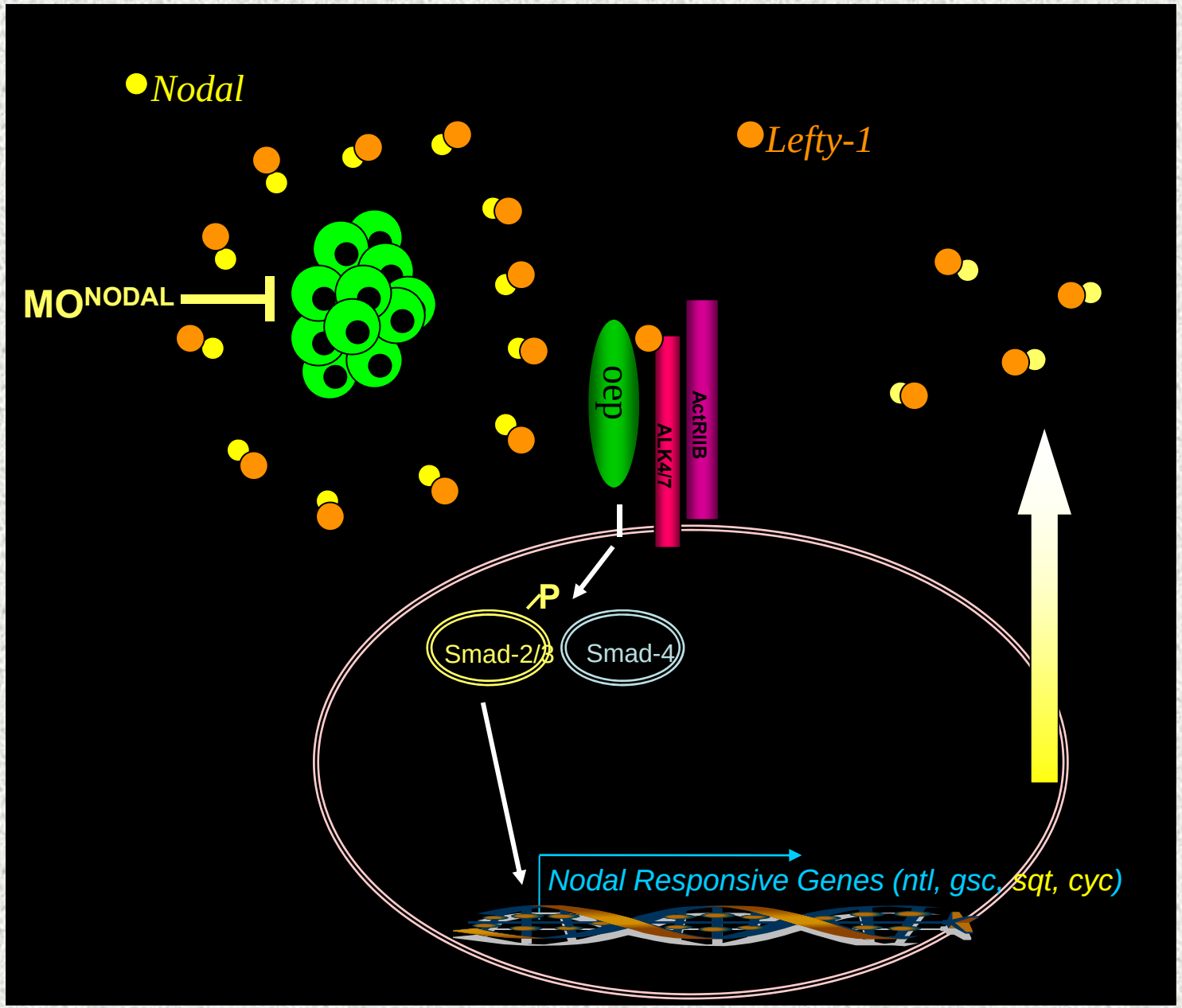
# Aggressive Melanoma Cells Induce an Ectopic Body Axis (Blastoderm Margin)





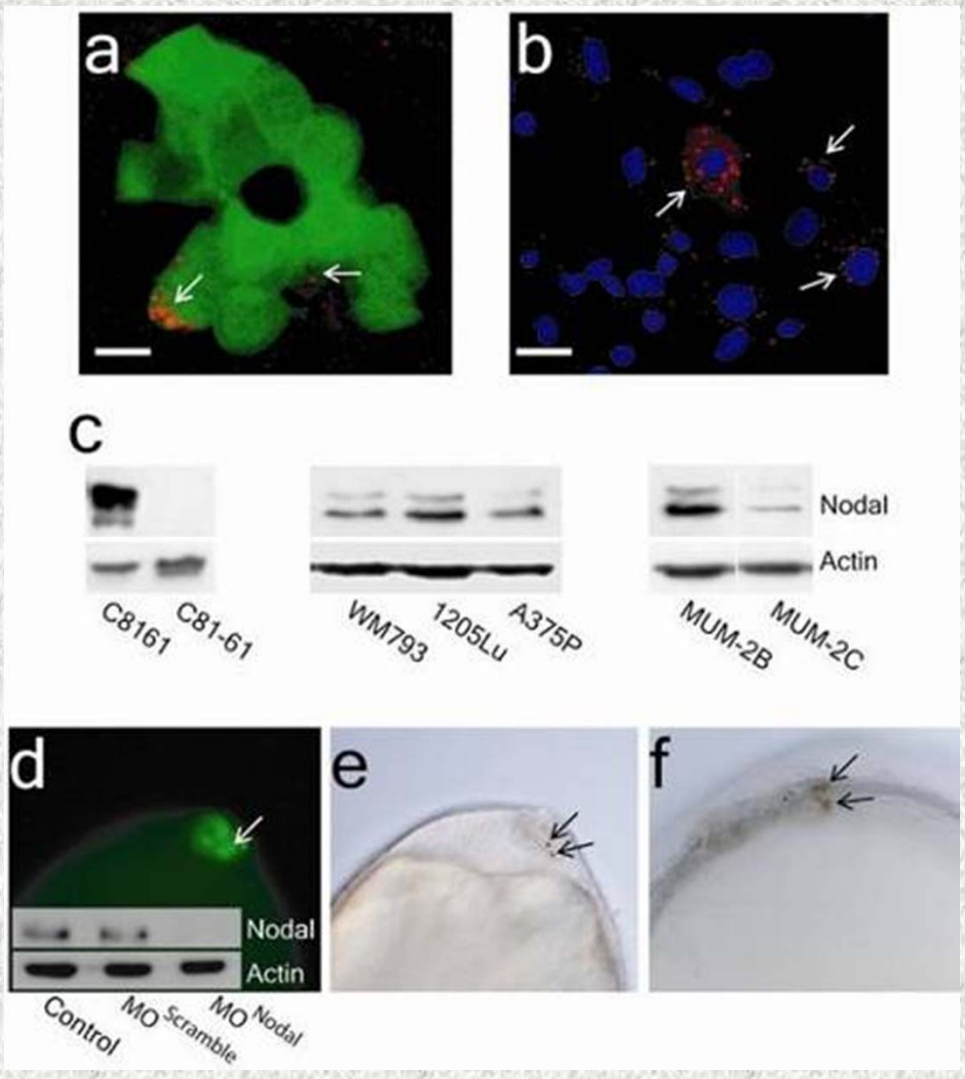
12h

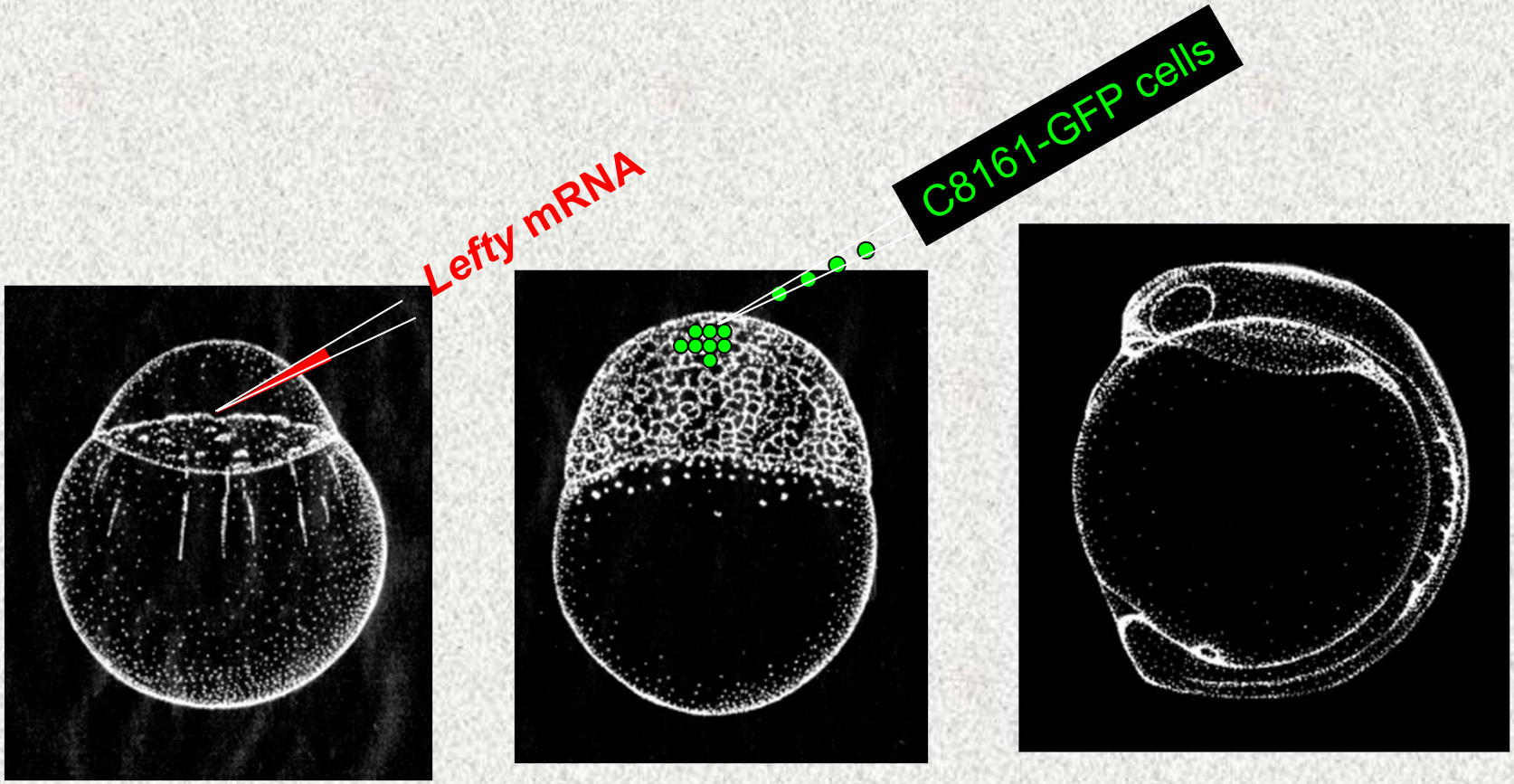
*pax2b*/*krox20*/*myoD*



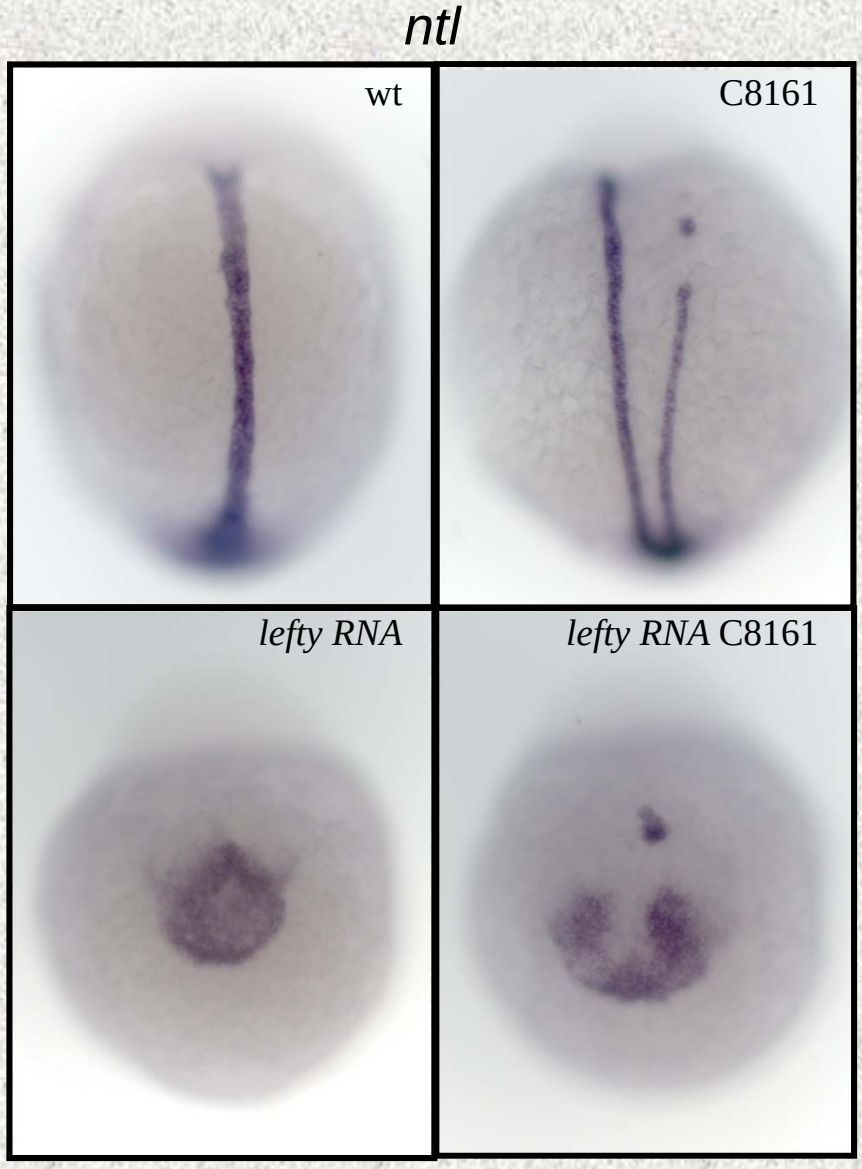

Oep: One-eyed pinhead; zf homolog of human Cripto

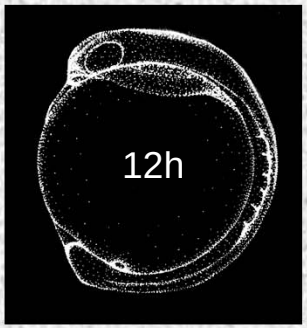
# Aggressive melanoma expresses Nodal: $MO^{NODAL}$ inhibits the induction of ectopic outgrowths in zebrafish



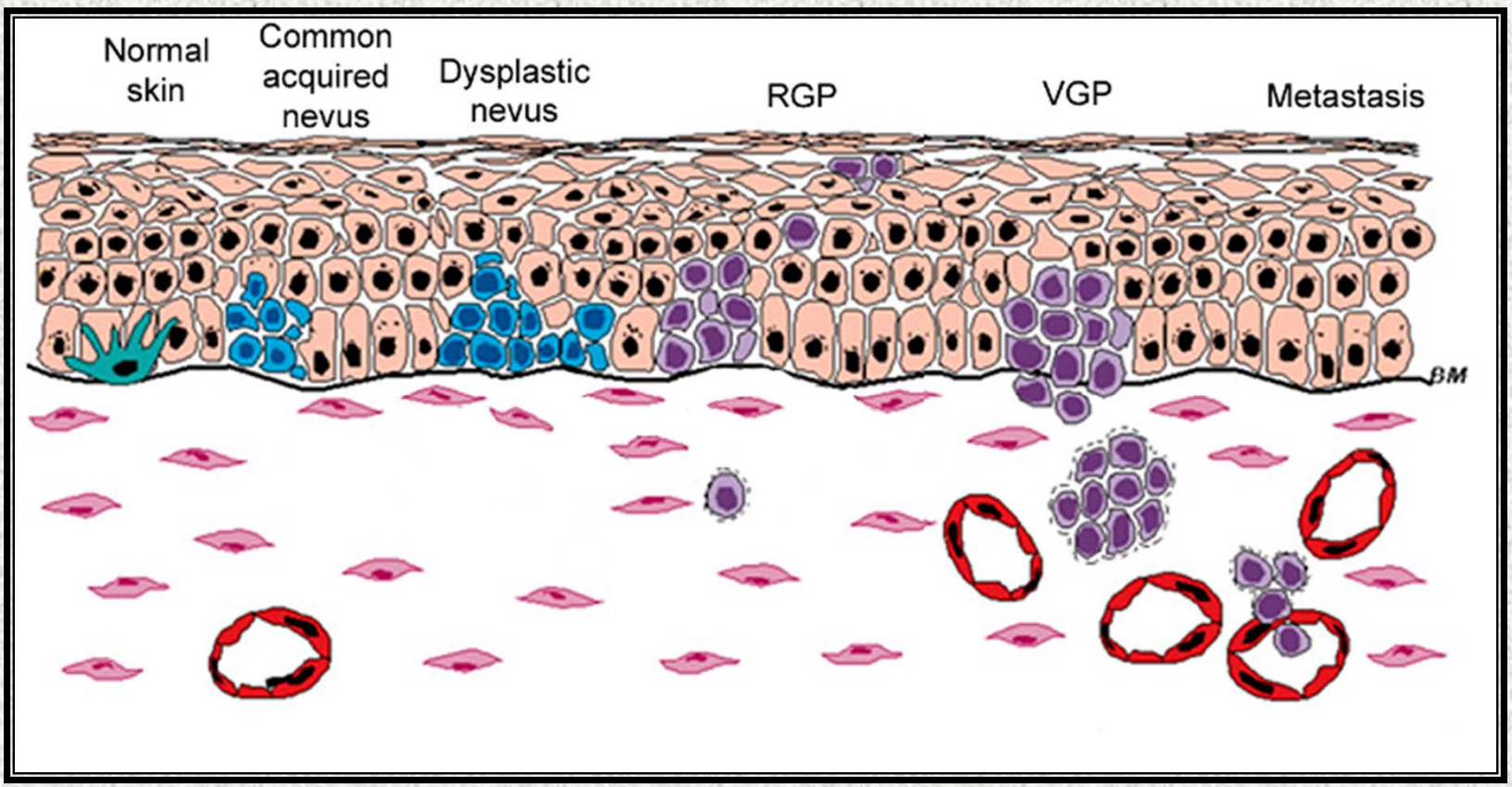

Lefty mRNA
C8161-GFP cells
0.2h
3.5h
12h

# Embryos Overexpressing *Lefty* Do Not Form Secondary Axes



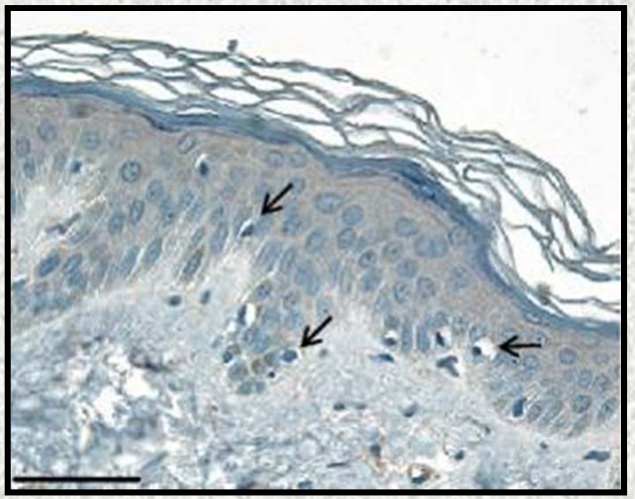
# Cutaneous Melanoma: Nodal as a New Biomarker?

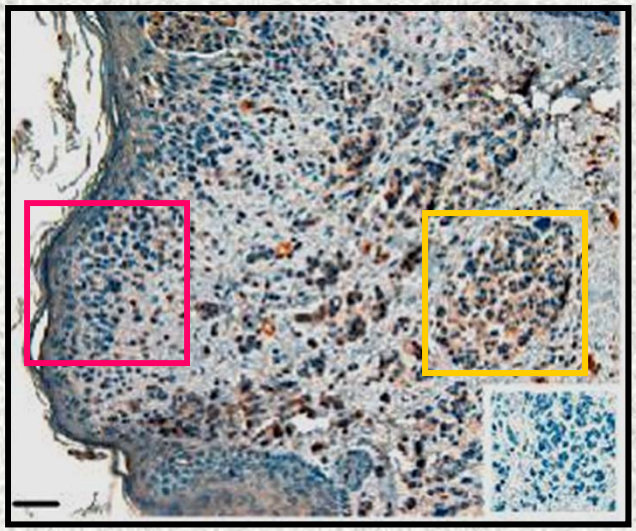



http://www.wistar.upenn.edu/herlyn/

# Nodal Protein Expression Correlates with Melanoma Progression

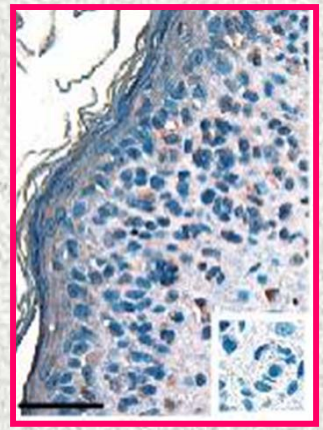
*Normal Skin*

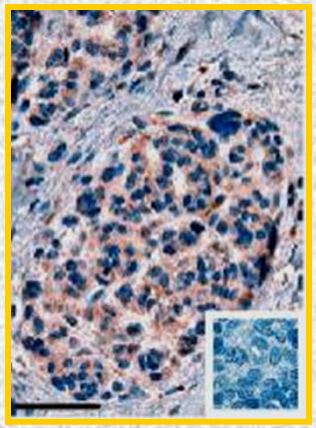




*Primary Melanoma*

*Radial Growth Phase*

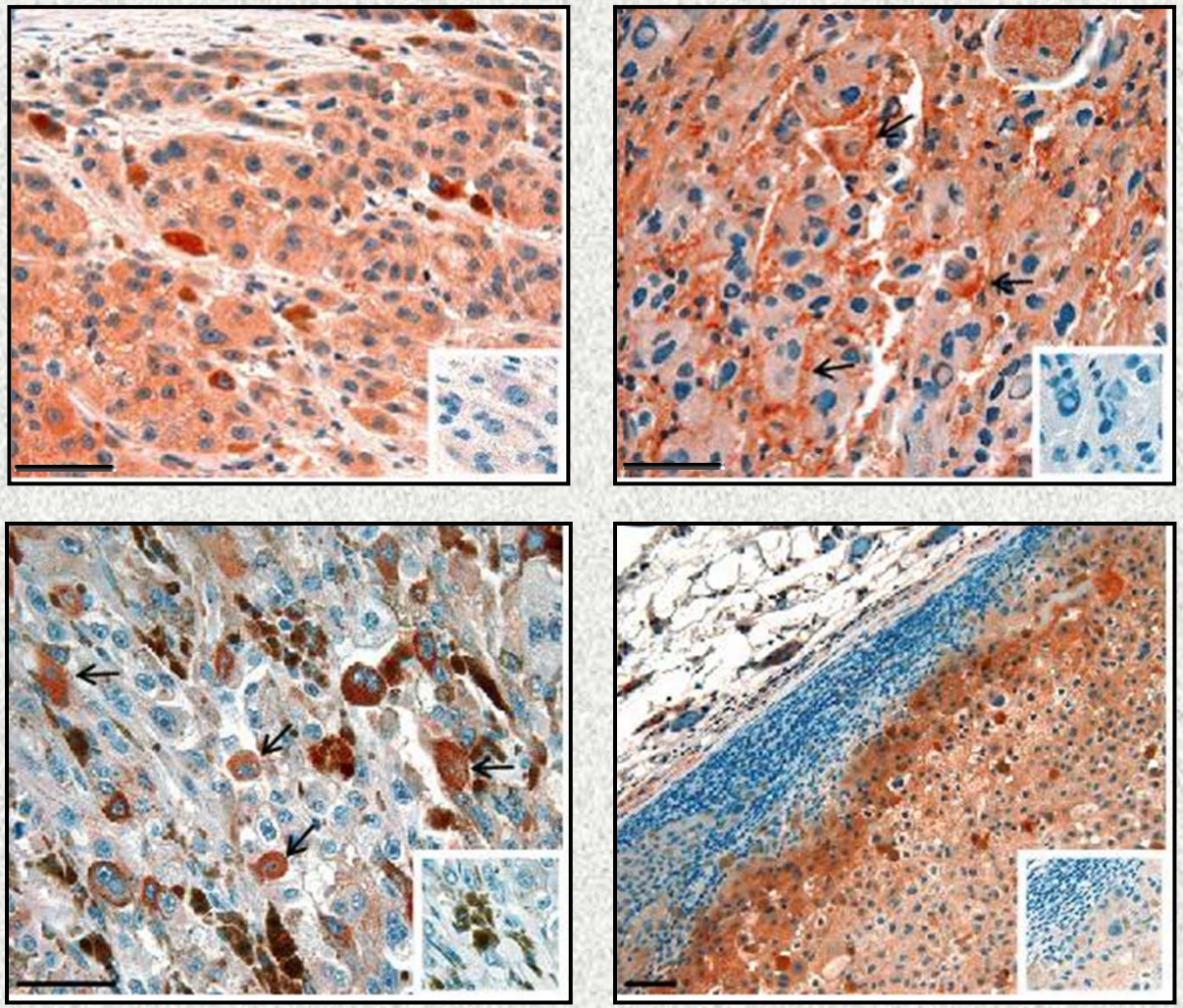




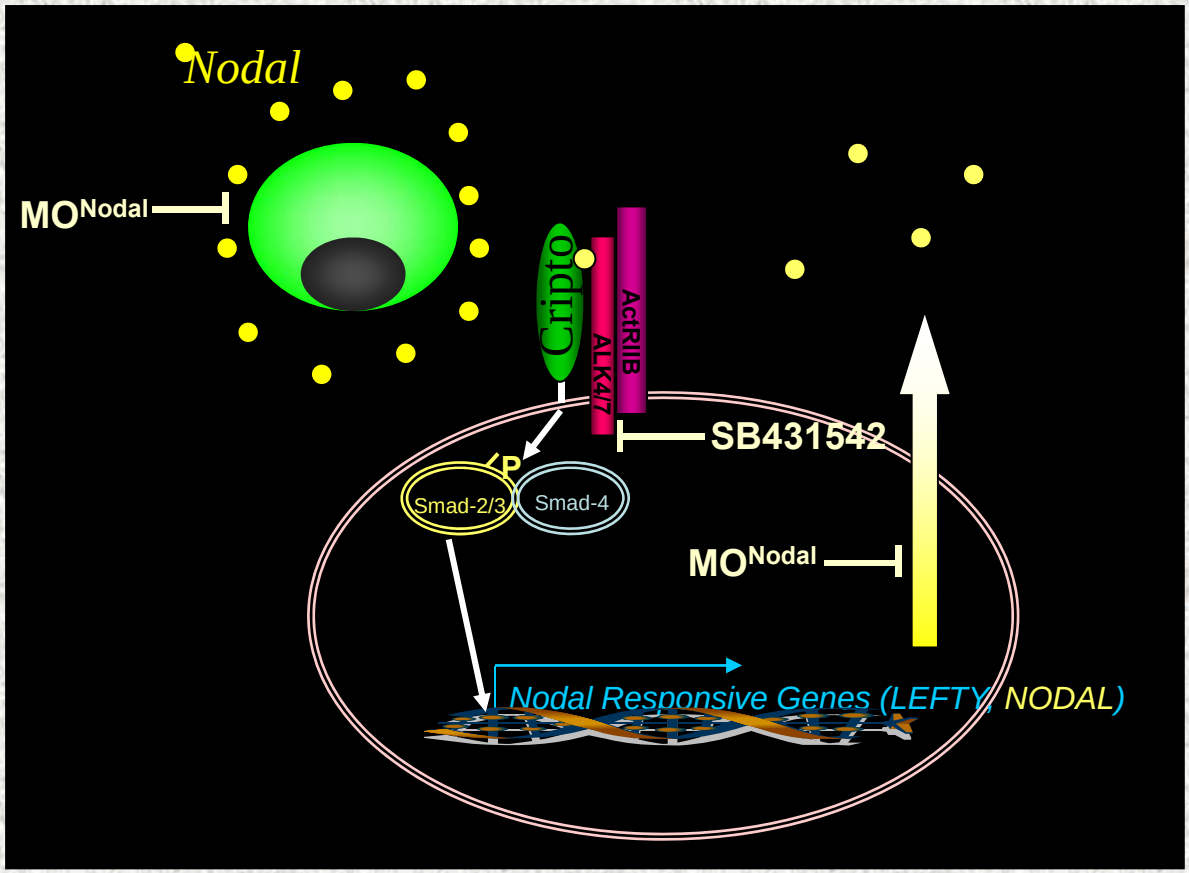
*Vertical Growth Phase*

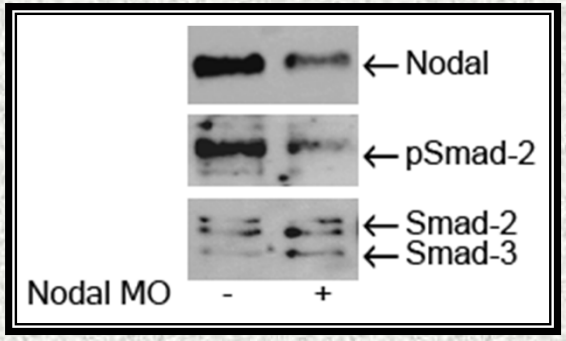
# Nodal Protein Expression Correlates with Melanoma Metastasis

*Metastatic Melanoma*

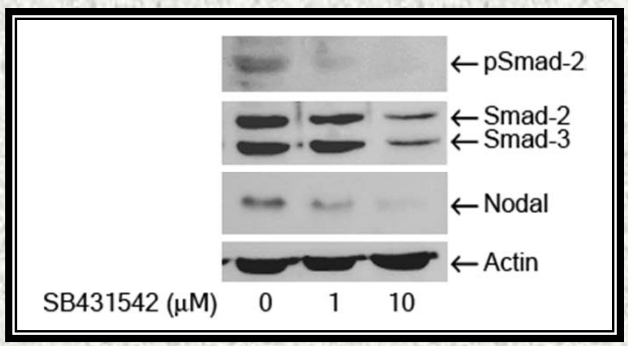


# Nodal Expression is Regulated by a Smad-2-Dependent Positive Feedback Loop



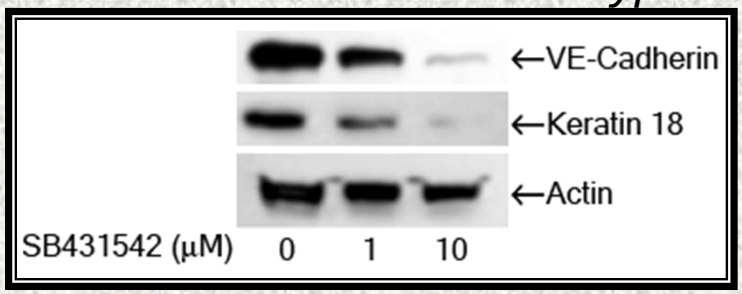

*MO$^{Nodal}$ Treatment*



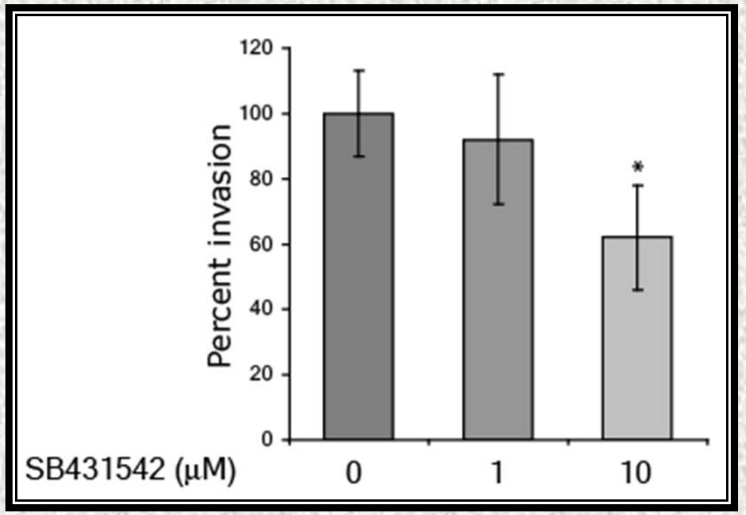

*Alk 4/7 Inhibition with SB431542*

# Nodal Inhibition Promotes the Reversion of Aggressive Melanoma Cells Toward a Less Aggressive Melanocyte-Like Phenotype

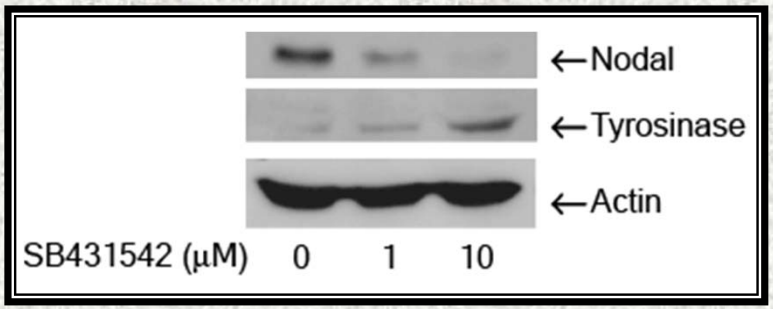
*Interconverted Phenotype*



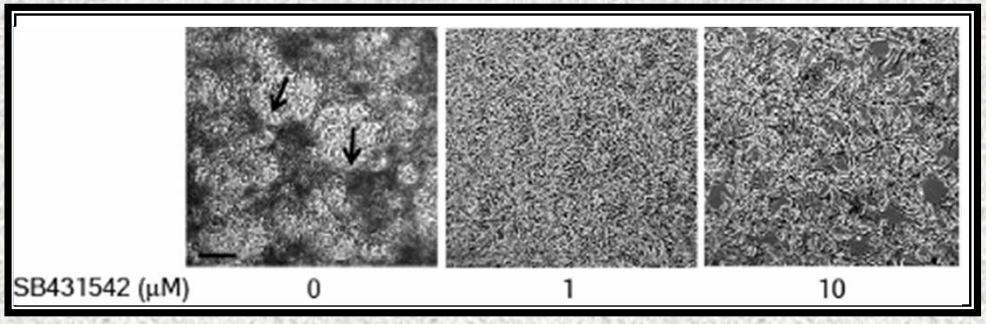

*Cellular Invasion*



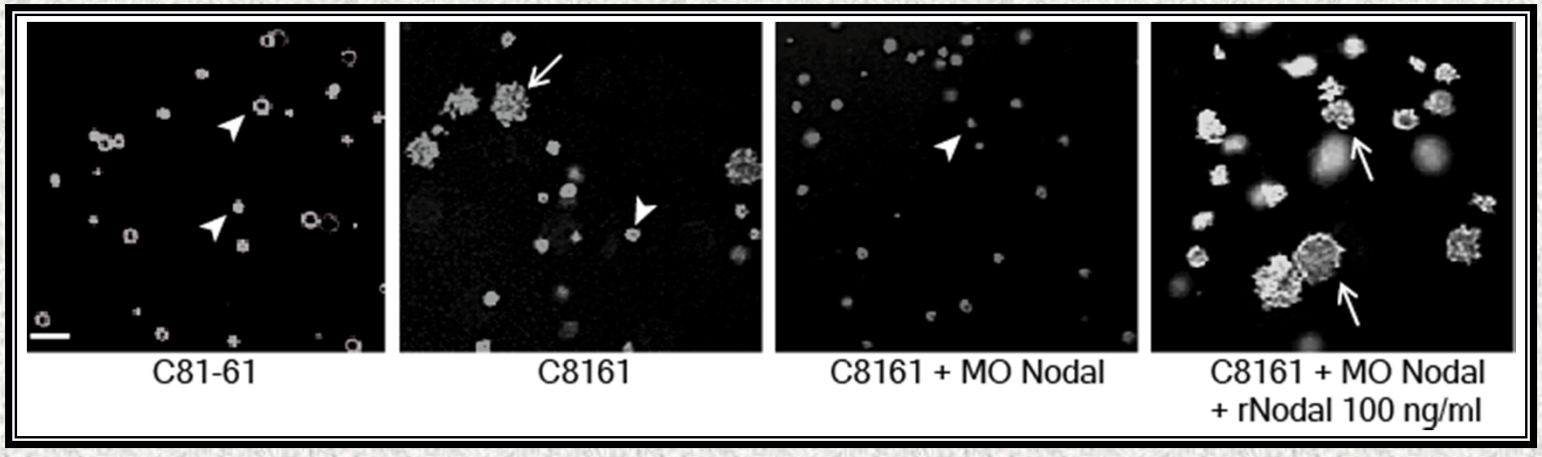

*Aggressive Amelanotic C8161 Cells Appear Pigmented and Re-express Tyrosinase when Nodal is Inhibited*



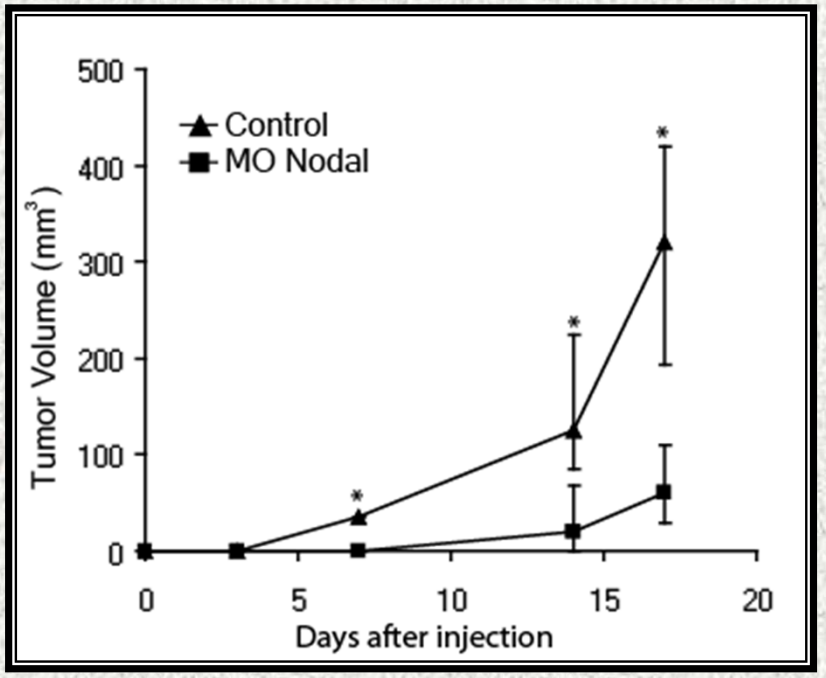

*Vasculogenic Mimicry*

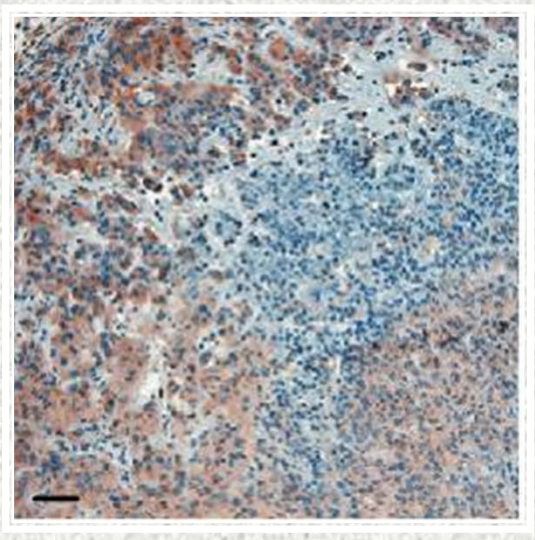


# Nodal Inhibition Abrogates Melanoma Cell Anchorage Independent Growth



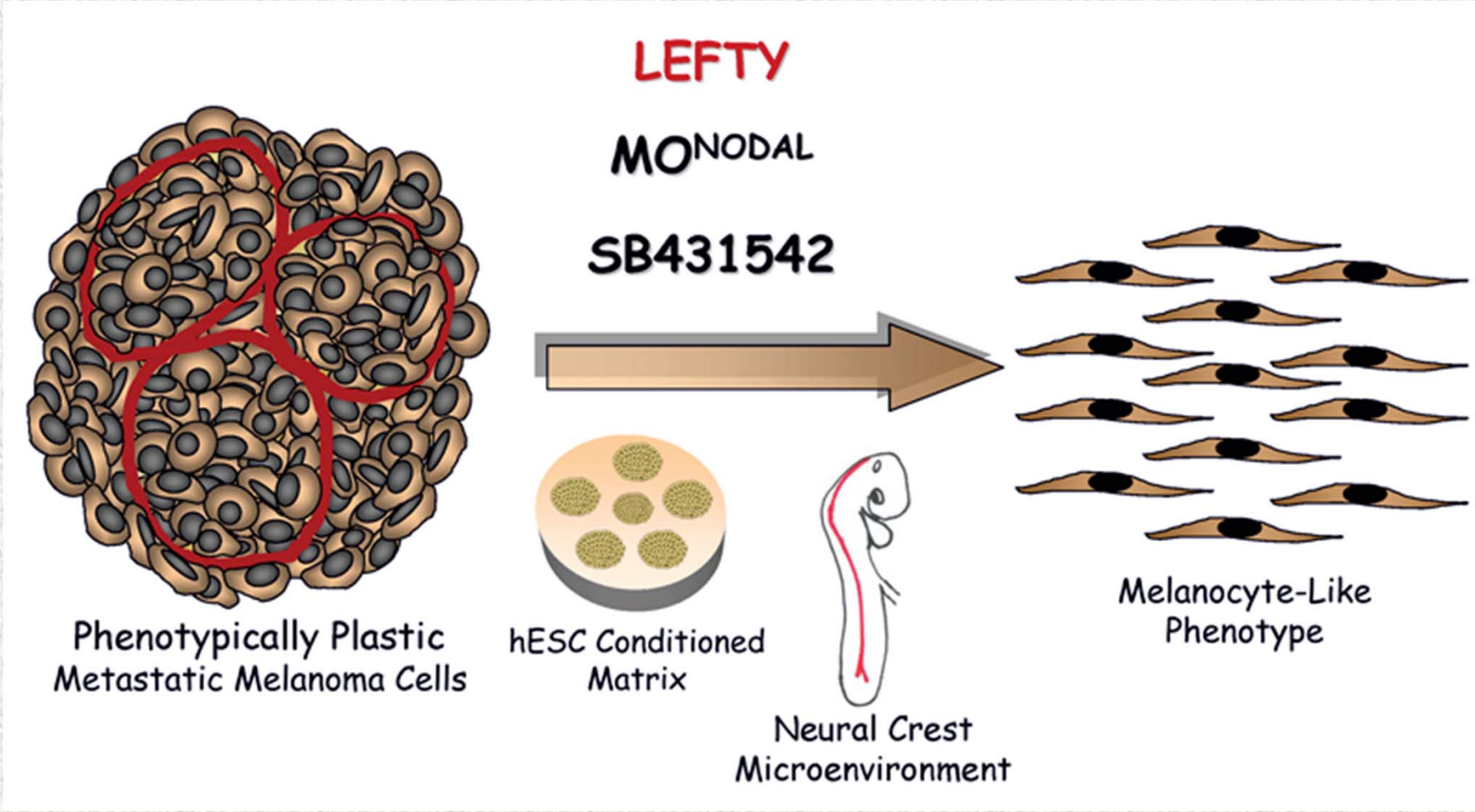
# Nodal Inhibition Reduces Melanoma Tumorigenicity in an Orthotopic Nude Mouse Model



*Nodal is re-expressed by day 17*

# Reprogramming multipotent metastatic melanoma cells



# TARGETING THE PLASTICITY OF AGGRESSIVE MELANOMA CELLS

- Aggressive melanoma cells express a plastic, dedifferentiated, multipotent phenotype and share many characteristics with embryonic progenitor cells.
- An embryonic zebrafish model, when used as a biosensor for tumor-derived signals, revealed that metastatic melanoma cells secrete Nodal (a potent embryonic morphogen) and consequently can induce ectopic formation of a zebrafish embryonic axis.
- In addition, Nodal is present in human metastatic tumors, but not in normal skin, and thus may be involved in melanoma pathogenesis. Inhibition of Nodal signaling reduces melanoma cell invasiveness, colony formation, tumorigenicity, and promotes the reversion of melanoma cells toward a melanocytic phenotype -- Nodal may represent a new biomarker and therapeutic target for plasticity and disease pathogenesis.

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