EPIGENOMETASTASIS

Epigenometastasis is defined as generation of metastatic tumor by epigenetic activation of growth of mesenchymal cells that are in the vicinity as well as at a distance from the primary tumor by Epigenetic Tumor/Growth Inducer (ETI/EGI) released from dying tumor cells.

Metastasis: Current Understanding

- **Metastasis is not a single disease**
- **Most solid primary tumors are epithelial cells**
- **Metastasis occurs mostly in tissues and organs abound with mesenchymal cells**
- **Metastasized tumors are functionally and morphologically quite different from the primary tumor**
- **Metastatic tumor cells are mostly mesenchymal cells**
- **Metastasis occurs through migration of primary tumor cells to distant niche sites**
- **The initial event that triggers migration of tumor cells is the**
- **Epithelial –Mesenchymal Transition (EMT) of primary tumor cells**
- **Subsequent steps include tumor cell detachment, intravasation, migration, extravasation, lodging and proliferation at secondary niche sites**

Unresolved Issue on EMT:

There is no direct unequivocal proof that a single primary epithelial tumor cell that has undergone EMT is actually the one that has migrated and formed metastatic tumor. There is no *in vitro* experiment that clearly demonstrates EMT from an epithelial cell tumor.

Background of Epigenometastasis discovery:

Clonexpress, a provider of human primary central nervous system cells for research, is engaged in development of different cell lines for drug discovery, toxicology, and cell therapy applications. Clonexpress has developed a patent pending technology that does not use any oncogenes and gene transfer approaches to develop permanent cell lines. This method is based upon an observation that introduction of only the cytoplasmic content of certain tumor cell lines into non dividing human primary cells, such as hepatocytes, pancreatic beta cells, and dopaminergic neuron cells, etc., results in activation of growth of these cell types. Permanent cell lines of the above cell types have been established.

Experimental Methods:

Tumor cell lines used:

Hep-T (a derivative of human hepatoma cell line Hep-G2), T-84 (human colon carcinoma), Daudi (human B cell lymphoma) and Jurkat (human T cell leukemia).

Functional enucleation of human tumor cell lines:

Various cell lines were treated with 200 ug/ml mitomycin C for three hours. Mitomycin C treatment causes inactivation and destruction of both nuclear and mitochondrial DNA. Therefore, these cells do not contribute any DNA component when they are fused with different human primary cells. The resulting hybrid between functionally enucleated cell (mitomycin C treated cell) and another whole cell has the genotype of the whole cell. This has been tested and confirmed by STR analysis.

Transwell assay:

Mitomycin C treated or untreated tumor cells are placed in the inner well and the whole primary cells are placed at the outer well with appropriate growth media for 2 days. The cells are physically separated by 0.4u membrane. The outer well primary cells are collected and plated in growth media containing specific growth factors that have been identified necessary for activation of growth of primary cells. This experiment is carried out to test whether mitomycin C treated and untreated tumor cells release factors into the medium that can activate growth of primary cells placed at a distance and physically separated from the tumor cells.

Results:

Epigenetic activation of growth of primary cells:

Functionally enucleated hepatoma cell line when fused with human primary cells (hepatocytes, pancreatic beta cells, and dopaminergic neuron) brings about activation of

growth of all three cell types in the presence of cell type specific growth factors. However, for continuous growth of all three cell types, two additional factors are absolutely necessary in addition to cell type specific growth factors.

Activation of growth of primary cells by other tumor cell cytoplasm:

Is activation of growth of primary cells specific to only functionally enucleted hepatoma cell line? Functionally enucleated T-84, Daudi, and Jurkat cell lines, when fused with human primary cells, were also capable of bringing about activation of growth of different human primary cells. In all cases, two factors (proprietary information) were absolutely essential for continuous growth of different primary cells. None of the human primary cells ever grew in cell type specific growth medium containing these two proprietary factors in the absence of any cell fusion. Several human hepatocyte cell lines, pancreatic beta cell lines, and dopaminergic neuron cell lines have been established in this manner. These studies show that cytoplasm of many tumor cell lines is capable of bringing about two specific factors dependent epigenetic activation of growth of human primary cells. These results suggest that activation of specific pathways may be involved during epigenetic activation of growth of primary cells by functionally enucleated tumor cells.

Diffusible nature of epigenetic growth activation factor:

Transwell assay was used to show that functionally enucleated hepatoma and Daudi cell lines release some factors into the medium that can activate growth of primary cells placed at a distance and separated from functionally enucleatd tumor cells by a membrane. However, such activation of growth was not seen when tumor cell lines without mitomycin C treatment were used in the transwell assay. This suggests that only dying tumor cells release some factors that activate growth of primary cells through activation of two factor dependent pathways. This factor is termed as Epigenetic Tumor/Growth Inducer (ETI/EGI).

Conclusion 1:

- **Human tumor cell lines contain factors that bring about activation of growth of human primary cells**
- **Growth activation occurs through activation of specific pathways**
- **The growth activation factor is called Epigenetic Tumor/Growth Inducer (ETI/EGI)**
- **ETI/EGI is released only from dying tumor cells and bring about activation of growth of primary cells placed at a distance and separated from dying tumor cells by a membrane**

Questions to be addressed:

- **Do fresh tumor cells contain ETI/EGI?**
- **Biochemical nature of ETI/EGI**
- **Mechanism of action of ETI/EGI**

Assay system for EGI:

A biochemical and cell based assay is needed to identify the biochemical nature of EGI. The end point of the assay is activation of growth of normally non-dividing human primary cells in the presence of two proprietary factors identified by Clonexpress. Many of the cell types described earlier are hard to isolate as they are all mostly from fetal tissues. It is necessary to use human primary cell that can be easily isolated for such cell based assays to identify the chemical nature of EGI. Clonexpress has developed a simpler assay system using human PBMNC as the test cells and activation of growth of these cells following fusion with functionally enucleated tumor cell lines as well as using them in a transwell assay. This assay will facilitate the identification and isolation of EGI.

Hypothesis and predictions:

- **Dying (from necrosis or by other mechanisms) primary tumors may release EGI that can activate growth of mesenchymal cells that are in the vicinity as well as at a distance from the primary tumor through activation of specific pathways.**
- **Mesenchymal cells can thus become secondary metastatic tumor.**
- **This can appear like EMT in tumor biopsies.**
- **Most tumor cells die after chemotherapy or radiation treatment. This may be the time when EGIs may be present most in circulation. This could increase the incidence of metastasis.**
- **It may be possible to prevent metastasis when patients undergoing chemotherapy or radiation treatment are treated with inhibitors that specifically block the pathways identified by Clonexpress during and immediately following chemotherapy treatment.**
- **Identification of the biochemical nature and mechanism of action of EGI can lead to potential novel targets for anti-metastatic drug development.**

Immediate applications of the technology:

- ## Development of hepatocyte cell lines

  Clonexpress has developed more than ten human hepatocyte cell lines using this approach. All these cell lines resemble human primary hepatocytes and all of them grow only in suspension. These cell lines express many functions characteristic of primary human hepatocytes including albumin, α-1 anti-trypsin, cytochrome P450 (CYP 1A, CYP3A4), ammonia clearance etc. These cell lines are well suited for toxicology, drug metabolism, and bioartificial liver development.

- ## Development of pancreatic beta cell lines

  Two adult and eight fetal human pancreatic beta cell lines (all from different donor primary beta cells) have been developed thus far. These cell lines produce insulin in response to increasing concentration of glucose and GLP-1 peptide. They also express other beta cell specific functions, such as Glut-2, pdx-1 etc. These cell lines also grow in suspension. These are very unique cell lines and resemble very close to normal beta cells. They will be suitable for drug discovery and cell therapy

applications. These cell lines have been used to develop patient-specific human beta like cell from PBMNC (See patient-specific beta cells and dopaminergic neurons).

- ## Development of dopaminergic neuron cell lines

  Four human dopaminergic neuron cell lines have been developed from dopaminergic neuron cells isolated from human fetal brain tissue. These cell lines express a number of dopaminergic neuron specific functions, such as expression of tyrosine hydroxylase, Nurr-1, etc. One dopaminergic neuron cell line has been used to generate patient-specific human dopaminergic neuron from primary human fibrolast.

Generation of patient-specific dopaminergic neuron and pancreatic beta cells:

- **Primary human fibroblast or concanavalin A stimulated PBMNC from patient is treated with 5-aza deoxycytidine and valproic acid (methylase and deacetylase inhibitors) for 4-5 days**
- **Functional enucleation of cytoplasmic donor cells – dopaminergic neuron and pancreatic beta cell lines**
- **Fusion of functionally enucleated cell lines with chemically treated patient fibroblast or PBMNC**
- **Growth of fused cells in cell type specific growth medium**
- **Functional characterization for the expression of cell type specific function in growing population of cells**

Results:

- **Eipegenetically- induced dopaminergic neuron (EIDN) like cells from fusion of chemical inhibitor treated fibroblast with functionally enucleated human dopaminergic neuron cell line**
- **EIDN cells express dopaminergic neuron specific functions**
- **Epigenetically induced beta (EIB) like cells from fusion of chemical inhibitor treated PBMNC with functionally enucleated human pancreatic beta cell lines**
- **EIB cells produce insulin and express other beta cell specific functions**
- **EIDN and EIB cell genotype is the same as that of patient fibroblast and PBMNC**

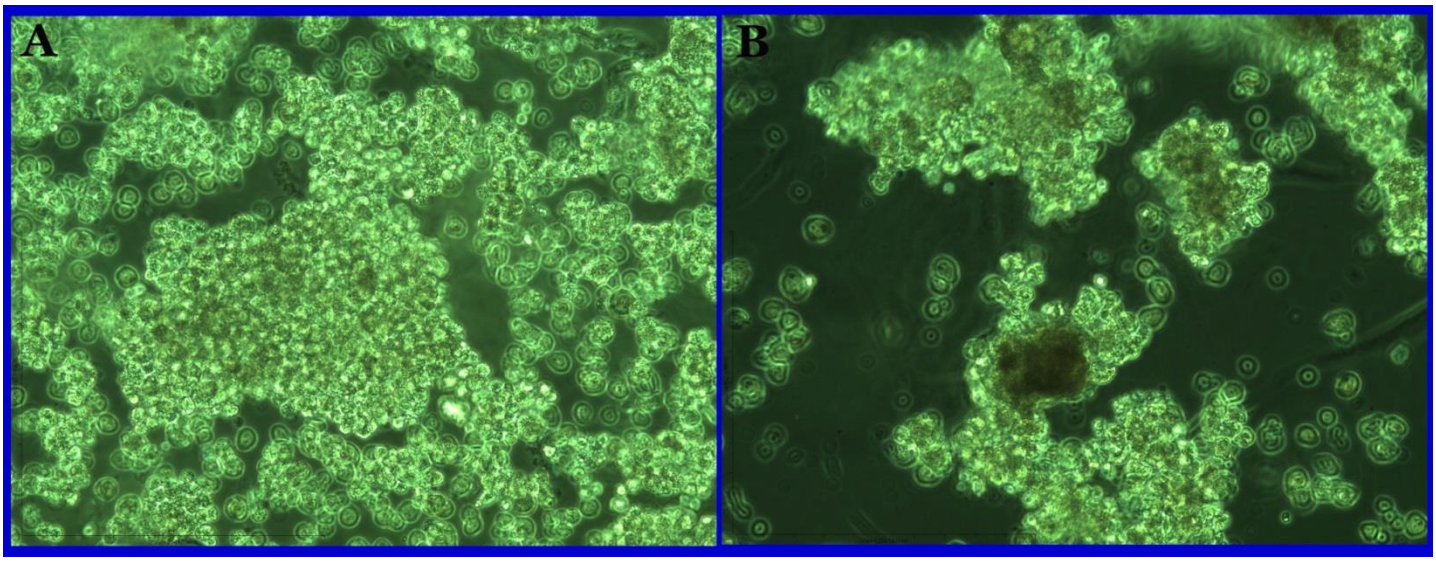
Conclusion 2:

- **Cell lines developed by epigenetic activation of growth by EGI continue to express EGI**
- **EGI from the cell lines can be transferred to chemically treated fibroblast or PBMNC to activate growth**
- **Inhibitor treatment of fibroblast and PBMNC is essential to activate cytoplasmic donor cell specific phenotype**
- **Epigenetically activated differentiated cell types derived from fibroblast and PBMNC have the same genotype as the original fibroblast and PBMNC**

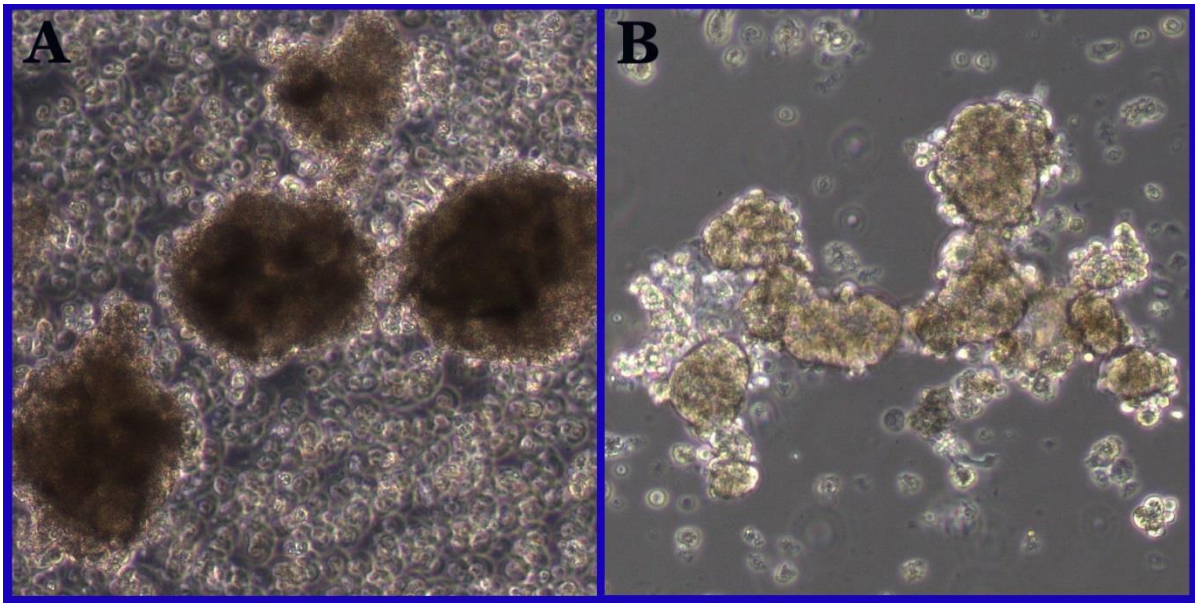
- **New approach to generate patient-specific differentiated cell types without a need to develop patient-specific iPS cells**

Future:

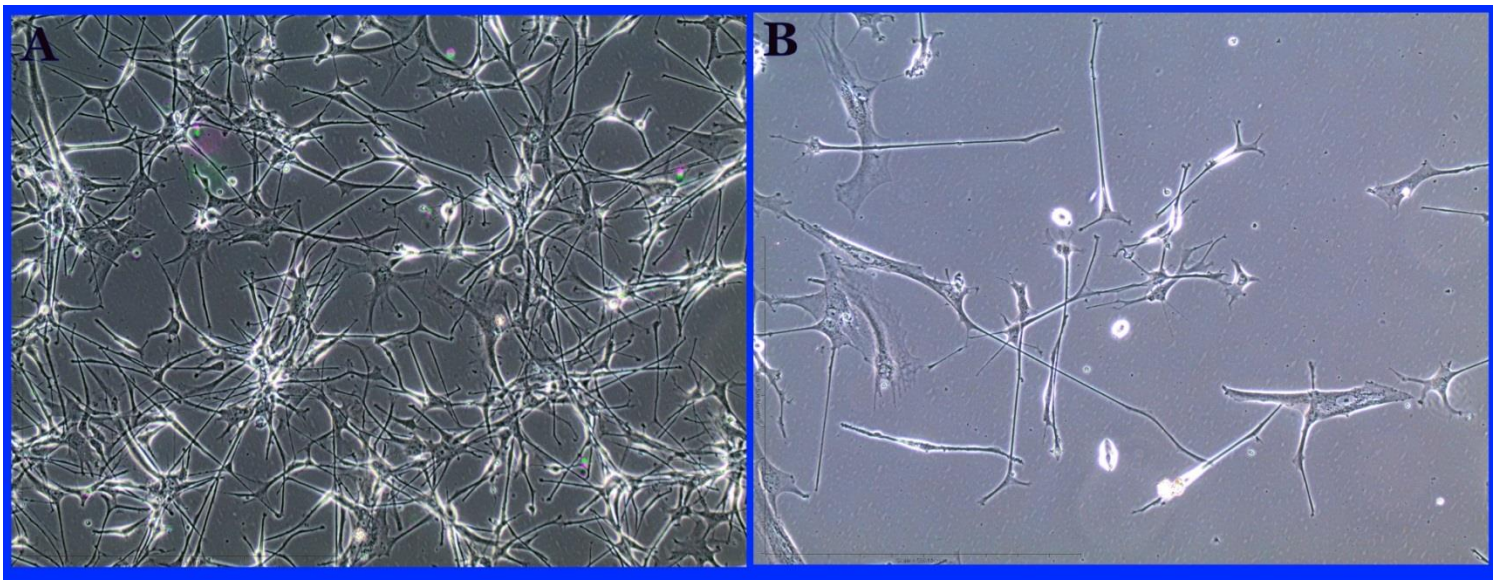
Patent, Publish, and Partnership to develop Clonexpress's technology platform for drug discovery, drug development, and cell therapy applications.



Two different human fetal liver hepatocyte cell lines.



A. **Human fetal pancreatic beta cell line.**
B. **Epigenetically induced beta (EIB) cells derived from fusion of chemically treated concanavalin A stimulated PBMNC with functionally enucleated beta cell line.**



A. Human dopaminergic neuron cell line.

B. Epigenetically induced dopaminergic neuron (EIDN) cells obtained by fusion of chemically treated human dermal fibroblast with functionally enucleated dopaminergic neuron cell line.