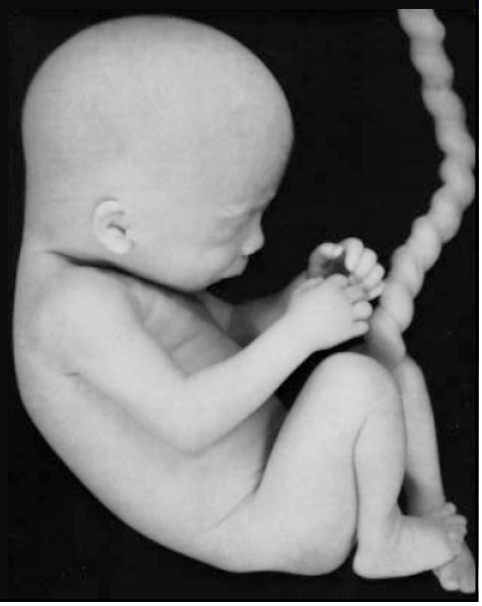
Βιολογία Βλαστοκυττάρων και Αναγέννησης



Εισαγωγή

Μ. Γρηγορίου 2022

1

Βιολογία Βλαστοκυττάρων και Αναγέννησης

Στόχοι του μαθήματος είναι οι φοιτητές:

- Να μελετήσουν τους βασικούς μοριακούς μηχανισμούς που διέπουν τη βιολογία των **εμβρυϊκών & των επαγόμενων βλαστοκυττάρων**.
- Να μελετήσουν τους βασικούς μοριακούς μηχανισμούς που διέπουν τη βιολογία των **ιστοειδικών βλαστοκυττάρων**.
- Να μελετήσουν τις εφαρμογές των παραπάνω στην Υγεία και να αντιληφθούν τις **δυνατότητες ανάπτυξης καινοτόμων θεραπευτικών κυτταρικών προσεγγίσεων**.
- Να μελετήσουν βασικούς μοριακούς μηχανισμούς που διέπουν τη βιολογία των **καρκινικών βλαστοκυττάρων**.
- Να μελετήσουν τις βασικές αρχές **της Μηχανικής Ιστών**.
- Να αναγνωρίζουν τους **βασικούς ηθικούς/νομικούς προβληματισμούς** που προκύπτουν από τη μελέτη των βλαστοκυττάρων και των ερφαρμογών τους

2

Βιολογία Βλαστοκυττάρων και Αναγέννησης

Περιεχόμενο του μαθήματος

- Εισαγωγή στη Βιολογία των βλαστοκυττάρων (Stem Cells- SC).
- Γενικές Αρχές Απομόνωσης, καλλιέργειας και διαφοροποίησης SC.
- Η Μοριακή βάση της πολυδυναμίας.
- Απομόνωση, καλλιέργεια και διαφοροποίηση των ESC
- Επαγόμενα πολυδύναμα βλαστοκύτταρα (iPS).
- Εφαρμογές των ESC.
- Αρχές Μηχανική ιστών.
- Βλαστοκύτταρα και κοινωνία

Καρκινικά βλαστοκύτταρα (CSC) - βιολογία και νέες προσεγγίσεις στη θεραπευτική του καρκίνου

3

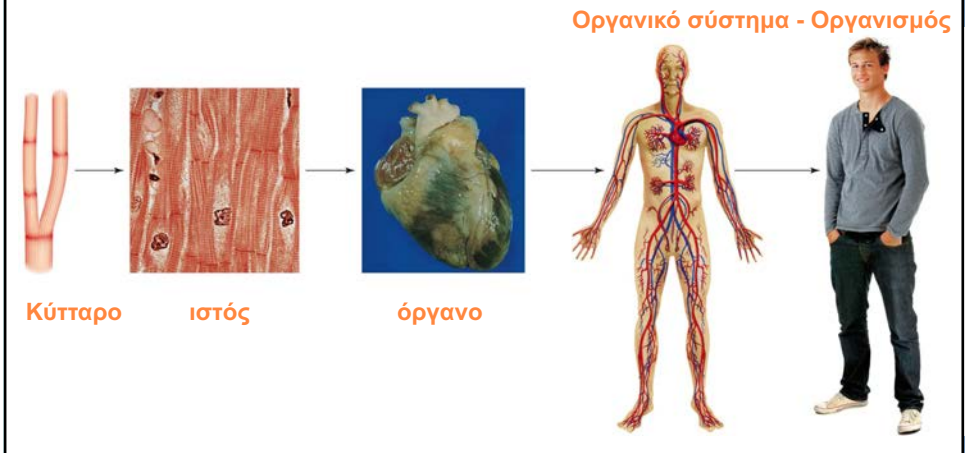
4

Περίγραμμα

- Τι είναι τα βλαστοκύτταρα;
- Γιατί είναι τα βλαστοκύτταρα στο επίκεντρο του ενδιαφέροντος;
- Πού εντοπίζονται στον οργανισμό τα βλαστοκύτταρα;
- Με ποιο τρόπο γίνεται η απομόνωση των βλαστοκυττάρων;
- Ποιο είναι το ενδιαφέρον στην έρευνα στα βλαστοκύτταρα;
- Ποια είναι η κατάσταση σήμερα στον τομέα των θεραπειών που χρησιμοποιούν βλαστοκύτταρα;

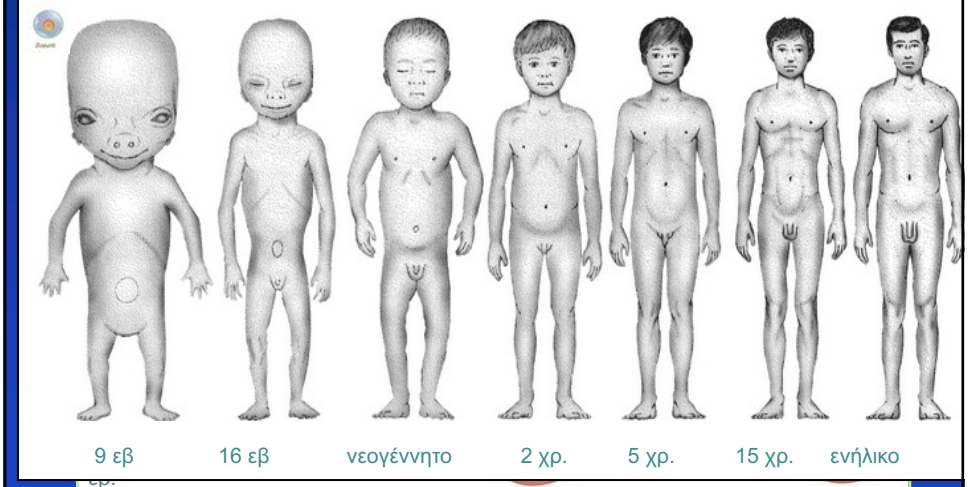
5

Από το κύτταρο στον οργανισμό



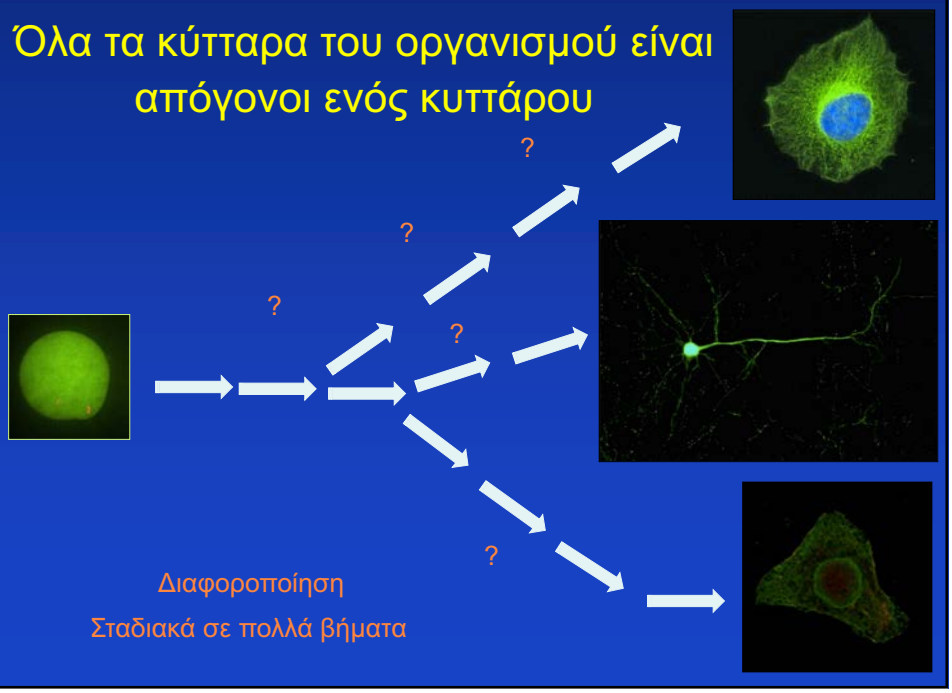
6

Όλα τα κύτταρα του οργανισμού είναι απόγονοι ενός κυττάρου



7

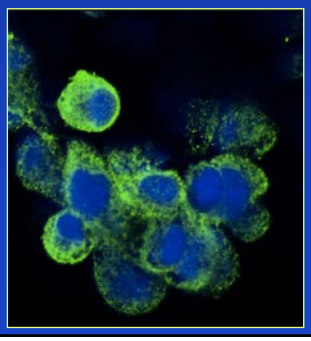
Όλα τα κύτταρα του οργανισμού είναι απόγονοι ενός κυττάρου



8

Τι είναι τα βλαστοκύτταρα;

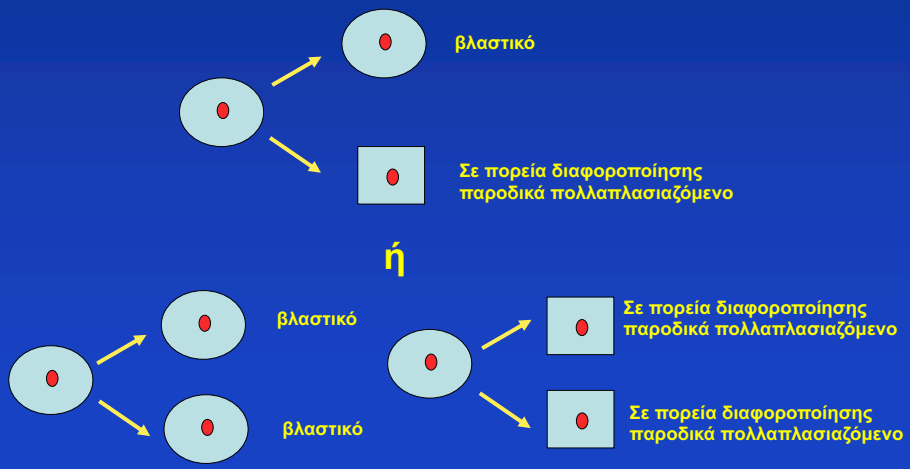
- Είναι κύτταρα που έχουν τη δυνατότητα να **αυτοανανεώνονται**
- Είναι κύτταρα που δεν έχουν διαφοροποιηθεί και **οι απόγονοί τους ανήκουν σε διαφορους κυτταρικούς τύπους**
- Είναι κύτταρα που δεν έχουν ακόμα εξειδικευτεί ή προκαθοριστεί
- Κάθε κύτταρο του σώματος είναι απόγονος «βλαστοκυττάρων»



9

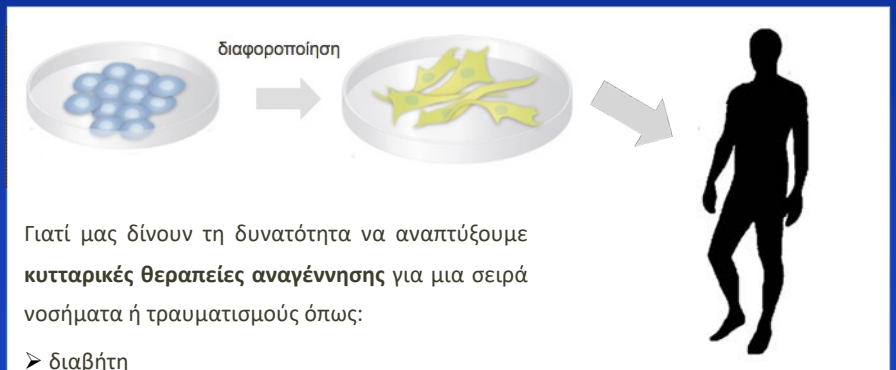
Τι είναι τα βλαστοκύτταρα;

Αυτοανανέωση – παραγωγή απογόνων που ανήκουν σε διαφορετικούς κυτταρικούς τύπους



10

Γιατί είναι τα βλαστοκύτταρα στο επίκεντρο του ενδιαφέροντος;



Γιατί μας δίνουν τη δυνατότητα να αναπτύξουμε **κυτταρικές θεραπείες αναγέννησης** για μια σειρά νοσήματα ή τραυματισμούς όπως:

- διαβήτη
- νευροεκφυλιστικές ασθένειες
- τραυματισμούς του νωτιαίου μυελού
- αναπτυξιακές διαταραχές

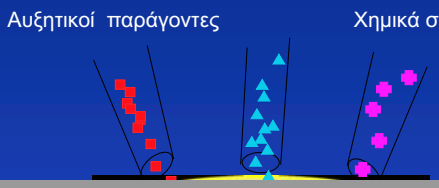

BIOTECH

Lab-Grown Blood Stem Cells Produced at Last

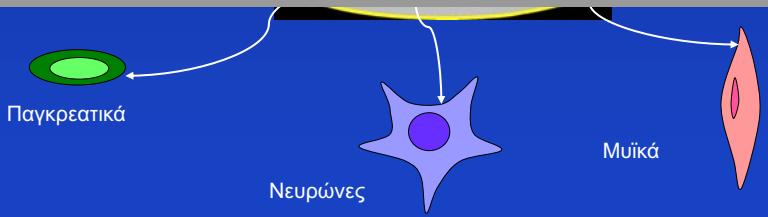
Two research teams cook up recipe to make long-sought cells in mice and people

11

Γιατί είναι τα βλαστοκύτταρα στο επίκεντρο του ενδιαφέροντος;



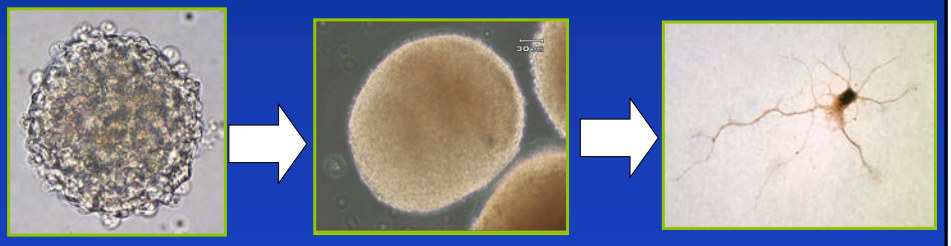
Προσπαθούμε να επαναλάβουμε τα γεγονότα που φυσιολογικά συμβαίνουν κατά τη διαφοροποίηση!



Τα βλαστοκύτταρα μπορούν με κατάλληλους χειρισμούς να διαφοροποιηθούν *in vitro* σε διάφορους κυτταρικούς τύπους

12

Κατευθυνόμενη διαφοροποίηση σε νευρώνες

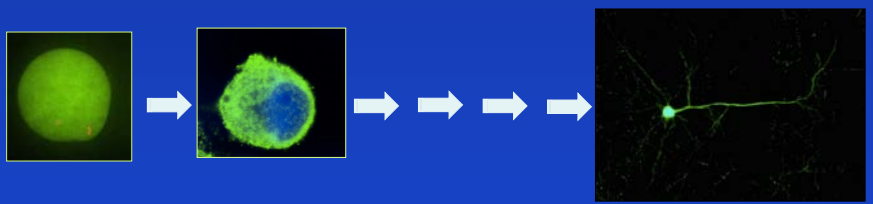


βλαστοκύτταρα Νευροσφαιρίδια Νευρώνες

13

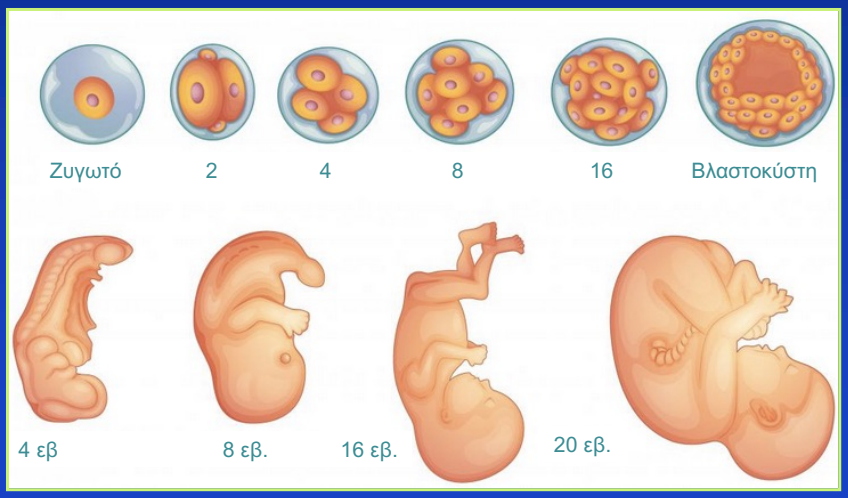
Πού εντοπίζονται τα βλαστοκύτταρα;

Κάθε κύτταρο του σώματος είναι απόγονος βλαστοκυττάρων



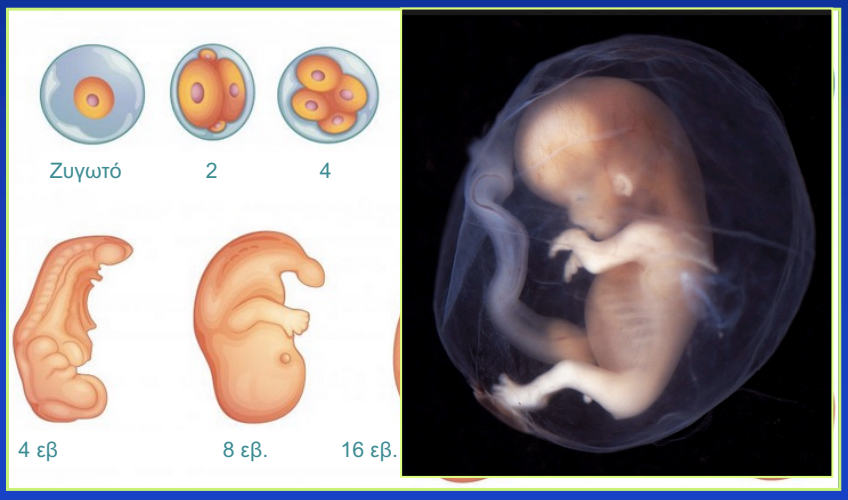
14

Πού εντοπίζονται τα βλαστοκύτταρα;



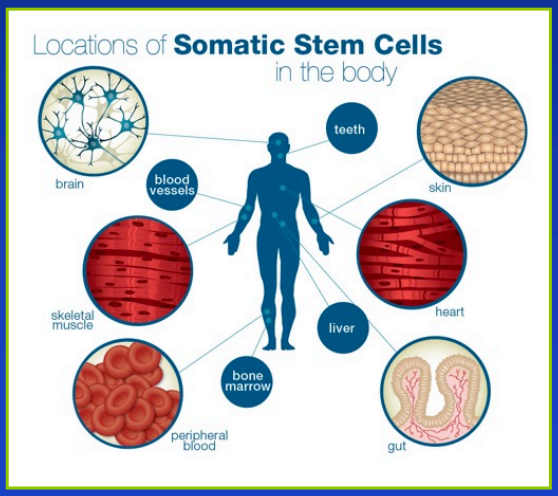
15

Πού εντοπίζονται τα βλαστοκύτταρα;



16

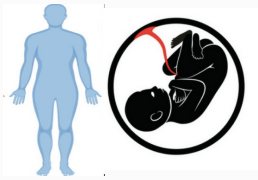
Πού εντοπίζονται τα βλαστοκύτταρα;



Ιστοί με έντονη κυτταρική ανανέωση διαθέτουν αρκετά βλαστοκύτταρα (π.χ αίμα) ενώ όσοι δεν ανανεώνονται πολύ λίγα (π.χ. Καρδιά)

17

Βλαστοκύτταρα: τύποι (α)



Απόγονοί τους διαφοροποιούνται σε **μερικούς κυτταρικούς τύπους** του σώματος

ΟΛΙΓΟΔΥΝΑΜΑ

Απόγονοί τους διαφοροποιούνται σε **πολλούς κυτταρικούς τύπους** του σώματος

ΠΟΛΥΔΥΝΑΜΑ

Απόγονοί τους διαφοροποιούνται σε **όλους τους κυτταρικούς τύπους**

ΟΛΟΔΥΝΑΜΑ

Αυτοανανέωση – παραγωγή απογόνων που ανήκουν σε διαφορετικούς κυτταρικούς τύπους

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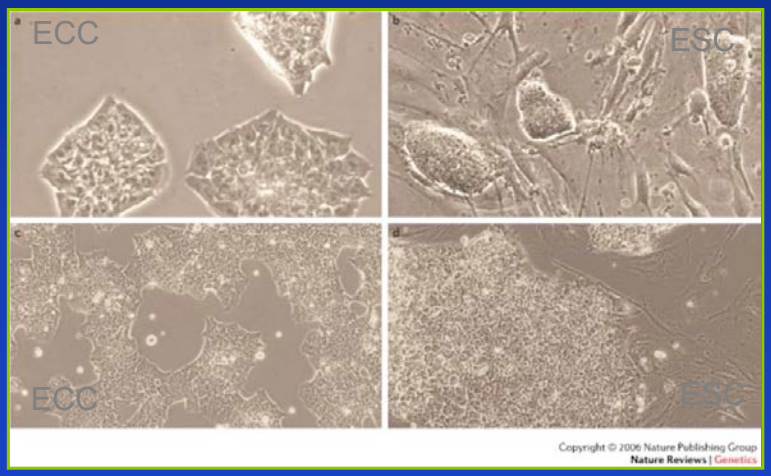
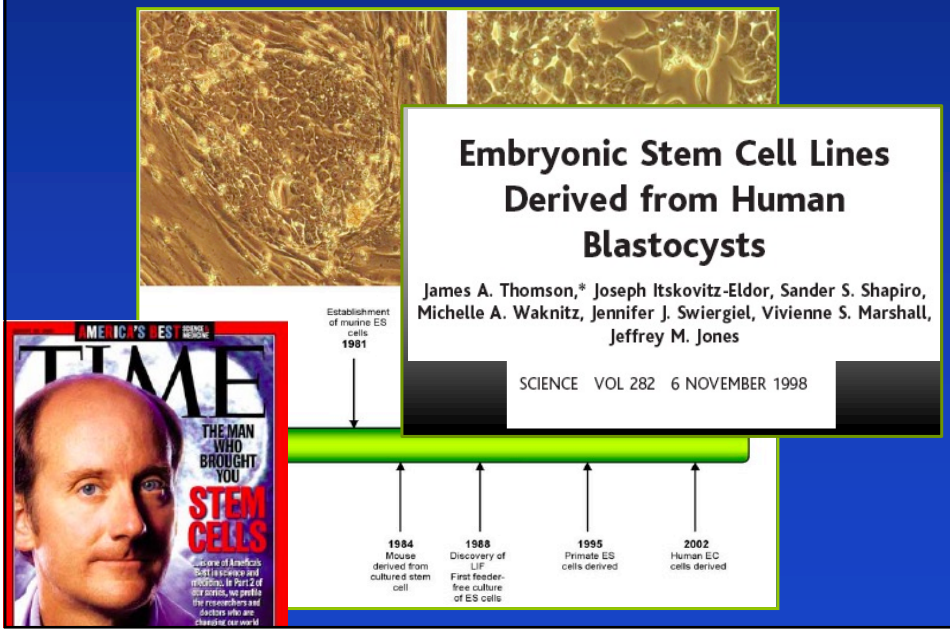
Εμβρυϊκά βλαστοκύτταρα



Το 1981 απομονώθηκαν τα εμβρυϊκά βλαστικά κύτταρα (Evans and Kaufman, 1981; Martin, 1981) – η αναζήτηση ενός τέτοιου κυτταρικού τύπου βασίστηκε στα δεδομένα από την μελέτη των τερατοκαρκινωμάτων.

19

Εμβρυϊκά βλαστοκύτταρα



20

Από πού απομονώνονται τα βλαστοκύτταρα;

Στον άνθρωπο

εμβρυϊκά βλαστοκύτταρα από βλαστοκύστεις

σωματικά βλαστοκύτταρα απομονώνονται από:

- Μυελό των οστών (χρήση σχεδόν 60 χρό
- Κύτταρα περιφερικού αίματος


The NEW ENGLAND JOURNAL of MEDICINE

Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling

BONE MARROW DONORS WORLDWIDE

Η Ελλάδα έχει ένα από τα **χαμηλότερα ποσοστά εθελοντών δοτών** / πληθυσμό (30.000 στόχος 160.000 = 2% για 80%)

21

Τράπεζες βλαστοκυττάρων

Στον άνθρωπο σωματικά βλαστοκύτταρα απομονώνονται από:

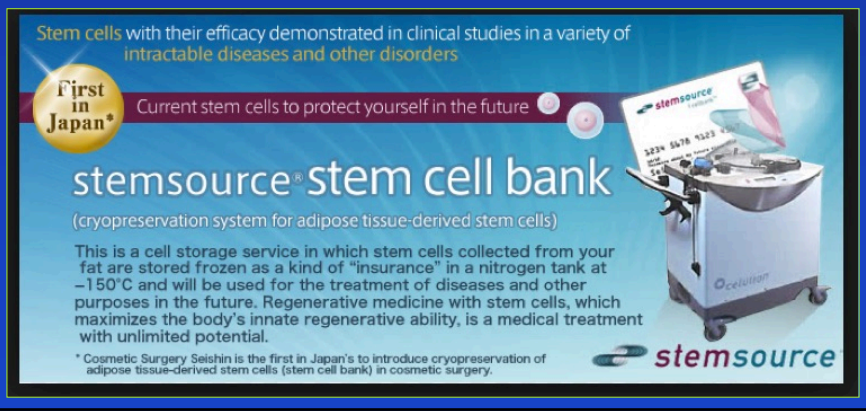
- Ομφαλοπλακουντιακό αίμα
- Τράπεζες φύλαξης των βλαστοκυττάρων του ΟΠΑ είτε ως εναλλακτική πηγη μυελού ή για μελλοντικές κυτταρικές θεραπείες βασισμένες σε βλαστοκύτταρα
- Τράπεζες ιδιωτικές ή δημόσιες
- Αποκλειστική χρήση ή δωρεά)?
- Στην ΕΕ οι οδηγίες το 2004.

22

Τράπεζες βλαστοκυττάρων

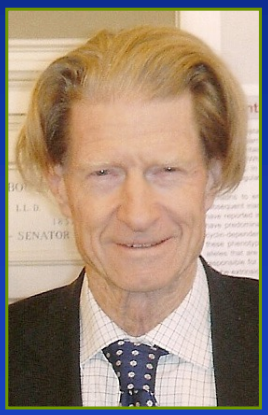
Στον άνθρωπο σωματικά βλαστοκύτταρα απομονώνονται από:

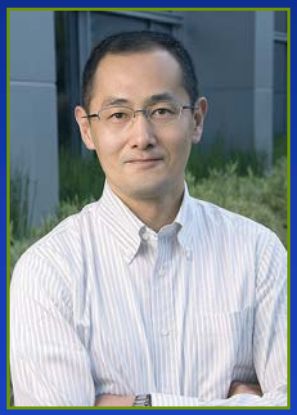
- Νεογιλά δόντια
- Αλλους ιστούς πχ λιποκύτταρα , κ.α



23

2012 Nobel Prize in Medicine





J. Gurdon
Department of Zoology, University of Cambridge

S. Yamanaka
Institute of Cardiovascular Disease, San Francisco

"revolutionized our understanding of how cells & organisms develop"

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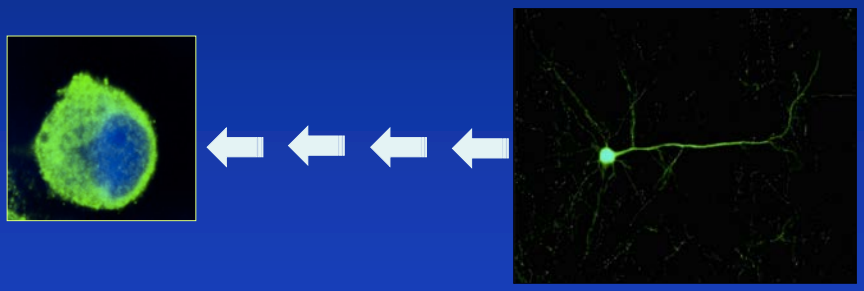
Επαναπρογραμματισμός



Από ένα διαφοροποιημένο φτιάχνουμε στο εργαστήριο ένα εμβρυϊκό βλαστοκύτταρο = επαναπρογραμματισμός

- Δεν χρειάζονται έμβρυα
- Εξατομικευμένη προσέγγιση - ιστοσυμβατότητα
- Διάφορες τεχνικές – επανάσταση!

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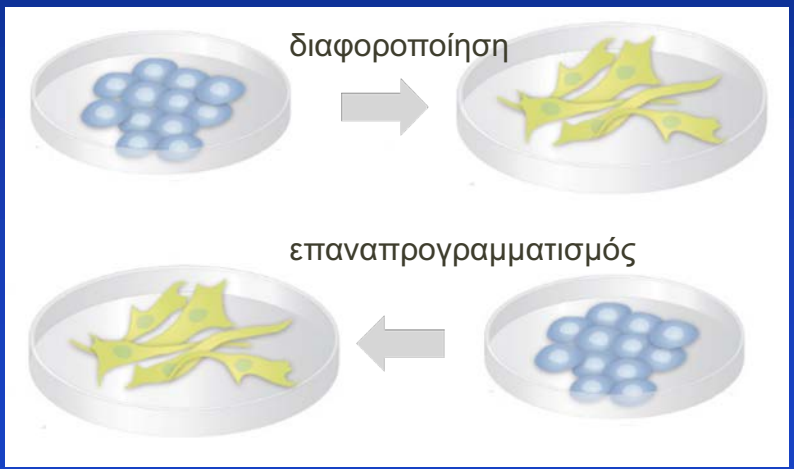
Επαναπρογραμματισμός



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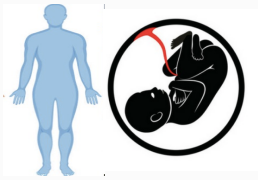
Βλαστοκύτταρα: τύποι (β)



Απόγονοί τους διαφοροποιούνται σε **μερικούς κυτταρικούς τύπους** του σώματος	Απόγονοί τους διαφοροποιούνται σε **πολλούς κυτταρικούς τύπους** του σώματος	Απόγονοί τους διαφοροποιούνται σε **όλους τους κυτταρικούς τύπους**

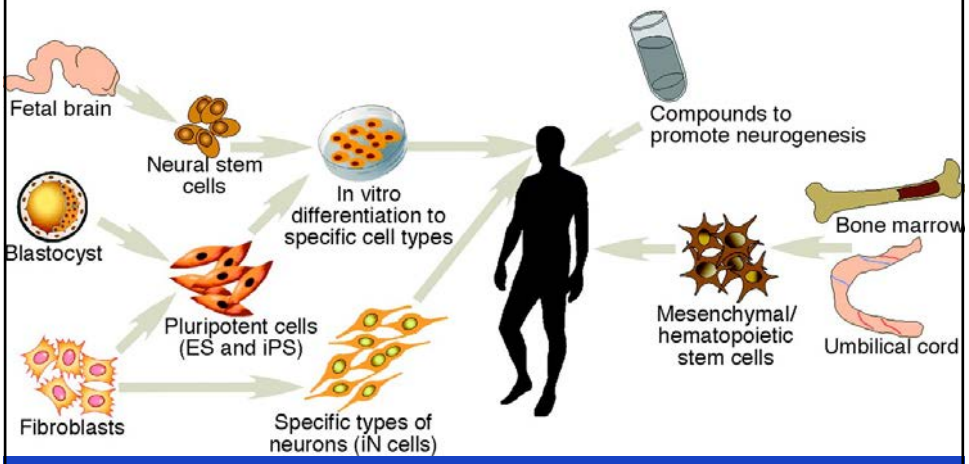
27

Ποιο είναι το ενδιαφέρον για την έρευνα στα βλαστοκύτταρα;

- Να καταλάβουμε πώς λειτουργούν τα βλαστοκύτταρα, να βρούμε τα ιδιαίτερα χαρακτηριστικά του και πώς τα διατηρούν
- Να ανακαλύψουμε με ποιους μηχανισμούς γίνεται η διαφοροποίηση
- Να καταλάβουμε τη βιολογία πίσω από ένα νόσημα
- Να προσπαθήσουμε να φτιάξουμε διαφοροποιημένα κύτταρα στο εργαστήριο
- Να σχεδιάσουμε και να δοκιμάσουμε κυτταρικές θεραπείες σε σειρά παθολογικών καταστάσεων

28

Πώς χρησιμοποιούνται τα βλαστοκύτταρα και ποιες είναι οι δυνατότητες;

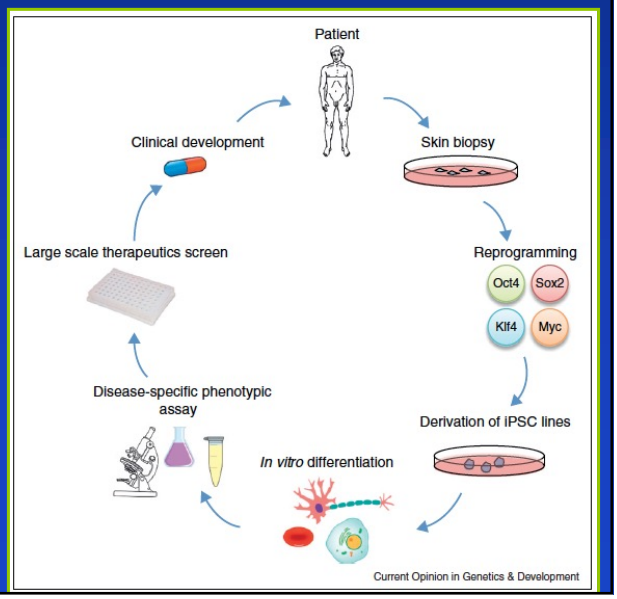


Κυτταρικές θεραπείες αναγέννησης οι δυνατότητες είναι πολλές!

29

Πώς χρησιμοποιούνται τα βλαστοκύτταρα και ποιες είναι οι δυνατότητες;

- Μηχανισμοί ασθενειών
- Έλεγχος φαρμάκων



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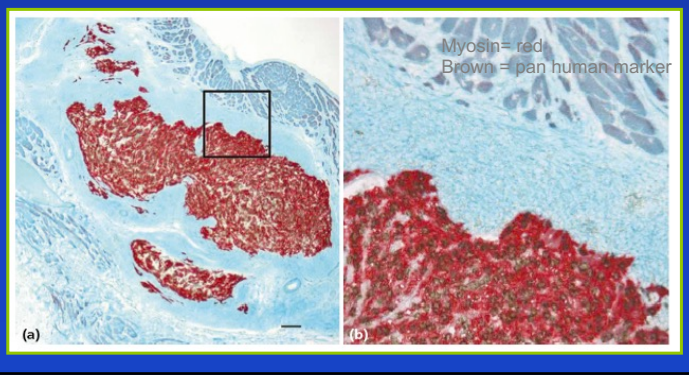
Κυτταρικές θεραπείες

Ανάπλαση καρδιακού ιστού ποντικού μετά από τραυματισμό και μεταμόσχευση ανθρώπινων ES διαφοροποιημένων σε καρδιομυοκύτταρα. Τα ανθρώπινα κύτταρα καφέ.

Έχουν δοκιμαστεί με επιτυχία κυτταρικές θεραπείες σε μοντέλα ασθενειών στον ποντικό



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Χρηματοδότηση

Ιδρύματα
Η οικογένεια του Reeve μέσω του Christopher & Dana Reeve Foundation έχει επιδοτήσει ερευνητκά προγράμματα στο πεδίο των βλαστοκυττάρων με περίπου 22 εκατομμύρια δολλάρια.

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Χρηματοδότηση

Εταιρείες Βιοτεχνολογίας που επενδύουν στην έρευνα και στην ανάπτυξη προϊόντων από βλαστο-κύτταρα

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Χρηματοδότηση

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Εμβρυϊκά βλαστοκύτταρα και κυτταρικές θεραπείες - Κλινικές δοκιμές

2021 μόνο ένα Ινστιτούτο!

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The Washington Post

Democracy Dies in Darkness

FDA OKs 1st Embryonic Stem Cell Trial

Timothy J. Atchison

By Steven Reinberg
HealthDay Reporter
Friday, January 23, 2009; 12:00 AM

FRIDAY, Jan. 23 (HealthDay News) -- The first human trial using embryonic stem cells as a medical treatment has been approved by the U.S. Food and Drug Administration.

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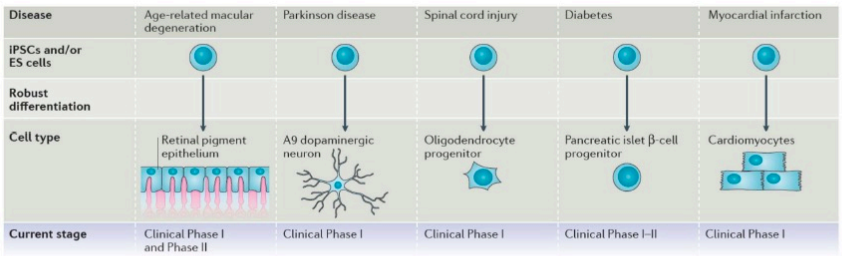
Εμβρυϊκά βλαστοκύτταρα και κυτταρικές θεραπείες - Κλινικές δοκιμές



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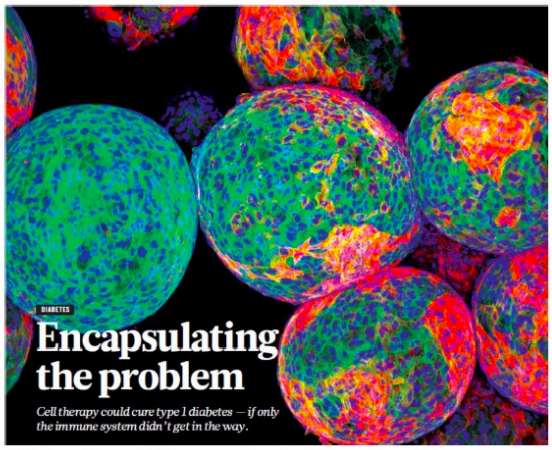
Εμβρυϊκά βλαστοκύτταρα και κυτταρικές θεραπείες - Κλινικές δοκιμές



35 days of carefully swap-ping 5 different growth media and mixing in 11 different factors, including sugars and proteins.

Nature 2016, 540, 560-562

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Βλαστοκύτταρα, Πολιτική και Νομοθεσία

Philippines Investigating 3 Politician Deaths Allegedly From Stem Cells in Germany

Posted on June 23, 2013

Stem cell doctor forced to close his clinic after child's death is back in business

The boss behind Europe's largest stem cell clinic, which was shut down following the death of a child in its care, is back in business working in partnership with a British laboratory.

Γερμανία 2012 – η περίπτωση της XCell-Center (τώρα στο Λίβανο)

Παρά το ότι σε πολλές περιπτώσεις δεν έχουν γίνει οι απαραίτητες δοκιμές, υπάρχουν κλινικές που δοκιμάζουν κάποιου τύπου θεραπείες συνήθως βασισμένες σε βλαστοκύτταρα μυελού, αμφιλεγόμενες όχι μόνο ως προς το αποτέλεσμα αλλά και ως προς τα κύτταρα που μεταμοσχεύονται

Το νομοθετικό πλαίσιο διαφέρει από χώρα σε χώρα.

Ιταλία 2013 – η περίπτωση της Stamina

40

Τα βλαστοκύτταρα και εμείς..

Stem cell doctor forced to close his clinic after child's death is back in business

The boss behind Europe's largest stem cell clinic, which was shut down following the death of a child in its care, is back in business working in partnership with a British laboratory.

Don't market stem-cell products ahead of proof

The controversy over an unproven stem-cell therapy in Italy highlights the dangers of doing translational medicine in reverse, argues **Paolo Bianco.**

Η Πολιτεία πρέπει να ρυθμίσει....

Philippines Investigating 3 Politician Deaths Allegedly From Stem Cells in Germany

Posted on June 23, 2013

41

Μαθήματα κάθε Τρίτη 15-17 μ.μ. (και κάποια έξτρα ίσως.....)

- Η παρουσία σας στο μάθημα αποτελεί βασική προϋπόθεση για να μάθετε! .....γιαυτό .... **Είναι υποχρεωτική!**

42

Μαθήματα κάθε Τρίτη 15-17 μ.μ. (και κάποια έξτρα ίσως)

•Η παρουσία σας στο μάθημα αποτελεί βασική προϋπόθεση για να μάθετε!
.....γιαυτό .... **Είναι υποχρεωτική!**

•Πώς θα δουλέψουμε?

Κατά κύριο λόγο στην τάξη – φέτος στην ψηφιακή !

Παρακολουθώντας συστηματικά μέσα από τις δημοσιεύσεις την ερευνητική πορεία ομάδων με σημαντική συμβολή στο πεδίο!

➢Από τη μια σειρά πειραμάτων στην επόμενη!

➢Ερμηνεία-Συμπέρασμα-Σχεδιασμός επόμενης φάσης – Νέα πειράματα

Ορισμένες ενότητες με κλασσικό τρόπο (κάλυψη ύλης)

1-2 ενότητες μέσω ανεστραμμένης τάξης

43

Μαθήματα κάθε Τρίτη 15-17 μ.μ. (και κάποια έξτρα) πρόγραμμα σήμερα στο eclass!

•Η παρουσία σας στο μάθημα αποτελεί βασική προϋπόθεση για να μάθετε!
.....γιαυτό .... Είναι υποχρεωτική!

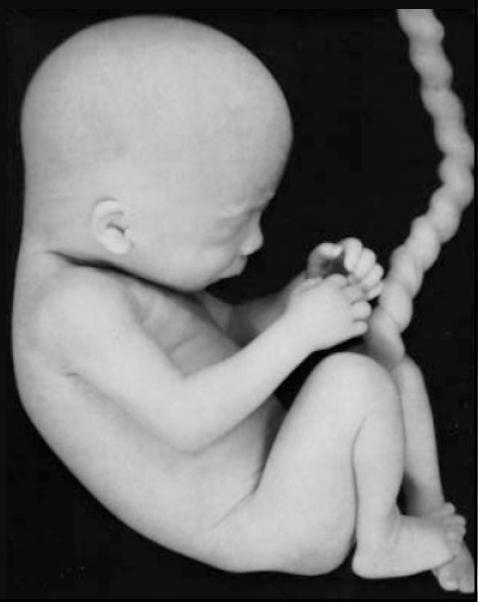
Βαθμολογία

- Εργασία στην ψηφιακή τάξη
- Εργασία στο σπίτι
- Εργασία – παρουσίαση σε ομάδες των τριών

Δεν υπάρχουν εξετάσεις τον Ιούνιο!

44

Βιολογία Βλαστοκυττάρων και Αναγέννησης



Martin Evans' saga

Μ. Γρηγορίου 2022

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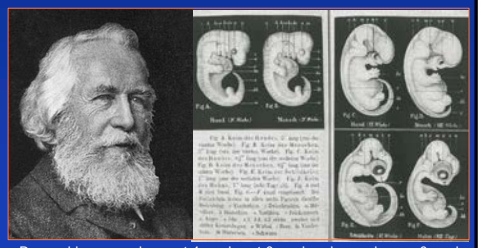
Βλαστοκύτταρα

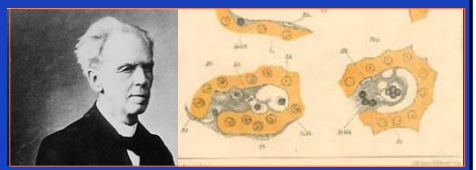
- Ως **βλαστοκύτταρο** χαρακτηρίζεται ένα κύτταρο το οποίο έχει την ικανοτητα να **αυτοαναγεννάται** (δηλ. να δίνει απογόνους ίδιους με αυτό) αλλά και να δίνει απογόνους που είναι **διαφοροποιημένα κύτταρα**.

•Ο όρος εμφανίζεται από τον Ernst Haeckel ο οποίος τον χρησιμοποιεί για το γονιμοποιημένο αυγό αλλά και για ένα πρόδρομο αρχαϊκό μονοκύτταρο οργανισμό.



Dog and human embryos at 4 weeks, at 6 weeks, shown above a 6-week turtle embryo and 8-day hen embryo (Haeckel 1868) as convincing proof of evolution.

•Αργοτερα ο Ernst Neumann (υιός) χρησιμοποίησε αυτόν τον όρο για να υποδηλώσει ένα κύτταρο του μυελού των οστών από το οποίο παράγονται τα κύτταρα του αιμοποιητικού συστήματος.



Draft by Ernst Neumann showing the development of erythropoiesis in embryonic liver 1914; GrLK: great lymphozyt (stem cell); Erblk: Erythroblast; Rk: original nucleus of reticulum cell(another picture see button stem cell).

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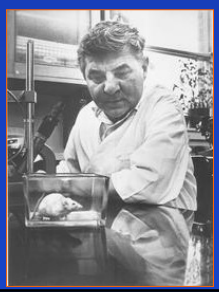
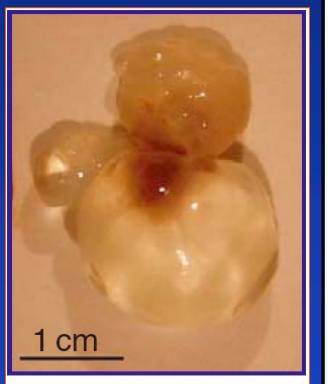
Τα τερατώματα και τα τερατοκαρκινώματα

- Τα τερατώματα και τα τερατοκαρκινώματα είναι καρκίνοι των κυττάρων της αναπαραγωγικής σειράς (Germ cell tumors -GCT).
- Τα GCT χαρακτηρίζονται από την παρουσία πολλών διαφορετικών κυτταρικών τύπων (και των τριών βλαστικών στοιβάδων) αυτό δείχνει ότι οι καρκίνοι αυτοί προέρχονται από **πολυδύναμα κύτταρα**.
- Ο Stevens έδειξε ότι στο στέλεχος 129 παρατηρείται αυξημένη συχνότητα τερατωμάτων και τερατοκαρκινωμάτων.





Teratoma

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Τα τερατώματα και τα τερατοκαρκινώματα

There is much confusion regarding the terminology of teratoma/ teratocarcinoma in the experimental setting, partially owing to inconsistencies in the use of medical terminology (Damjanov and Andrews, 1987).

From a histopathological point of view, **benign GCTs with differentiation** to all embryonic germ layers are termed "**teratomas**." These can be mature teratomas (which contain only mature, well-differentiated tissues) or immature teratomas (which contain tissues of more embryonic, less-differentiated nature).

If the tumors also contain **clusters of totally undifferentiated**, highly malignant embryonic carcinoma cells, than they are defined as "**teratocarcinomas**" (Gonzalez-Crussi, 1982; Pierce et al., 1960).

Lensch and Ince, 2007

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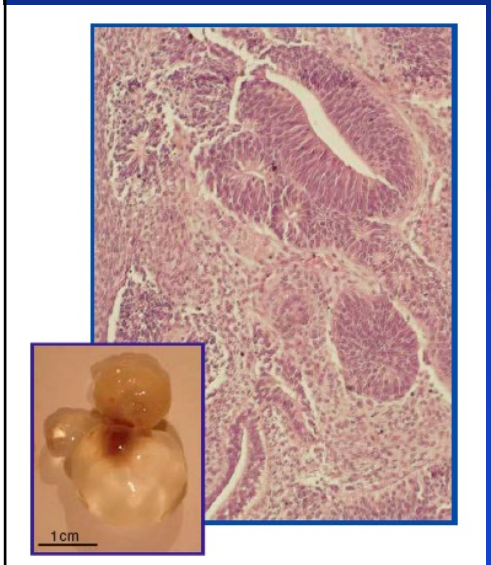
Τα ECC



Ονόμασαν το πολυδύναμο κύτταρο που ευθύνονταν για την καρκινογένεση pluripotent tumor initiating cell embryonal carcinoma cell (ECC).

Τα ECC είναι τα πρώτα βλαστοκύτταρα που χαρακτηρίστηκαν με κριτήριο το αναπτυξιακό δυναμικό και την αυτο-ανανέωση (1964)

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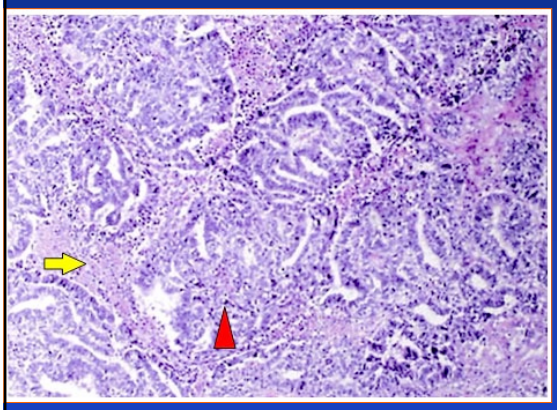
Τα ECC



Απαντούν σε μικρούς θύλακες (nests) μέσα στον όγκο.

Ο καρυότυπός τους συνήθως είναι ανώμαλος (ανευπλοειδία)

Όγκοι που δεν περιλαμβάνουν τέτοιους θύλακες δεν είναι δυνατόν να δώσουν μετά από μεταμόσχευση νέους όγκους -και γενικά τέτοιοι όγκοι αναπτύσσονται πολύ αργά.

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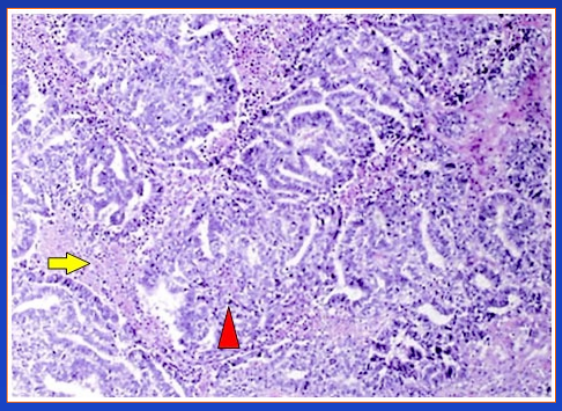
Τα ECC

Μεταμόσχευση **ενός ECC** (Kleinsmith and Pierce (1964) έχει ως αποτέλεσμα το σχηματισμό ενός όγκου που περιλαμβάνει:

- ECC (κόκκινο βέλος) αλλά και
- πολλούς άλλους κυτταρικούς τύπους.
- Τα πειράματα αυτά κατέδειξαν την πολυδυναμία των ECC .



Τα ECC είναι τα πρώτα βλαστικά κύτταρα που χαρακτηρίστηκαν (1964) με κριτήριο το αναπτυξιακό δυναμικό και την αυτο-ανανέωση (1964)

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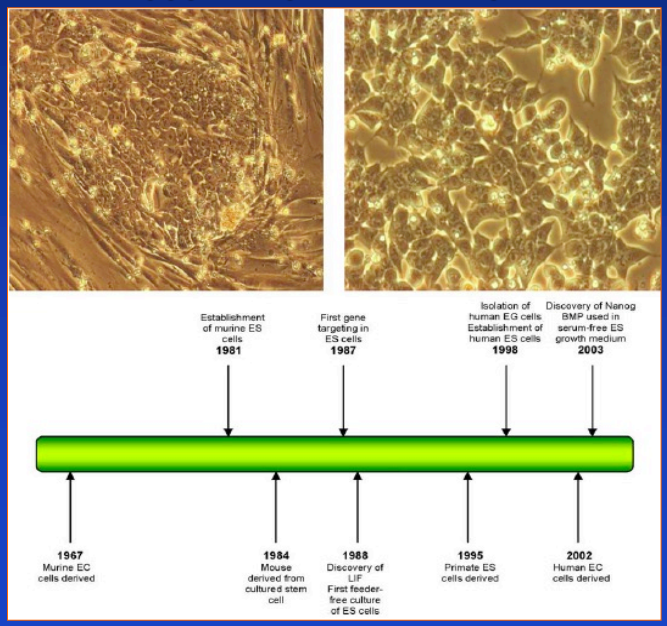
Εμβρυϊκά βλαστοκύτταρα



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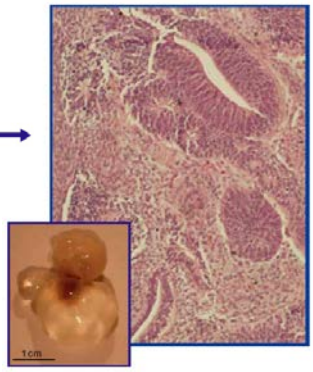
Origin of Testicular Teratomas From Primordial Germ Cells in Mice [1,2]

LEROY C. STEVENS, *The Jackson Laboratory, Bar Harbor, Maine 04609*

JOURNAL OF THE NATIONAL CANCER INSTITUTE

SUMMARY—Teratomas were experimentally induced in testes of strain 129 mice when 12½-day genital ridges from fetuses were grafted into the testes of adults. Seventy-five percent of 194 testes developing from implanted $Sl^{d}/+$, $Sl^{4}/+$, and $+/+$ genital ridges contained teratomas. These gonads had normal numbers of primordial germ cells. In contrast, only 3% of 75 Sl^{1}/Sl^{1} and Sl^{4}/Sl^{4} testes contained teratomas. These gonads had very few primordial germ cells. This finding supports strongly the hypothesis that testicular teratomas in mice are derived from primordial germ cells.—J Nat Cancer Inst 38: 549–552, 1967.



Pierce *et al.* (*19*) and I (*21*) have observed structures in a transplantable teratoma of the mouse with epithelia resembling neural fold, amnion, yolk sac, and condensations of mesodermal cells resembling somites, all in their proper relationships to one another. I believe that these structures in the mouse are embryoid, and Pierce *et al.* (*19*) think that human teratocarcinomas are similar to those found in the mouse.

Τα πειράματα αυτά έδειξαν ότι στις γεννητικές ακρολοφίες απαντούν κύτταρα με ιδιότητες παρόμοιες με αυτές των ECC.

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DEVELOPMENTAL BIOLOGY 21, 364–382 (1970)

The Development of Transplantable Teratocarcinomas from Intratesticular Grafts of Pre- and Postimplantation Mouse Embryos[1]

LEROY C. STEVENS
The Jackson Laboratory, Bar Harbor, Maine 04609

Accepted August 8, 1969

When 3- and 6-day mouse embryos are grafted into the testes of adults they become disorganized and develop into growths composed of many kinds of tissues. In some grafts, some of the cells remain undifferentiated for remarkably long periods of time. These cells are pluripotent and they may continue to proliferate indefinitely and serve as stem cells of teratomas. These teratomas can be transplanted serially, and they may be composed of many kinds of tissues, or they may progress along one of several morphological lines. For example, they may become predominantly embryonic, neural, or parietal yolk sac tumors. When some of these transplantable teratomas were grafted intraperitoneally, they produced embryoid bodies which morphologically resemble early normal mouse embryos. Teratomas were derived from embryos of strains 129/Sv, A/He, and F, hybrids between these strains. The tumors resemble in every respect the spontaneous testicular teratomas characteristic of strain 129/Sv.

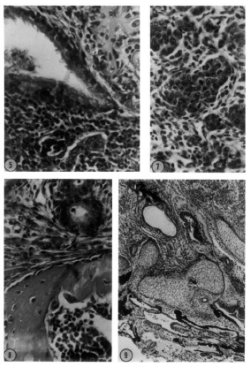
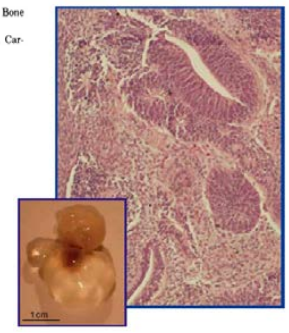


FIG. 5. Metastatic growth in the left renal lymph node derived from a 6-day embryo grafted to the testis for 30 days. Note ciliated epithelium and bone (upper right). Lymphoid cells below.

FIG. 7. Transplantable teratoma OTT 5568 derived from a 3-day embryo. Solid clump of undifferentiated cells surrounded by mesenchyme. Fifteenth transplant generation.

FIG. 8. Transplantable teratoma OTT 5938 derived from a 3-day embryo. Bone with marrow, immature muscle fibers, and ciliated epithelium.

FIG. 9. Primary graft which gave rise to transplantable teratoma OTT 6196. Cartilage, muscle, epithelium, neural tissue, pigment, and embryonic cells.



Τα πειράματα αυτά έδειξαν ότι **στα φυσιολογικά έμβρυα απαντούν κύτταρα με ιδιότητες παρόμοιες με αυτές των ECC.**

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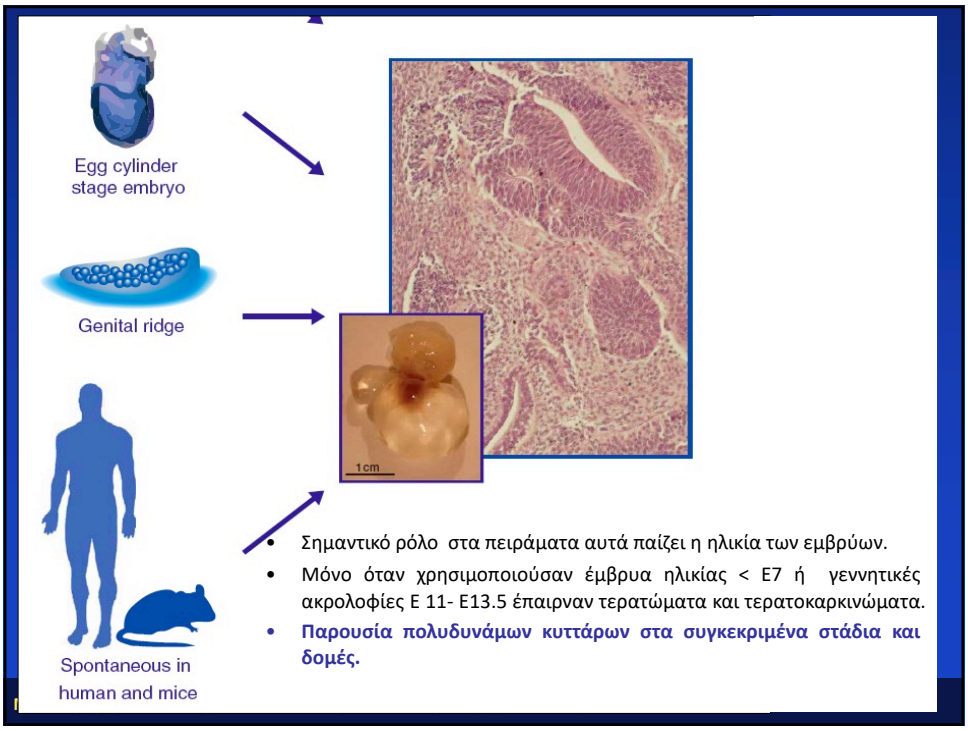

- Σημαντικό ρόλο στα πειράματα αυτά παίζει η ηλικία των εμβρύων.
- Μόνο όταν χρησιμοποιούσαν έμβρυα ηλικίας < Ε7 ή γεννητικές ακρολοφίες Ε 11- Ε13.5 έπαιρναν τερατώματα και τερατοκαρκινώματα.
- **Παρουσία πολυδυνάμων κυττάρων στα συγκεκριμένα στάδια και δομές.**

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Multipotentiality of Single Embryonal Carcinoma Cells*

LEWIS J. KLEINSMITH† AND G. BARRY PIERCE, JR.‡
(Department of Pathology, The University of Michigan, Ann Arbor, Michigan)

In order to test the hypothesis that embryonal carcinoma cells are multipotential stem cells of a teratocarcinoma, an *in vivo* cloning technic was designed. Small embryoid bodies containing mostly embryonal carcinoma were obtained from ascitic conversion of a murine teratocarcinoma and were dissociated with trypsin to form a suspension of single cells; the single cells were picked up in small capillary tubes and transplanted directly into mice.

From over 1700 single cell grafts, 44 clonal lines were obtained; 43 of these lines were teratocarcinomas composed of as many as fourteen well differentiated somatic tissues in addition to embryonal carcinoma. These 43 lines varied in their degree of differentiation, capacity to produce embryoid bodies, and in growth rate. The remaining clonal line showed limited potential for differentiation, producing only yolk sac, trophoblast, and embryonal carcinoma.

The results demonstrated the multipotentiality of single embryonal carcinoma cells, as well as the heterogeneity of the embryonal carcinoma of a teratocarcinoma. The capacity of single embryonal carcinoma cells to differentiate into benign tissues supports neither the dogma of the irreversibility of the malignant transformation nor the somatic cell mutation theory of cancer. These findings were interpreted as giving strong support to the stem cell theory of cancer.

TABLE 2
INCIDENCE (PER CENT) OF DIFFERENTIATED TISSUES, GROWTH RATE, AND EMBRYOID BODY PRODUCTION OF SEVERAL CLONES

TISSUES PRESENT	STOCK TERATOCARCINOMA	CLONES			
		NRS-C39	NRS-Cl 18	NRS-Cl 35	NRS-Cl 38
Number of tumors:*	14	10	7	8	10
Embryonal carcinoma	100	100	100	100	100
Astrocytes	100	100	100	100	100
Ependyma	100	100	100	100	100
Simple glands	76	40	14	100	60
Trophoblast	39	20	29	100	40
Squamous epithelium	79	10	0	75	0
Mesenchyme	71	30	14	100	60
Cartilage	50	0	14	25	40
Bone	7	0	14	25	30
Smooth muscle	57	0	0	38	30
Striated muscle	43	0	0	25	30
Notochord	0	0	0	0	10
Ciliated epithelium	71	30	14	88	40
Visceral yolk sac	100	100	0	100	100
Parietal yolk sac	79	10	29	50	70
Growth rate (days)	28	35	33	54	37
Embryoid bodies	2-layered	2-layered	None	3-layered	3-layered

* At least five generations.

This paper demonstrates the multipotentiality of single embryonal carcinoma cells and presents the technique which was successful in cloning embryonal carcinoma by enzymatic dissociation of cells from fresh tissue, followed by *in vivo* transplantation of single cells.

Ένα μοναδικό κύτταρο που μεταμορφώνεται *in vivo* να ανασυστήσει έναν όγκο που περιλαμβάνει ως και 14 διαφορετικά είδη διαφοροποιημένων κυττάρων.

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Τα Εμβρυϊκά Βλαστοκύτταρα

Οι μελέτες που είχαν γίνει στα τερατοκαρκινώματα έδειχναν την ύπαρξη πολυδύναμων κυττάρων παρόμοιων με αυτά των τερατοκαρκινωμάτων.

Δημιουργήθηκαν αρκετές κυτταρικές σειρές που προέρχονταν από κύτταρα τερατοκαρκινωμάτων.

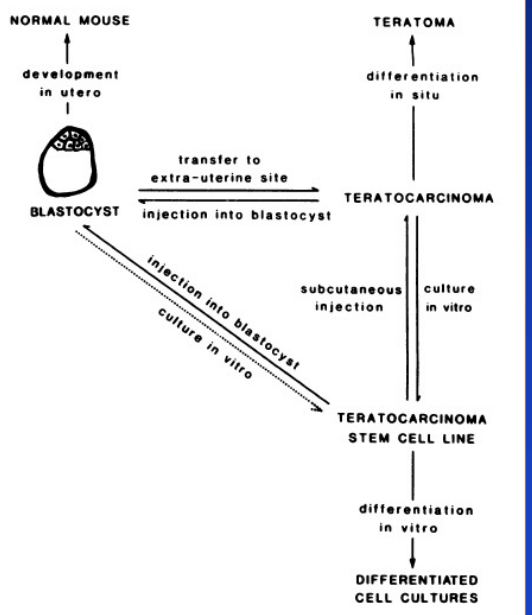

FIG. 1. Relationship between normal embryos and teratocarcinoma stem cells. Adapted from Martin (10).

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J. Embryol. exp. Morph. Vol. 28, 1, pp. 163–176, 1972
Printed in Great Britain

The isolation and properties of a clonal tissue culture strain of pluripotent mouse teratoma cells

By MARTIN J. EVANS[1]
From the Department of Anatomy and Embryology, University College London

SUMMARY

A clonal tissue culture strain of pluripotent cells has been isolated from a transplantable teratoma of inbred strain of mice 129 Sv-Sl[i] CP. This cell strain SIKR when re-inoculated into mice produces teratomas containing at least ten types of tissue. Sub-clones have been isolated and two types distinguished.

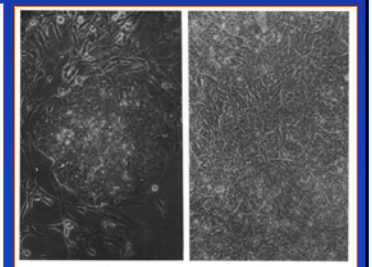
(1) 'C-type' with a densely-piled *in vitro* growth. These are tumourigenetic and pluripotent displaying a comparable range of differentiation to the original SIKR.

(2) 'E-type' spreading, often epithelioid growth. These grow to a lower density in culture than 'C-type'. Mostly non-tumourigenetic; in those cases where a tumour has been obtained it did not display multiple differentiations.

The results are interpreted as demonstrating that the culture consists of equivalently pluripotent cells which may become determined and differentiate spontaneously *in vitro* into slower growing cell types which are continuously overgrown by the culture.

Table 2. *Tissues found in the tumours produced after subcutaneous inoculation of mice with SIKR and 10 different sub-clones of SIKR*

Tissue	SIKR tumours	Tumours from SIKR sub-clones 1	2	3	4	5	6	7	8	9	10
Embryonal carcinoma	+	+	+	+	+	+	+	+	+	+	+
Mesenchyme	+	+	+	+	+	+	+	+	+	+	+
Epithelium	+	+	+	+	+	+	+	+	+	+	+
Embryonal yolk sac	+	+	+	+	+	+	+	+	.	+	+
Neural tissue	+	+	+	+	+	+	+	+	+	+	.
Cartilage	+	+	+	+	+	.	+	.	+	+	+
Keratinizing epithelium	+	+	+	.	.	+	.	.	.	.	.
Trophoblast	+	+	+	+	+	+	.	.	.	.	.
Muscle	+	+	.	.	.	.	+	+	+	.	.
Adipose tissue	+	+	+	+	.	.	.	.	.	.	.
Sebaceous gland	+	.	.	.	.	.	.	.	.	.	.

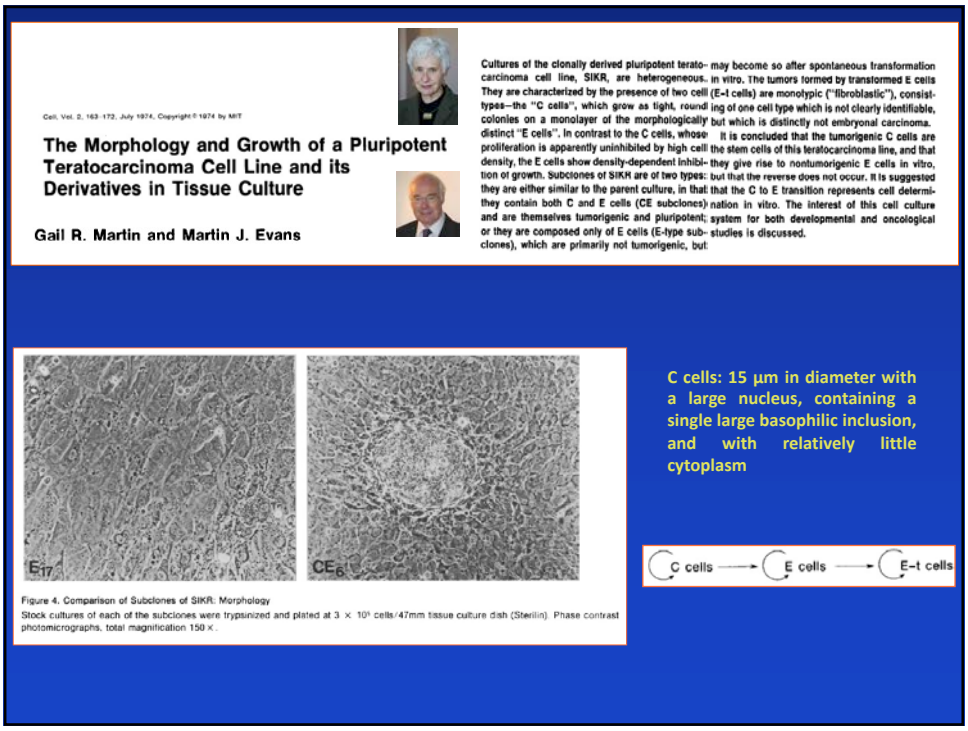


A B

Fig. 3A. A typical C-type colony of teratoma cells *in vitro*; phase contrast. ×125.
Fig. 3B. An area from a clone of E-type teratoma cells *in vitro*; phase contrast. ×125.

Ο Evans είναι ο πρώτος που θα καλλιεργήσει κύτταρα τερατοκαρκινωμάτων προσπαθώντας να τα μελετήσει και ***in vitro*** και όχι μόνο *in vivo* μετά από μεταμόσχευση.

58



Cell, Vol. 2, 163-172, July 1974, Copyright © 1974 by MIT

The Morphology and Growth of a Pluripotent Teratocarcinoma Cell Line and its Derivatives in Tissue Culture

Gail R. Martin and Martin J. Evans

Cultures of the clonally derived pluripotent terato- may become so after spontaneous transformation carcinoma cell line, SIKR, are heterogeneous. in vitro. The tumors formed by transformed E cells They are characterized by the presence of two cell (E-t cells) are monotypic ("fibroblastic"), consist- types—the "C cells", which grow as tight, roundi ing of one cell type which is not clearly identifiable, colonies on a monolayer of the morphologically but which is distinctly not embryonal carcinoma. distinct "E cells". In contrast to the C cells, whose It is concluded that the tumorigenic C cells are proliferation is apparently uninhibited by high cell the stem cells of this teratocarcinoma line, and that density, the E cells show density-dependent inhibi- they give rise to nontumorigenic E cells in vitro, tion of growth. Subclones of SIKR are of two types: but that the reverse does not occur. It is suggested they are either similar to the parent culture, in that that the C to E transition represents cell determi- they contain both C and E cells (CE subclones) nation in vitro. The interest of this cell culture and are themselves tumorigenic and pluripotent; system for both developmental and oncological or they are composed only of E cells (E-type sub- studies is discussed. clones), which are primarily not tumorigenic, but

Figure 4. Comparison of Subclones of SIKR: Morphology
Stock cultures of each of the subclones were trypsinized and plated at 3 × 10⁵ cells/47mm tissue culture dish (Sterilin). Phase contrast photomicrographs, total magnification 150×.

C cells: 15 µm in diameter with a large nucleus, containing a single large basophilic inclusion, and with relatively little cytoplasm

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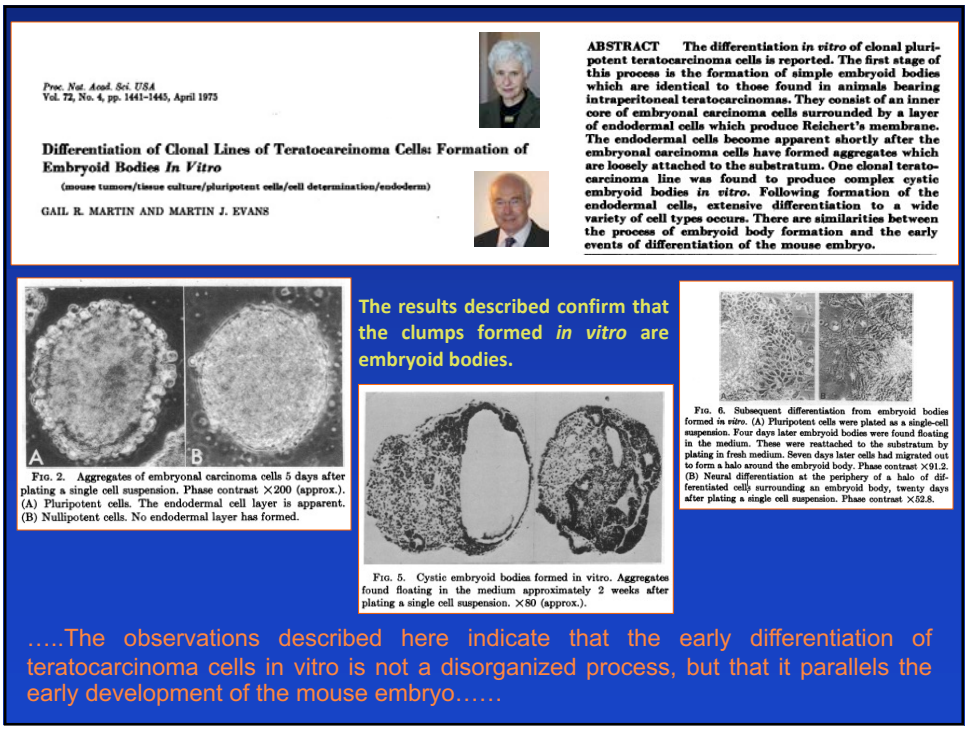

Proc. Nat. Acad. Sci. USA
Vol. 72, No. 4, pp. 1441–1445, April 1975

Differentiation of Clonal Lines of Teratocarcinoma Cells: Formation of Embryoid Bodies *In Vitro*

(mouse tumors/tissue culture/pluripotent cells/cell determination/endoderm)

GAIL R. MARTIN AND MARTIN J. EVANS

ABSTRACT **The differentiation *in vitro* of clonal pluripotent teratocarcinoma cells is reported. The first stage of this process is the formation of simple embryoid bodies which are identical to those found in animals bearing intraperitoneal teratocarcinomas. They consist of an inner core of embryonal carcinoma cells surrounded by a layer of endodermal cells which produce Reichert's membrane. The endodermal cells become apparent shortly after the embryonal carcinoma cells have formed aggregates which are loosely attached to the substratum. One clonal teratocarcinoma line was found to produce complex cystic embryoid bodies *in vitro*. Following formation of the endodermal cells, extensive differentiation to a wide variety of cell types occurs. There are similarities between the process of embryoid body formation and the early events of differentiation of the mouse embryo.**

FIG. 2. Aggregates of embryonal carcinoma cells 5 days after plating a single cell suspension. Phase contrast ×200 (approx.). (A) Pluripotent cells. The endodermal cell layer is apparent. (B) Nullipotent cells. No endodermal layer has formed.

The results described confirm that the clumps formed *in vitro* are embryoid bodies.

FIG. 5. Cystic embryoid bodies formed in vitro. Aggregates found floating in the medium approximately 2 weeks after plating a single cell suspension. ×80 (approx.).

FIG. 6. Subsequent differentiation from embryoid bodies formed *in vitro*. (A) Pluripotent cells were plated as a single-cell suspension. Four days later embryoid bodies were found floating in the medium. These were reattached to the substratum by plating in fresh medium. Seven days later cells had migrated out to form a halo around the embryoid body. Phase contrast ×91.2. (B) Neural differentiation at the periphery of a halo of differentiated cells surrounding an embryoid body, twenty days after plating a single cell suspension. Phase contrast ×52.8.

…..The observations described here indicate that the early differentiation of teratocarcinoma cells in vitro is not a disorganized process, but that it parallels the early development of the mouse embryo……

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I had turned my anxiety into my profession.

Proc. Nat. Acad. Sci. USA
Vol. 70, No. 10, pp. 2988–2992, October 1973

Surface Antigens Common to Mouse Cleavage Embryos and Primitive Teratocarcinoma Cells in Culture

(cytotoxic test/peroxidase-coupled antibody against mouse immunoglobulin/cell differentiation)

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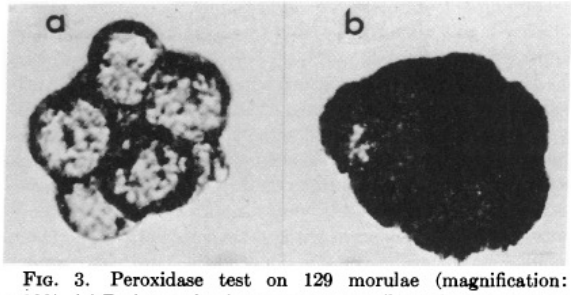

FIG. 3. Peroxidase test on 129 morulae (magnification: ×800`. (*a*) Preimmunization serum 1:800; (*b*) antiserum against F9, 1:800.

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Fate of teratocarcinoma cells injected into early mouse embryos

V. E. Papaioannou
M. W. McBurney
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Nature Vol. 258 November 6 1975

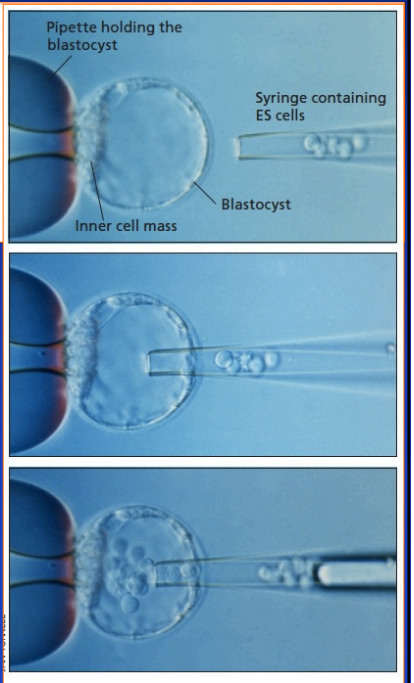

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Table 3 Chimaeric animals

Animal code	Sex	Injected cell line	Animal fate	Age at death (d)	Pigment: Coat	Pigment: Eyes	Blood	Brain	Gonads	Gut	Heart	Kidney	Liver	Lung	GPI: Skeletal muscle	GPI: Spinal cord	Spleen	Thymus	Tumour	Tumour location
T120	f	SIKR-OSB	k	42	C	?	C	H	C	—	C	C	H	C	C	—	H	C		None
T84	f	C17	l		H	C	H	—	—	—	—	—	—	—	—	—	—	—		None
T43	m	C86	d	8	H	H	—	H	C	—	H	H	C	—	C	—	C	—	C	s.c. neck
T65	f	C86	d	26	H	H	H	H	—	—	H	H	H	H	H	—	H	—	C	s.c. thorax
																			C	s.c. below eye
																			C	abdominal cavity
T72	m	C86	d	5	H	H	—	H	H	H	H	H	H	C	C	—	—	—	C	s.c. under ear

k, Killed; d, died; l, live; H, host; C, chimaeric; —, not analysed; ?, not analysable; s.c., subcutaneous.

" I should like to suggest that it may be quite feasible to obtain cultures of pluripotent cells directly from the embryo now that experience has been obtained handling such cells"

Evans 1975

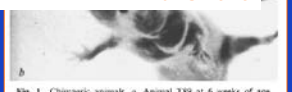

Fig. 1 Chimaeric animals. *a*, Animal T89 at 6 weeks of age showing pigmented area of coat. *b*, Animal T38 at 2 weeks of age showing three subcutaneous tumours in the neck and throat regions.

……that some of these animals showed later-origin tumours of differentiated cell types.

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Proc. Nat. Acad. Sci. USA
Vol. 72, No. 9, pp. 3585–3589, September 1975
Cell Biology

Normal genetically mosaic mice produced from malignant teratocarcinoma cells

(embryonal carcinoma/teratoma/embryoid body core cells/blastocyst injection/allophenic mice)

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Institute for Cancer Research, Fox Chase, Philadelphia, Pennsylvania 19111

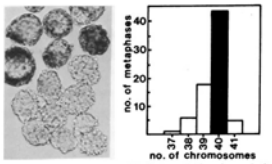

FIG. 3 (*left*). Living embryoid body "cores" (clear, bottom) of embryonal carcinoma cells after removal of the yolk sac "rinds". Intact embryoid bodies (dark, top) are shown for comparison.

FIG. 4 (*right*). The normal chromosome number of 40 is the modal number in metaphases of embryonal carcinoma cells taken from the "cores" of embryoid bodies grown only *in vivo*.

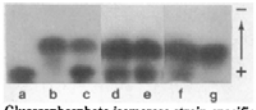

FIG. 9. Glucosephosphate isomerase strain-specific allelic variants in starch gel electrophoresis of tissue homogenates from 129 (alot a) and C57 (b) controls, and a 1:1 control mixture (c). Female *mosaic mouse no.* 2 has tumor-derived (129-strain) cells in thymus (d), kidneys (e), and reproductive tract (f), but not in blood (g).

ABSTRACT Malignant mouse teratocarcinoma (or embryonal carcinoma) cells with a normal modal chromosome number were taken from the "cores" of embryoid bodies grown only *in vivo* as an ascites tumor for 8 years, and were injected into blastocysts bearing many genetic markers, in order to test the developmental capacities, genetic constitution, and reversibility of malignancy of the core cells. Ninety-three live normal pre- and postnatal animals were obtained. Of 14 thus far analyzed, three were cellular genetic mosaics with substantial contributions of tumor-derived cells in many developmentally unrelated tissues, including some never seen in the solid tumors that form in transplant hosts. The tissues functioned normally and synthesized their specific products (e.g., immunoglobulins, adult hemoglobin, liver proteins) coded for by strain-type alleles at known loci. In addition, a tumor-contributed color gene, *steel*, not previously known to be present in the carcinoma cells, was detected from the coat phenotype. Cells derived from the carcinoma, which is of X/Y sex chromosome constitution, also contributed to the germ line and formed reproductively functional sperms, some of which transmitted the *steel* gene to the progeny. Thus, after almost 200 transplant generations as a highly malignant tumor, embryoid body core cells appear to be developmentally totipotent and able to express, in an orderly sequence in differentiation of somatic and germ-line tissues, many genes hitherto silent in the tumor of origin. This experimental system of "cycling" teratocarcinoma core cells through mice, in conjunction with experimental mutagenesis of those cells, may therefore provide a new and useful tool for biochemical, developmental, and genetic analyses of mammalian differentiation.

The results also furnish an unequivocal example in animals of a non-mutational basis for transformation to malignancy and of reversal to normalcy. The origin of this tumor from a disorganized embryo suggests that malignancies of some other, more specialized, stem cells might arise comparably through tissue disorganization, leading to developmental aberrations of gene expression rather than changes in gene structure.

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DEVELOPMENTAL BIOLOGY 21, 364–382 (1970)

The Development of Transplantable Teratocarcinomas from Intratesticular Grafts of Pre- and Postimplantation Mouse Embryos[1]

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The Jackson Laboratory, Bar Harbor, Maine 04609

Accepted August 8, 1969

When 3- and 6-day mouse embryos are grafted into the testes of adults they become disorganized and develop into growths composed of many kinds of tissues. In some grafts, some of the cells remain undifferentiated for remarkably long periods of time. These cells are pluripotent and they may continue to proliferate indefinitely and serve as stem cells of teratomas. These teratomas can be transplanted serially, and they may be composed of many kinds of tissues, or they may progress along one of several morphological lines. For example, they may become predominantly embryonic, neural, or parietal yolk sac tumors. When some of these transplantable teratomas were grafted intraperitoneally, they produced embryoid bodies which morphologically resemble early normal mouse embryos. Teratomas were derived from embryos of strains 129/Sv, A/He, and F, hybrids between these strains. The tumors resemble in every respect the spontaneous testicular teratomas characteristic of strain 129/Sv.

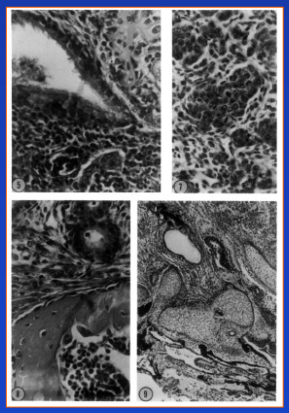

FIG. 5. Metastatic growth in the left renal lymph node derived from a 6-day embryo grafted to the testis for 30 days. Note ciliated epithelium and bone (upper right). Lymphoid cells below.

FIG. 7. Transplantable teratoma OTT 5568 derived from a 3-day embryo. Solid clump of undifferentiated cells surrounded by mesenchyme. Fifteenth transplant generation.

FIG. 8. Transplantable teratoma OTT 5938 derived from a 3-day embryo. Bone with marrow, immature muscle fibers, and ciliated epithelium.

FIG. 9. Primary graft which gave rise to transplantable teratoma OTT 6196. Cartilage, muscle, epithelium, neural tissue, pigment, and embryonic cells.

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Proc. Nat. Acad. Sci. USA
Vol. 70, No. 10, pp. 2988–2992, October 1973

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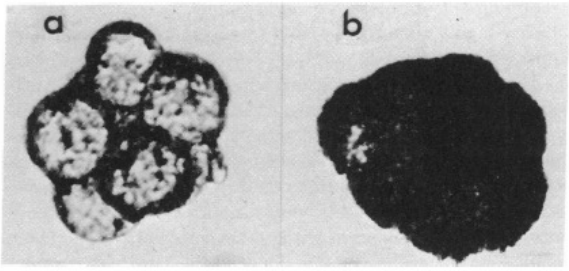

FIG. 3. Peroxidase test on 129 morulae (magnification: ×800`. (*a*) Preimmunization serum 1:800; (*b*) antiserum against F9, 1:800.

The same specific cell surface antigens are present upon the cells of early mouse embryos and germ cells !

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J. Embryol. exp. Morph. Vol. 59, pp. 187–206, 1980
Printed in Great Britain © Company of Biologists Limited 1980

Changes in protein synthesis during differentiation of embryonal carcinoma cells, and a comparison with embryo cells

By R. H. LOVELL-BADGE[1] AND M. J. EVANS[1]
From the Department of Genetics, University of Cambridge

Two-dimensional electrophoresis was used to find changes in protein synthesis occurring as pluripotent embryonal carcinoma (EC) cells differentiate to give embryoid bodies *in vitro*. 2-D patterns from other embryonic cell lines, and from the inner cell mass (ICM) cells of mouse embryos, were also analysed for the expression of those proteins showing some change during embryoid body formation and for overall differences between these and the EC cells.

Most changes in protein synthesis occurred before 12 h but endoderm was not discerned morphologically on the outside of EC cell clumps until at least 18 h after their suspension. The number of changes occurring is small compared with the number of polypeptides resolved, but is in line with similar studies. Comparisons with nullipotent EC cells and an endodermal cell line have allowed these changes to be assigned, tentatively, to the different cell types within embryoid bodies, and may allow them to be used as markers of differentiation.

Comparisons between the 2-D patterns derived from ICMs and EC cells reveal substantial differences between the two that might not have been expected from their developmental homology. The importance of these differences to their pluripotentiality is discussed.

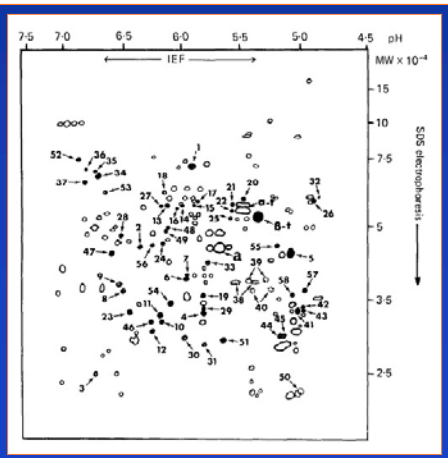

Fig. 4. Diagrammatic representation of the protein changes occurring during EC cell differentiation and the expression of these proteins in other cell types. [1] Refers to lower M.W. form. [2] Refers to higher M.W. form. ....., Undetectable; ——, just detectable.

EC cells very similar to early embryo cell types but in particular 5 day ectoderm

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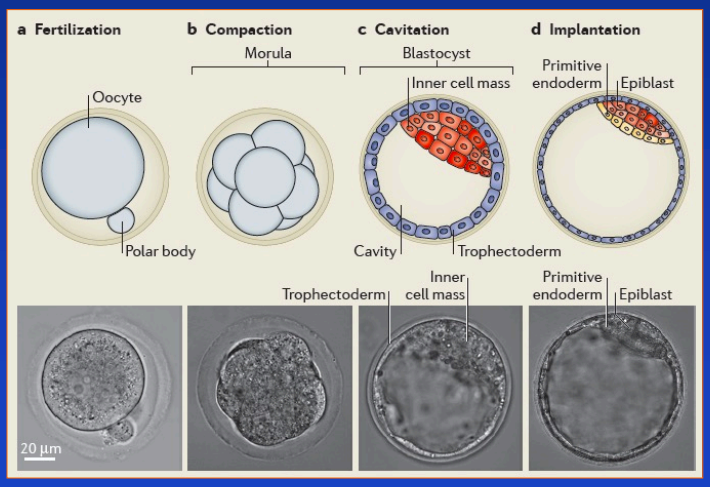
....και τώρα λίγη Ανάπτυξη!



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Establishment in culture of pluripotential cells from mouse embryos

M. J. Evans* & M. H. Kaufman†
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Nature Vol. 292 9 July 1981

Pluripotential cells are present in a mouse embryo until at least an early post-implantation stage, as shown by their ability to take part in the formation of chimaeric animals[1] and to form teratocarcinomas[2]. Until now it has not been possible to establish progressively growing cultures of these cells *in vitro*, and cell lines have only been obtained after teratocarcinoma formation *in vivo*. We report here the establishment in tissue culture of pluripotent cell lines which have been isolated directly from *in vitro* cultures of mouse blastocysts. These cells are able to differentiate either *in vitro* or after innoculation into a mouse as a tumour *in vivo*. They have a normal karyotype.

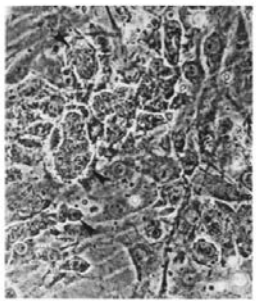

Fig. 1 Groups of pluripotential embryo cells (arrowed) growing in monolayer culture on a background of mitomycin C-inhibited STO cells. The isolation of a definite cell line from a blastocyst takes only ~3 weeks and the pluripotential cell colonies are visible within 5 days of passage. We have had 30% yield of lines from blastocysts in one experiment. Two of the lines have been rigorously cloned by single-cell isolation but most were only colony-picked—this makes no difference.

Efforts to isolate normal conterpart of EC cell

Potential problems:

Only very small numbers of founder cells available? Improve cloning / media. (Martin's Magic Medium" or MMM)

•Timing? Early stages

•Conditions to retain pluripotency – normal cells differentiate quickly in culture? implantational delay - diapause.

Kaufman used **implantational delay** to grow parthenogenetic embryos to an early post-implantation stage.

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Διάπαυση Diapause

Εμβρυϊκή διάπαυση: η παροδική διακοπή της ανάπτυξης του εμβρύου.

✓Πολύ συνηθισμένη στα αρθρόποδα.

✓Παρατηρείται σε πολλά είδη θηλαστικών περίπου 100 έχουν περιγραφεί

✓Ο ρυθμός πολλαπλασιασμού των κυττάρων της βλαστοκύστης επιβραδύνεται (σχεδόν σταματά)

✓Μερικές μέρες μέχρι και μερικούς μήνες

✓Χαμηλή μεταβολική και συνθετική ενεργότητα

✓Υποχρεωτική σε μερικά είδη (νυχτερίδες, εντομοφάγα)- εξασφάλιση γέννησης του μικρού την άνοιξη

✓Περιστασιακή σε άλλα (τρωκτικά, μαρσιποφόρα) (διάφοροι περιβαλλοντικοί λόγοι)

✓Επανέναρξη της ανάπτυξης μετά από αλλαγή στο ορμονικό προφίλ της μητέρας.

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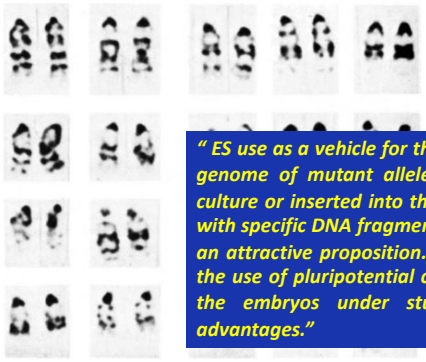
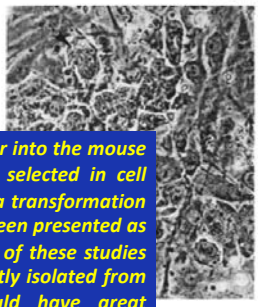


Fig. 2 Karyotype of an embryo-derived pluripotential cell line, 40XY. Over 80% of the spreads of this clonal line possessed 40 chromosomes and had a clearly identifiable Y chromosome.



…yo cells (arrowed) growing in … mitomycin C-inhibited STO … from a blastocyst takes only … 3 weeks and the pluripotential cell colonies are visible within 5 days of passage. We have had 30% yield of lines from blastocysts in one experiment. Two of the lines have been rigorously cloned by single-cell isolation but most were only colony-picked—this makes no difference.

" ES use as a vehicle for the transfer into the mouse genome of mutant alleles, either selected in cell culture or inserted into the cells via transformation with specific DNA fragments, has been presented as an attractive proposition. In many of these studies the use of pluripotential cells directly isolated from the embryos under study should have great advantages."

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Proc. Natl. Acad. Sci. USA
Vol. 78, No. 12, pp. 7634–7638, December 1981
Developmental Biology

Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells

(embryonic stem cells/inner cell masses/differentiation in vitro/embryonal carcinoma cells/growth factors)

GAIL R. MARTIN
Department of Anatomy, University of California, San Francisco, California 94143

Communicated by J. Michael Bishop, September 14, 1981

ABSTRACT **This report describes the establishment directly from normal preimplantation mouse embryos of a cell line that forms teratocarcinomas when injected into mice. The pluripotency of these embryonic stem cells was demonstrated conclusively by the observation that subclonal cultures, derived from isolated single cells, can differentiate into a wide variety of cell types. Such embryonic stem cells were isolated from inner cell masses of late blastocysts cultured in medium conditioned by an established teratocarcinoma stem cell line. This suggests that such conditioned medium might contain a growth factor that stimulates the proliferation or inhibits the differentiation of normal pluripotent embryonic cells, or both. This method of obtaining embryonic stem cells makes feasible the isolation of pluripotent cells lines from various types of noninbred embryo, including those carrying mutant genes. The availability of such cell lines should make possible new approaches to the study of early mammalian development.**

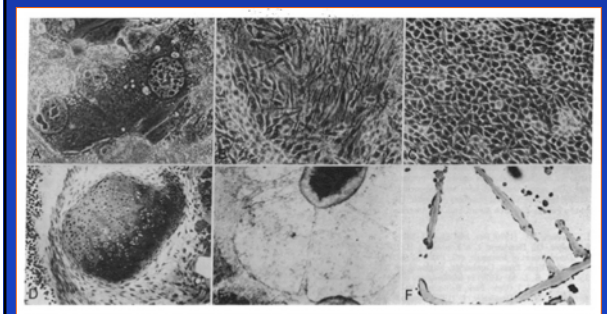

FIG. 5. Differentiation of ESC-ICR cells *in vitro*. A variety of cell types are apparent during the 6 weeks after the reattachment to tissue culture dishes of embryoid bodies formed by ESC-ICR cells. (*Upper*) Phase-contrast microscopy of live cells. (Approximately ×160). (*A*) Giant cells, (*B*) neuron-like cells, (*C*) endodermal cells. (*Lower*) (*D*) Section of plastic-embedded culture showing cartilage. (Approximately ×100.) (*E*) Live cells forming tubules. (Approximately ×35.) (*F*) Section of area shown in *E* after embedding in plastic. Tubules are filled with a granular, acellular deposit. (Approximately ×100.)

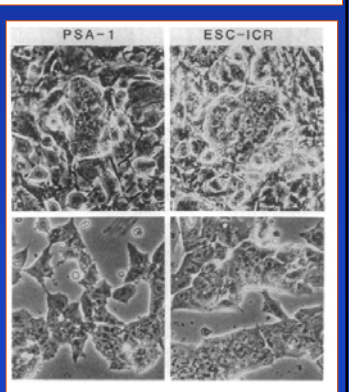

FIG. 2. Morphological similarity of embryo-derived ESC-ICR cells to PSA-1 embryonal carcinoma cells. (*Upper*) Cells growing on a fibroblastic feeder layer. (*Lower*) Mass cultures of the cells seeded in the absence of feeder cells. (Phase-contrast microscopy; approximately ×250.)

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Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines

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NATURE VOL. 309 17 MAY 1984

The recent availability in culture of embryo-derived pluripotential cells which exhibit both a normal karyotype and a high differentiative ability[1–3] has encouraged us to assess the potential of these cells to form functional germ cells following their incorporation into chimaeric mice. We report here the results of blastocyst injection studies using three independently isolated XY embryo-derived cell lines (EK.CP1, EK.CC1.1 and EKCC1.2) which produce a very high proportion (>50%) of live-born animals that are overtly chimaeric. Seven chimaeric male mice, derived from these three lines, have, so far, proved to be functional germ-line chimaeras.

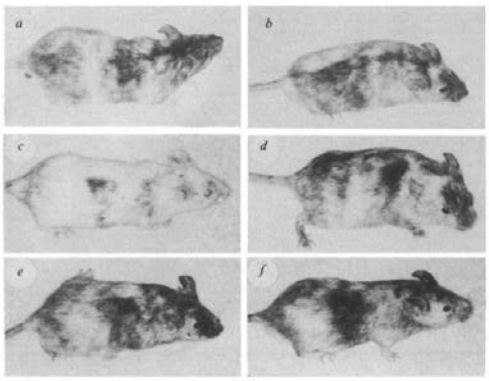
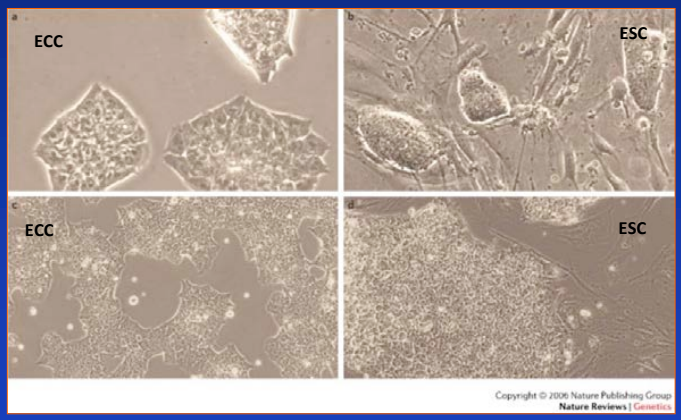


Fig. 1 Six of the seven germ-line chimaeras described in Tables 1 and 2. *a*, CP1.3; *b*, CP1.5; *c*, CP1.11; *d*, CC1.1.3; *e*, CC1.2.6; *f*, CC1.2.8. Between 8 and 12 embryo-derived cells were introduced into the blastocoelic cavity of host-fertilized blastocysts homozygous for the recessive albino locus. The blastocysts were then allowed to re-expand and were subsequently transferred to the uterine lumen of recipients on the third day of pseudopregnancy. All the conceptuses were allowed to develop to term, and live-born animals were scored for the presence of eye and coat pigmentation at or shortly after birth.

75

Εμβρυϊκά βλαστοκύτταρα



Τα ESC εμφανίζουν πολλές ομοιότητες με τα ECC :

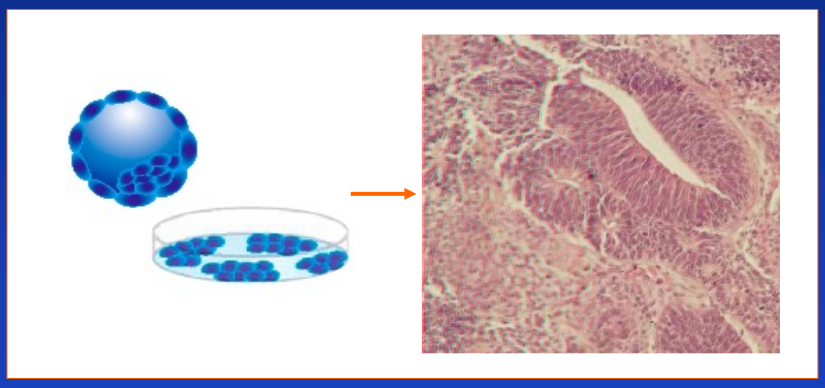
- ✓ στη μορφολογία,
- ✓ στις συνθήκες καλλιέργειας και
- ✓ στα επιφανειακά αντιγόνα που εκφράζουν

αλλά

- ✓ έχουν φυσιολογικό καρυότυπο
- ✓ μεγαλύτερο αναπτυξιακό δυναμικό

76

Εμβρυϊκά βλαστικά κύτταρα



- Τα ESC όπως τα ECC είναι ικανά να σχηματίσουν όγκους μετά από μεταμόσχευση σε ανοσοκατεσταλμένα ποντίκια.
- Ομως οι χίμαιρες από ESC δεν εμφανίζουν όγκους .
- Ενώ δεν αναπτύσονταν ποτε κύτταρα της γαμετικής σειράς από τα ECC από τα ESC σχηματίζονται- **germ line transmission!**

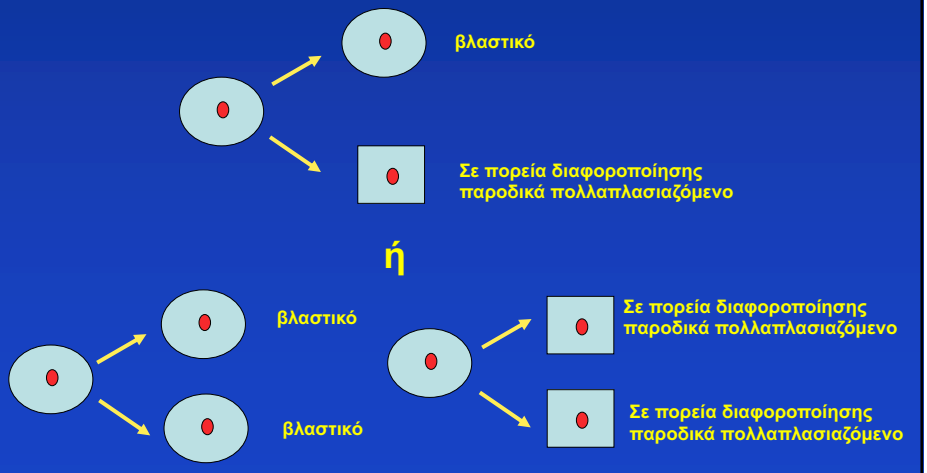
77

Εμβρυϊκά βλαστοκύτταρα

- Είναι κύτταρα που έχουν τη δυνατότητα να **αυτοανανεώνονται**
- Είναι κύτταρα που δεν έχουν διαφοροποιηθεί και **οι απόγονοί τους περιλαμβάνουν όλους τους κυτταρικούς τύπους του ατόμου (αλλά όχι εξωεμβρυϊκές δομές)**
- Είναι πολυδύναμα (ολοδύναμα μόνο το ζυγωτό και τα πρώιμα βλαστομερίδια)
- Είναι κύτταρα που δεν έχουν ακόμα εξειδικευτεί ή προκαθοριστεί
- Κάθε κύτταρο του σώματος είναι απόγονος των εμβρυϊκών βλαστοκυττάρων
- Απομονώνονται από την ΕΚΜ
- Για τη διατήρηση της αυτοδυναμίας τουςαπαιτούνται συγκεκριμένες συνθήκες αλλιώς διαφοροποιούνται αυθόρμητα
- Στο έμβρυο είναι παρόντα για μικρό χρονικό διάστημα in vitro πολύ μεγάλος αριθμός διαιρέσεων (συνθήκες!!)

78

Αυτοανανέωση – παραγωγή απογόνων που ανήκουν σε διαφορετικούς κυτταρικούς τύπους

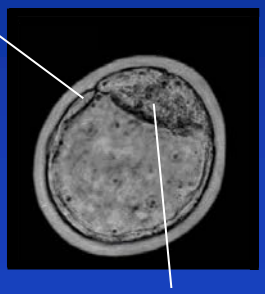


79

Ο διαχωρισμός των δύο κυτταρικών πληθυσμών της βλαστοκύστης αποτελεί σημαντικό διαφοροποιητικό γεγονός στην ανάπτυξη των θηλαστικών.

Τροφοβλάστη → χόριο :

α. Θρέψη
β. Παραγωγή ορμονών
γ. Παραγωγή ουσιών που αναστέλλουν την απόρριψη του εμβρύου από τη μητέρα.



Εσωτερική κυτταρική μάζα → έμβρυο (τελικά το ζώο)
εξωεμβρυϊκές δομές

Από το στάδιο των 64 κυττάρων και μετά, οι δύο στιβάδες ΔΕΝ συνεισφέρουν πια κανένα κύτταρο η μια στην άλλη.

80

Proc. Nat. Acad. Sci. USA
Vol. 71, No. 4, pp. 1250–1254, April 1974

Simian Virus 40 DNA Sequences in DNA of Healthy Adult Mice Derived from Preimplantation Blastocysts Injected with Viral DNA

(blastocyst microinjection *in vitro*/development/DNA reassociation kinetics of simian virus 40)

RUDOLF JAENISCH* AND BEATRICE MINTZ†

ABSTRACT Explanted mouse blastocysts were microinjected in the blastocoel cavity with simian virus 40 (SV40) viral DNA. After surgical transfer to the uteri of pseudopregnant surrogate mothers, approximately 40% of the blastocysts developed to term and became healthy adults without apparent tumors at 1 year of age. Molecular hybridization tests for the presence of SV40-specific DNA sequences were conducted on DNA extracted from various organs of these animals. Between 0.5 and 13 SV40 genome equivalents per diploid mouse DNA value were found in some organs of approximately 40% of the adult survivors; this represents a substantial augmentation of the amount administered per embryo. The results are consistent with the working hypothesis that the SV40 DNA may have been integrated into the host genome; alternatively, the viral DNA may have replicated as an extrachromosomal entity or by lytic infection in a few permissive cells. Persistence of the viral DNA from preimplantation stages to adult life may thus provide a new tool for experimental investigation of vertical transmission and expression of tumor viruses.

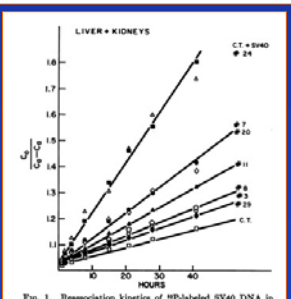


FIG. 1. Reassociation kinetics of ^{32}P-labeled SV40 DNA in the presence of unlabeled DNA extracted from pooled liver and kidneys of each of 7 (numbered) mice derived from blastocysts injected with SV40 DNA. The reaction mixtures, containing 0.5 ng/ml of ^{32}P-labeled SV40 DNA (1.6×10^6 cpm/μg), 680 μg/ml of mouse- or calf-thymus DNA in 0.01 M Tris·HCl, pH 7.4, 1 mM EDTA, 1 M NaCl, were heat-denatured and incubated at 68°. Samples were removed at different times and the fraction of SV40 DNA reassociated was determined by digestion with S1 nuclease. The results are plotted as the reciprocal of DNA remaining single-stranded as a function of time. Calf-thymus DNA (= C.T.) and calf-thymus DNA plus 1.8 ng/ml of unlabeled SV40 DNA (= C.T. + SV40) were included as controls. Each point represents a total of 2000 cpm.

TABLE 1. *Detection of SV40 DNA in DNA extracted from various mouse organs*

Mouse no.	No. of SV40 copies per diploid mouse DNA value: Liver and kidneys	Brain
1	0	13
3	0.62	0.7
7	1.5	0
8	0.72	n.t.
11	1.0	n.t.
15	0	8.5
19	0	1.95
20	1.6	0
24	3.6	n.t.
29	0.5	0

The number of SV40 genome equivalents in the DNA extracted from these mouse organs was calculated according to previous methods (5) from the data in Figs. 1 and 2, for each of the 10 mice with evidence of SV40 after injection of SV40 DNA in the preimplantation blastocyst stage. Liver and kidneys were pooled from each of 25 mice tested; brains of 15 of the 25 were tested. n.t. = not tested.

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Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector

Elizabeth Robertson, Allan Bradley, Michael Kuehn & Martin Evans

Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2 3EH, UK

NATURE VOL. 323 2 OCTOBER 1986

Embryonic stem cells isolated directly from mouse embryos[1] can be cultured for long periods *in vitro* and subsequently repopulate the germ line in chimaeric mice[2,3]. During the culture period these embryonic cells are accessible for experimental genetic manipulation[4–6]. Here we report the use of retroviral vectors to introduce exogenous DNA sequences into a stem-cell line and show that these modified cells contribute extensively to the somatic and germ-cell lineages in chimaeric mice. Compared with current methods for manipulation of the mouse genome, this approach has the advantage that powerful somatic-cell genetic techniques can be used to modify and to select cells with germ-line potential, allowing the derivation of transgenic strains with pre-determined genetic changes. We have by this means inserted many proviral vector sequences that provide new chromosomal molecular markers for linkage studies in the mouse and that also may cause insertional mutations.

"cultured embryonic cells provide an efficient means for the production of transgenic animals"

"it may also eventually be possible to produce specific alterations in endogenous genes through homologous recombination with cloned copies modified in vitro"

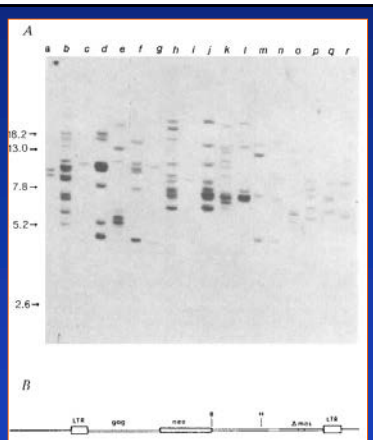

Fig. 2 *A*, Southern blot analysis of DNA samples from F_1 offspring of two germ-line chimaeric males. Samples are distributed as follows. *A*, V29.7 progeny; lanes *a*, *g*, V29.7.1; *b*, *h*, V29.7.2; *c*, *i*, V29.7.3; *d*, *j*, V29.7.4; *e*, *k*, V29.7.5; *f*, *l*, V29.7.6. 2A.5 progeny; lanes *m*, *p*, 2A.5.10; *n*, *q*, 2A.5.11; *o*, *r*, 2A.5.12. *B*, Chromosomal integration of MPSV mos$^-$neo retroviral vector. B, *Bam*HI; H, *Hin*dIII restriction sites; LTR, long terminal repeat. Single line, chromosomal DNA; double line, vector DNA.
Methods. *A*, The two restriction enzymes used here cleave once in the proviral vector outside the region of homology with the pSV2neo probe, consequently each restriction fragment on the autoradiograph corresponds to a fragment containing a unique integration site of a proviral vector genome. DNAs were prepared from whole newborn animals (V29.7 progeny) or from livers from 1-week-old animals (2A.5 progeny). Samples (3 μg) were digested with *Bam*HI (lanes *b–g*; *m–o*) or with *Hin*dIII (lanes *a*, *h–l*, *p–r*), fractionated on 0.7% agarose gels, transferred to nylon membranes, hybridized with nick-translated pSV2neo, washed and autoradiographed using standard procedures.

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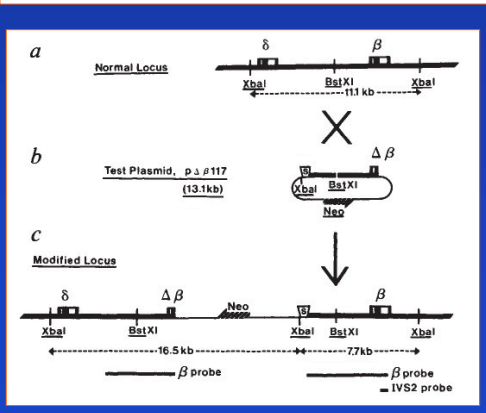
Insertion of DNA sequences into the human chromosomal β-globin locus by homologous recombination

Oliver Smithies*, Ronald G. Gregg*, Sallie S. Boggs*, Michael A. Koralewski* & Raju S. Kucherlapati†

A 'rescuable' plasmid containing globin gene sequences allowing recombination with homologous chromosomal sequences has enabled us to produce, score and clone mammalian cells with the plasmid integrated into the human β-globin locus. The planned modification was achieved in about one per thousand transformed cells whether or not the target gene was expressed.

NATURE VOL. 317 19 SEPTEMBER 1985



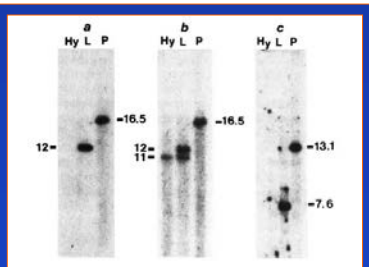


Fig. 3 Nitrocellulose blots of digests of DNA from two of the G418-resistant colonies from pool 1 (see text and Table 2) and from untreated Hu 11 hybrid cells. The blots were: *a*, *Xba*I digests hybridized to a probe specific for pSV2Neo-derived sequences; *b*, *Xba*I digests hybridized to probes specific for the IVS-2 of the human δ-globin gene and for pSV2Neo sequences; *c*, *Bst*XI digests hybridized to a probe specific for pSV2Neo sequences. Samples are: Hy, untreated Hu 11 hybrid cells; L, the G418-resistant colony L; P, the G418-resistant colony P. Measured fragment sizes are shown in kb.

83

Targetted correction of a mutant HPRT gene in mouse embryonic stem cells

Thomas Doetschman*, Ronald G. Gregg*, Nobuyo Maeda*, Martin L. Hooper†, David W. Melton‡, Simon Thompson‡ & Oliver Smithies*§

NATURE VOL. 330 10 DECEMBER 1987

Two recent developments suggest a route to predetermined alterations in mammalian germlines. These are, first, the characterization of mouse embryonic stem (ES) cells[1] that can still enter the germline after genetic manipulation in culture[2,3] and second, the demonstration that homologous recombination between a native target chromosomal gene and exogenous DNA can be used in culture to modify specifically the target locus[4]. We here use gene targetting functionally to correct the mutant hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene in the ES cell line which has previously been isolated and used to produce an HPRT-deficient mouse[5]. This modification of a chosen gene in pluripotent ES cells demonstrates the feasibility of this route to manipulating mammalian genomes in predetermined ways.

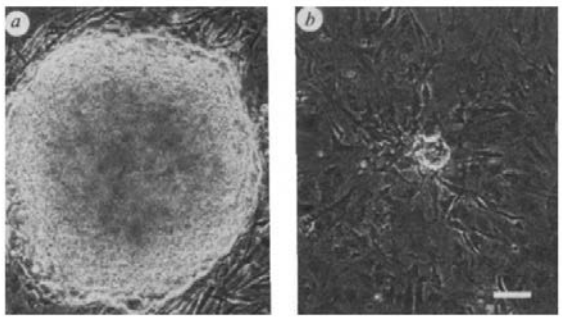


Fig. 2 ES cell colonies, growing on primary embryonic fibroblasts in the presence of HAT, 14 days after electroporation of HPRT⁻ ES cells in the presence of the correcting plasmid. *a*, A large colony scored as HPRT⁺ (see text). *b*, A small colony scored as HPRT⁻. Scale bar, 100 μm.

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Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 8927–8931, November 1989
Genetics

Germ-line transmissio
hypoxanthine phospho
homologous recombin

(gene targeting/chimeras)

BEVERLY H. KOLLER*, LORA J. HA
PHILLIP J. WILLIAMS*, NEAL L. FI

yonic stem cells (derived from 129/Ola ant hypoxanthine phosphoribosyltrans- been corrected *in vitro* in a planned recombination were injected into blas- 57BL/6J mice. The injected blastocysts seudopregnant female mice to complete ven surviving pups were obtained. Nine es and three females. Two of the males onic stem cell genome containing the anthine phosphoribosyltransferase gene high frequencies. These experiments planned alteration in a chosen gene can line of an experimental animal by tion in an embryonic stem cell.

Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 8932–8935, November 1989
Genetics

Inactivating the β_2-mi cells by homologous r

(class I antigens/gene targeting)

BEVERLY H. KOLLER AND OLIVER

ve inactivated, by gene targeting, the bulin gene in a mouse embryonic stem ent of the β_2-microglobulin gene with upted by the insertion of the neomycin- l to transfect the embryonic stem cells. were selected and then screened using eaction to identify those in which the egrated into the embryonic stem cell s recombination. Of a total of 234 screened, 2 correctly targeted colonies ric mice carrying the inactivated β_2- e been obtained from both of these lines. Breeding of offspring from such tigation of the effects of homozygous

Fig. 1. (*Upper*) Four fertile male chimeras (from left to right: 5.3, 4.1, 5.1, and 5.2 in Table 2) resulting from C57BL/6J blastocysts injected with ES98-12 cells. The two males in the center transmitted the ES98-12 genome to their progeny (see text and Table 2). (*Lower*) One of the transmitting males, 5.1, (*Right top*) mated to a C57BL/6J female (*Left top*). In this family, four nonvariegated agouti pups (brown in the photograph) received ES98-12 genomes from their lightly variegated father; six black pups received C57BL/6J genomes from him.

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Cell 51:503-12, 1987

Site-directed mutagenes
mouse embryo-derived

Thomas KR, Capecchi, M.R.

We mutated, by gene targeting, the endogenous hypoxanthine phosphoribosyl transferase (HPRT) gene in mouse embryo-derived stem (ES) cells. A specialized construct of the neomycin resistance (neor) gene was introduced into an exon of a cloned fragment of the Hprt gene and used to transfect ES cells. Among the G418r colonies, 1/1000 were also resistant to the base analog 6-thioguanine (6-TG). The G418r, 6-TGr cells were all shown to be Hprt- as the result of homologous recombination with the exogenous, neor-containing, Hprt sequences. We have compared the gene-targeting efficiencies of two classes of neor-Hprt recombinant vectors: those that replace the endogenous sequence with the exogenous sequence and those that insert the exogenous sequence into the endogenous sequence. The targeting efficiencies of both classes of vectors are strongly dependent upon the extent of homology between exogenous and endogenous sequences. The protocol described herein should be useful for targeting mutations into any gene.

Targeted disruption of t
***int-1* proto-oncogene re**
severe abnormalities in
and cerebellar developm

Kirk R. Thomas & Mario R. Capecchi*

Howard Hughes Medical Institute, Department of Bio
and Human Genetics, Salt Lake City, Utah 84112, US

THE *int-1* proto-oncogene was first identified as a gene activated in virally induced mouse mammary tumours[1,2]. Expression studies, however, suggest that the normal function of this gene may be in spermatogenesis and in the development of the central nervous system[3–5]. Genes sharing sequence similarity with *int-1* have been found throughout the animal kingdom. For example, *int-1* has 54% amino-acid identity to the *Drosophila* segment polarity gene *wingless* (*wg*)[6]. Both the *int-1* and *wg* gene products seem to be secreted proteins, presumably involved in cell–cell signalling[7–11]. We have now explored the function of *int-1* in the mouse by disrupting one of the two *int-1* alleles in mouse embryo-derived stem cells using positive–negative selection[12]. This cell line was used to generate a chimaeric mouse that transmitted the mutant allele to its progeny[13–16]. Mice heterozygous for the *int-1* null mutation are normal and fertile, whereas mice homozygous for the mutation may exhibit a range of phenotypes from death before birth to survival with severe ataxia. The latter pathology in mice and humans is often associated with defects in the cerebellum. Examination of *int-1*$^{-}$/*int-1*$^{-}$ mice at several stages of embryogenesis revealed severe abnormalities in the development of the mesencephalon and metencephalon indicating a prominent role for the *int-1* protein is in the induction of the mesencephalon and cerebellum.

FIG. 3 Comparison of brains from heterozygous and homozygous, *int-1*$^{-}$ mice. *a* and *b*, 17.5 day embryos were fixed in Bouin's reagent (Sigma). The yolk sacs were removed for DNA analysis as described in Table 1. Following two days in fixative, the brains were dissected, rinsed in PBS and photographed at ×6 magnification. The field of view is 5 × 10 mm. Arrows indicate the cerebellar region absent in the homozygote. *c* and *d*, The brains shown in *a* and *b* were embedded in paraffin, sectioned (10 μm), and stained by haematoxylin and eosin (H and E) regressive staining. The field of view is 6 × 4 mm. Arrows indicate mesencephalic tissue absent in the homozygote. *e* and *f*, Brains were dissected from adult (5 week) mice, fixed in PLP, embedded in paraffin, sectioned (8 μm) stained with H and E. The field of view is 6 × 4 mm. tc, telencephalon; msc, mesencephalon; mtc, metencephalon; pcp, posterior choroid plexus.

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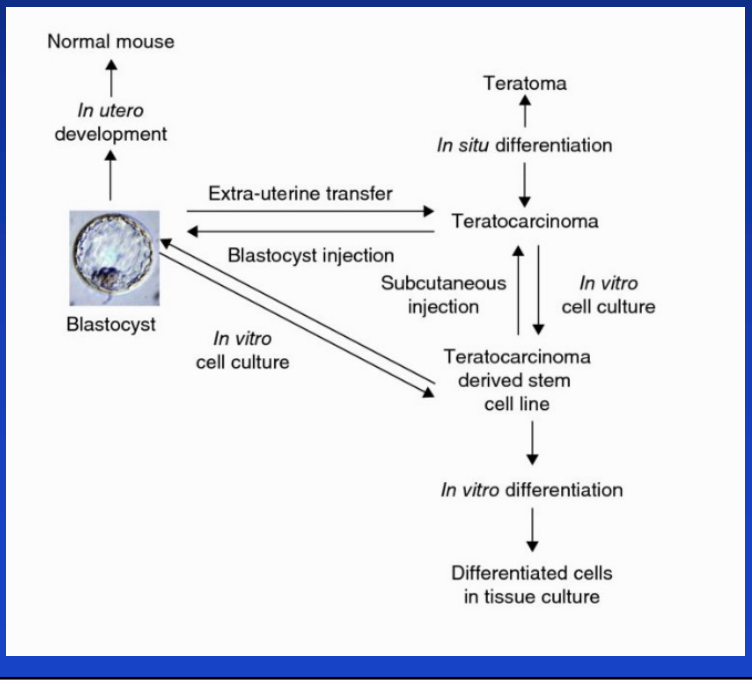
The Nobel Prize in Physiology or Medicine 2007

Mario R. Capecchi, Sir Martin J. Evans, Oliver Smithies

"for their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells"

87



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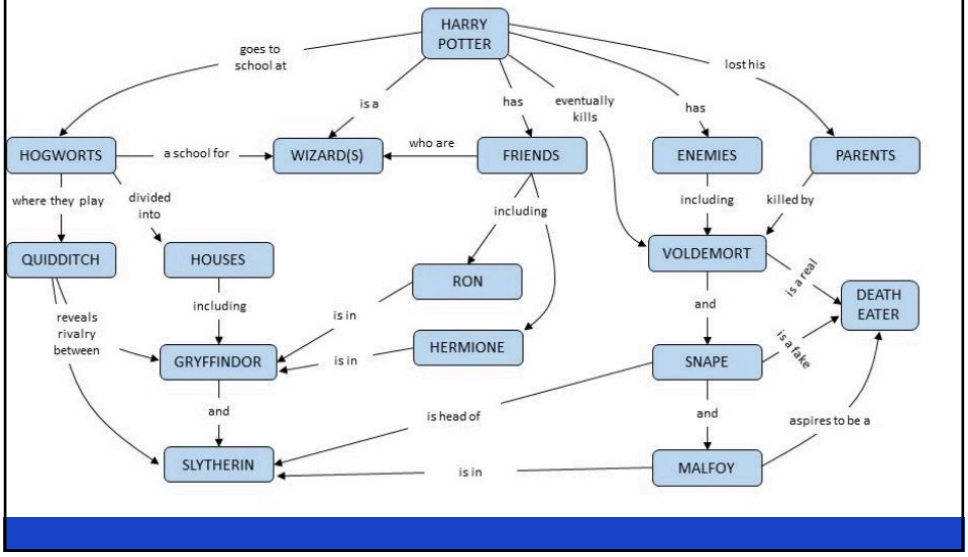
Χάρτες εννοιών



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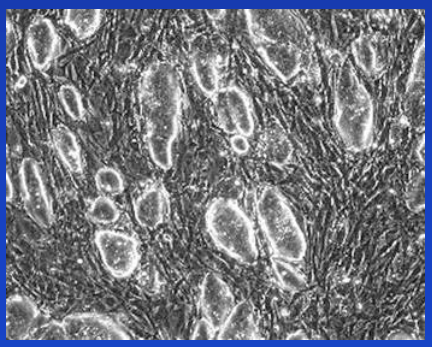
Καλλιέργεια ESC ποντικού

Οι καλλιέργειες ESC είναι στην ουσία *πρωτογενείς καλλιέργειες* επομένως οι συνθηκες καλλιέργειας θα πρέπει να διατηρούν αυτές τους ιδιότητες ώστε να μην απομονωθούν παραλαγγές με διαφορετικές ιδιότητες.

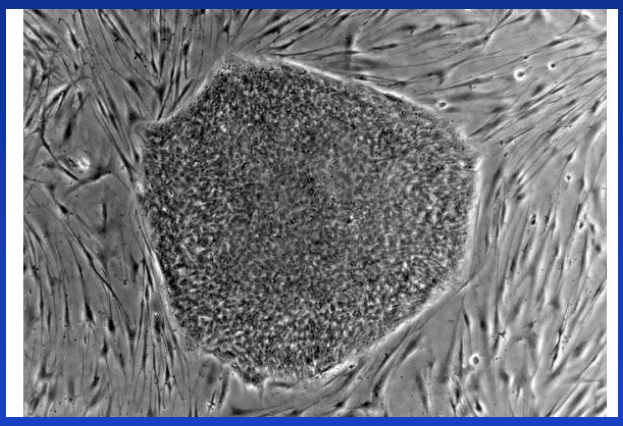
Αυτό σημαίνει ότι οι συνθήκες πρέπει να διατηρούνται άριστες για να μη δημιουργείται επιλεκτική πίεση. Δηλαδή:

1) Τα κύτταρα να παραμένουν σε μη διαφοροποιημένη κατάσταση
2) Τα κύτταρα να διατηρούν ακέραιο το αναπτυξιακό τους δυναμικό
3) Τα κύτταρα να διατηρούν φυσιολογικό καρυότυπο.



90

Καλλιέργεια ESC ποντικού



Τα ESC του ποντικού καλλιεργούνται:

1) πάνω σε τάπητα (μονή στοιβάδα) ινοβλαστών οι οποίοι έχουν ακτινοβοληθεί ώστε να μην διαιρούνται ή
2) Παρουσία LIF (βλ. επόμενο μάθημα!)

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Καλλιέργεια ESC ποντικού

Προκειμένου να διατηρήσουν τις ιδιότητές τους στις συνθήκες καλλιέργειας :

1) Αποφεύγουμε τις ακραίες καταστάσεις δηλ. Δεν απλώνουμε τα κύτταρα ούτε πολύ αραιά ούτε πολύ πυκνά για να μην προκαλέσουμε την ανάπτυξη ανευπλοειδικών κυττάρων.
2) Παρατηρούμε το χρόνο διπλασιασμού τους που πρέπει να είναι 15-20 h
3) Πρακτικά αυτό σημαίνει ότι κάθε τρεις ημέρες χρειάζονται ανακαλλιέργεια.

Οι κατάλληλες συνθήκες καλλιέργειας για κάθε σειρά ελέγχονται:

α) με τη χρήση της δοκιμής σχηματισμού κλώνων. Στη δοκιμή αυτή χρησιμοποιεί κανείς έναν πληθυσμό κυττάρων σε διαφορετικά θρεπτικά μέσα. Τα κύτταρα απλώνονται με τέτοιο τρόπο ώστε να σχηματίσουν κλώνους (δηλ πολύ αραιά). Μετά από 6-8 ημέρες οι καλλιέργειες μονιμοποιούνται και μελετάται ο αριθμός των κλώνων που αναπτύχθηκαν σε κάθε μέσο αλλά και η μορφολογία, έκφραση συγκεκριμένων αντιγόνων κλπ.

β) σχηματισμός χιμαιρας –(και γαμετικά κύτταρα!!)

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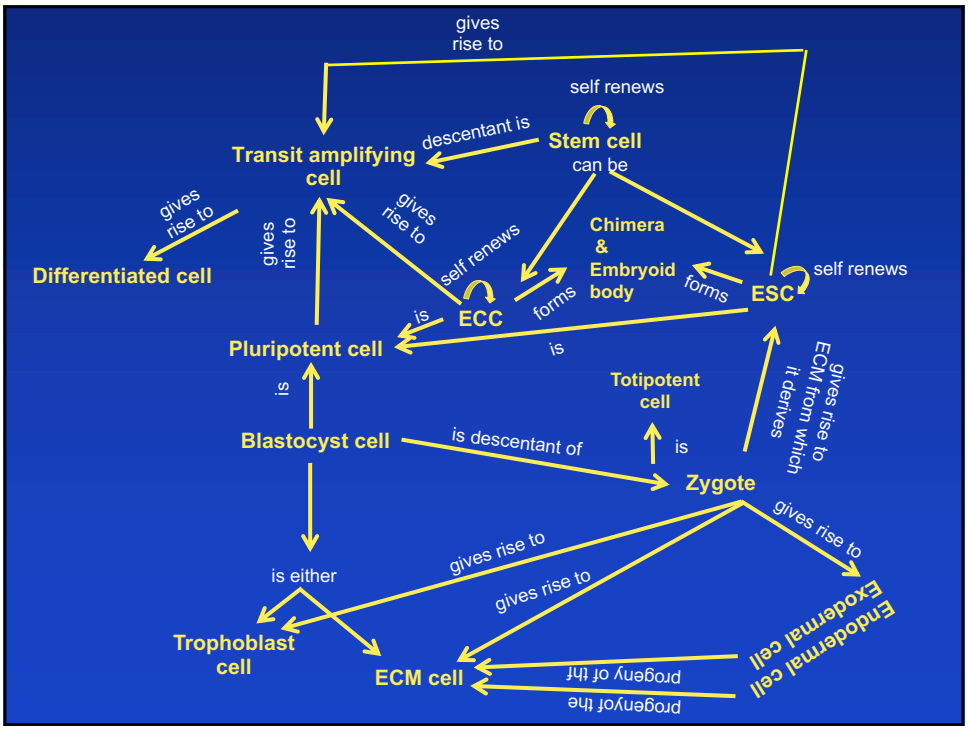

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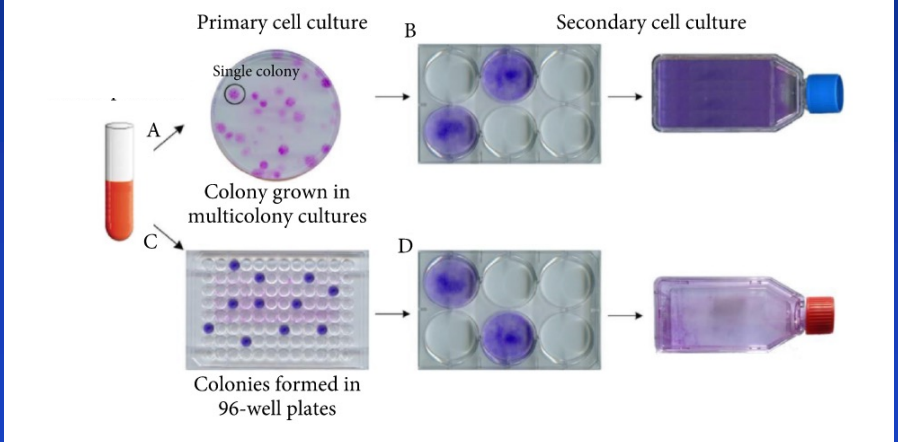
Clonal culture



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Chimeras

C2J4 line derived from **C57BL/6J-Tyrc-2J/J** mouse line, also known as **albino B6** mice. Being injected into **C57Bl/6** blasocysts it generates chimeras exhibiting white patches of coat color indicating ES cell contribution to the genotype.

Those chimeras should be judged according following guidelines:

Grade A chimeras exhibit some black coat color with high percentage of white 50% to completely 100% white coat color.

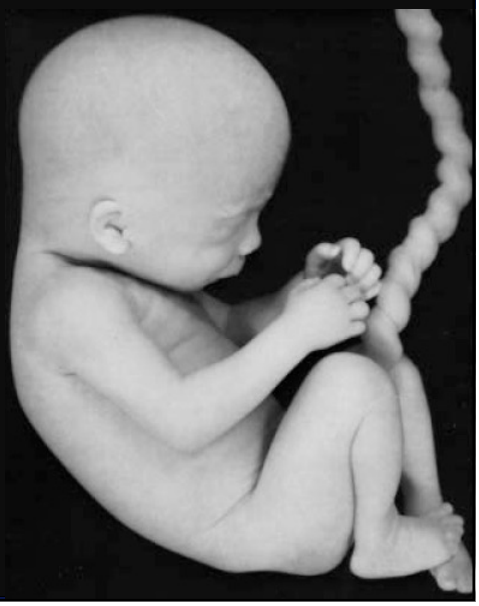
Grade B chimeras exhibit mostly black coat color with some patches of white 30%-40%.

Grade C chimeras exhibit mostly black coat color with small percentage of white 1%-20%.

https://www.rockefeller.edu/transgenics/links-guidelines/guidelines-for-chimeric-coat-color-estimation/

95

Βιολογία Βλαστοκυττάρων και Αναγέννησης



Austin Smith's saga

Μ. Γρηγορίου 2018

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Proc. Nat. Acad. Sci. USA
Vol. 72, No. 4, pp. 1441–1445, April 1975

Differentiation of Clonal Lines of Teratocarcinoma Cells: Formation of Embryoid Bodies *In Vitro*

(mouse tumors/tissue culture/pluripotent cells/cell determination/endoderm)

GAIL R. MARTIN AND MARTIN J. EVANS

ABSTRACT The differentiation *in vitro* of clonal pluripotent teratocarcinoma cells is reported. The first stage of this process is the formation of simple embryoid bodies which are identical to those found in animals bearing intraperitoneal teratocarcinomas. They consist of an inner core of embryonal carcinoma cells surrounded by a layer of endodermal cells which produce Reichert's membrane. The endodermal cells become apparent shortly after the embryonal carcinoma cells have formed aggregates which are loosely attached to the substratum. One clonal teratocarcinoma line was found to produce complex cystic embryoid bodies *in vitro*. Following formation of the endodermal cells, extensive differentiation to a wide variety of cell types occurs. There are similarities between the process of embryoid body formation and the early events of differentiation of the mouse embryo.

"Without added feeders they begin to die. The cells survive for longer periods when passaged on gelatin-coated dishes. Both the feeder layers and the gelatin coating of the substratum appear to increase the spreading and attachment of the cells to the substratum; however, the feeder cells must have some additional effect, since the cells do not survive indefinitely when passaged on gelatin-coated dishes"

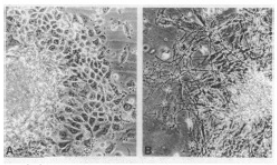

FIG. 6. Subsequent differentiation from embryoid bodies formed *in vitro*. (A) Pluripotent cells were plated as a single-cell suspension. Four days later embryoid bodies were found floating in the medium. These were reattached to the substratum by plating in fresh medium. Seven days later cells had migrated out to form a halo around the embryoid body. Phase contrast ×91.2. (B) Neural differentiation at the periphery of a halo of differentiated cells surrounding an embryoid body, twenty days after plating a single cell suspension. Phase contrast ×52.8.

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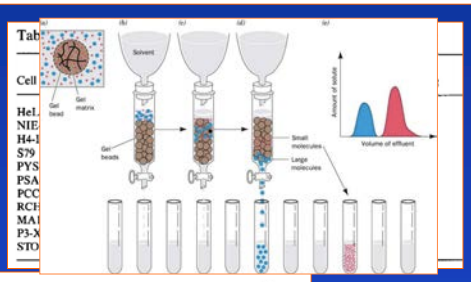
Experimental Cell Research 154 (1984) 233–242

A Factor Produced by Feeder Cells Which Inhibits Embryonal Carcinoma Cell Differentiation

Characterization and Partial Purification

PETER KOOPMAN and RICHARD G. H. COTTON

Medium conditioned by STO mouse fibroblast cells inhibited both the spontaneous differentiation of NG2 embryonal carcinoma cells and the differentiation of F9 embryonal carcinoma cells induced by retinoic acid. This effect was due to a differentiation retarding factor (DRF). Reduction in DRF activity in conditioned medium by boiling and by pronase treatment suggested the involvement of a polypeptide, which had an apparent molecular weight of 57 000 on gel filtration. A 28-fold purification of DRF was achieved. DRF delayed but did not prevent the extensive differentiation observed after prolonged culture of NG2 colonies. Conditioned medium could be successfully used to replace feeder cells in NG2 stock cultures. Media conditioned by a variety of other cell types also contained differentiation retarding activity.



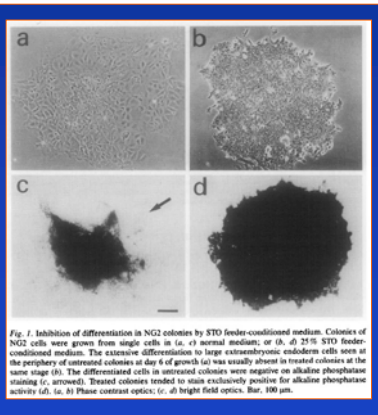


Fig. 1. Inhibition of differentiation in NG2 colonies by STO feeder-conditioned medium. Colonies of NG2 cells were grown from single cells in (*a*, *c*) normal medium; or (*b*, *d*) 25% STO feeder-conditioned medium. The extensive differentiation to large extraembryonic endoderm cells seen at the periphery of untreated colonies at day 6 of growth (*a*) was usually absent in treated colonies at the same stage (*b*). The differentiated cells in untreated colonies were negative on alkaline phosphatase staining (*c*, arrowed). Treated colonies tended to stain exclusively positive for alkaline phosphatase activity (*d*). (*a*, *b*) Phase contrast optics; (*c*, *d*) bright field optics. Bar, 100 μm.

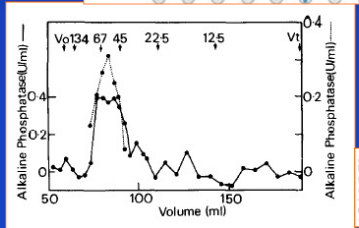


Fig. 3. Gel filtration of STO feeder-conditioned medium. Curves represent DRF activity, as detected by alkaline phosphatase activities of lysates of cells grown in the presence of (●—●) 0.2 ml or (●---●) 0.05 ml of column fractions. Elution volumes of molecular weight markers, determined from at least two runs, are indicated by arrows; values given are molecular weights in kD. Markers used were: blue dextran (V_o), bovine serum albumin dimer (MW 134 000), bovine serum albumin (MW 67 000), ovalbumin (MW 45 000) chymotrypsin (MW 22 500), cytochrome *c* (MW 12 500) and tritiated water (V_t).

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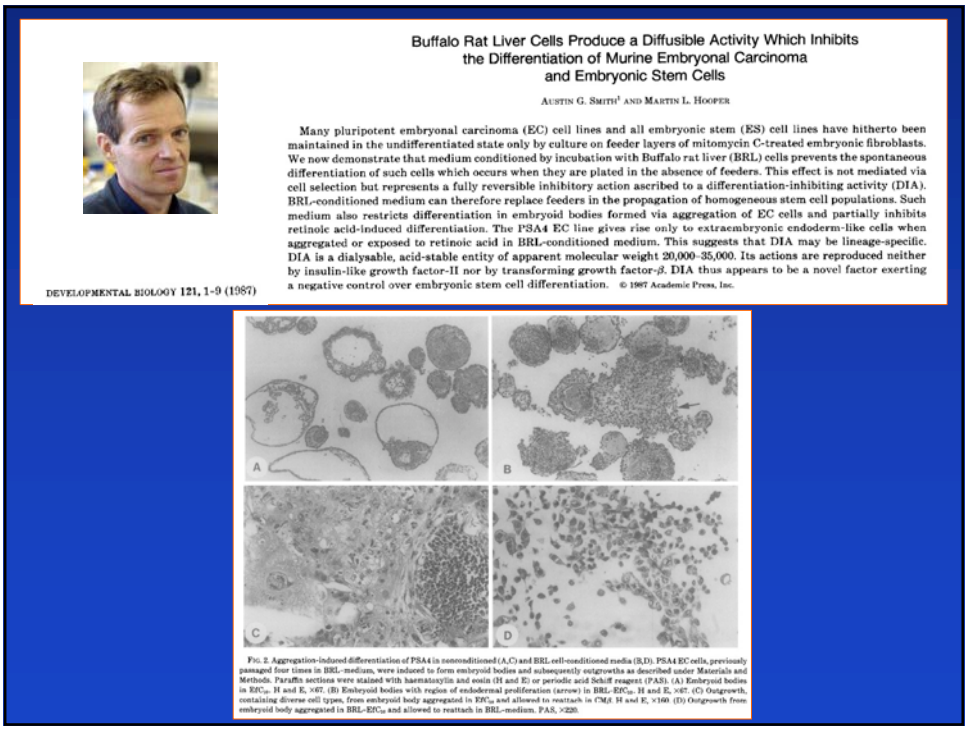

Buffalo Rat Liver Cells Produce a Diffusible Activity Which Inhibits the Differentiation of Murine Embryonal Carcinoma and Embryonic Stem Cells

AUSTIN G. SMITH[1] AND MARTIN L. HOOPER

Many pluripotent embryonal carcinoma (EC) cell lines and all embryonic stem (ES) cell lines have hitherto been maintained in the undifferentiated state only by culture on feeder layers of mitomycin C-treated embryonic fibroblasts. We now demonstrate that medium conditioned by incubation with Buffalo rat liver (BRL) cells prevents the spontaneous differentiation of such cells which occurs when they are plated in the absence of feeders. This effect is not mediated via cell selection but represents a fully reversible inhibitory action ascribed to a differentiation-inhibiting activity (DIA). BRL-conditioned medium can therefore replace feeders in the propagation of homogeneous stem cell populations. Such medium also restricts differentiation in embryoid bodies formed via aggregation of EC cells and partially inhibits retinoic acid-induced differentiation. The PSA4 EC line gives rise only to extraembryonic endoderm-like cells when aggregated or exposed to retinoic acid in BRL-conditioned medium. This suggests that DIA may be lineage-specific. DIA is a dialysable, acid-stable entity of apparent molecular weight 20,000–35,000. Its actions are reproduced neither by insulin-like growth factor-II nor by transforming growth factor-β. DIA thus appears to be a novel factor exerting a negative control over embryonic stem cell differentiation.

DEVELOPMENTAL BIOLOGY 121, 1–9 (1987)

FIG. 2. Aggregation-induced differentiation of PSA4 in nonconditioned (A,C) and BRL cell-conditioned media (B,D). PSA4 EC cells, previously passaged four times in BRL-medium, were induced to form embryoid bodies and subsequently outgrowths as described under Materials and Methods. Paraffin sections were stained with haematoxylin and eosin (H and E) or periodic acid Schiff reagent (PAS). (A) Embryoid bodies in EC_{20}. H and E, ×67. (B) Embryoid bodies with region of endodermal proliferation (arrow) in BRL-EC_{20}. H and E, ×67. (C) Outgrowth, containing diverse cell types, from embryoid body aggregated in EC_{20} and allowed to reattach in CM_0. H and E, ×160. (D) Outgrowth from embryoid body aggregated in BRL-EC_{20} and allowed to reattach in BRL-medium. PAS, ×220.

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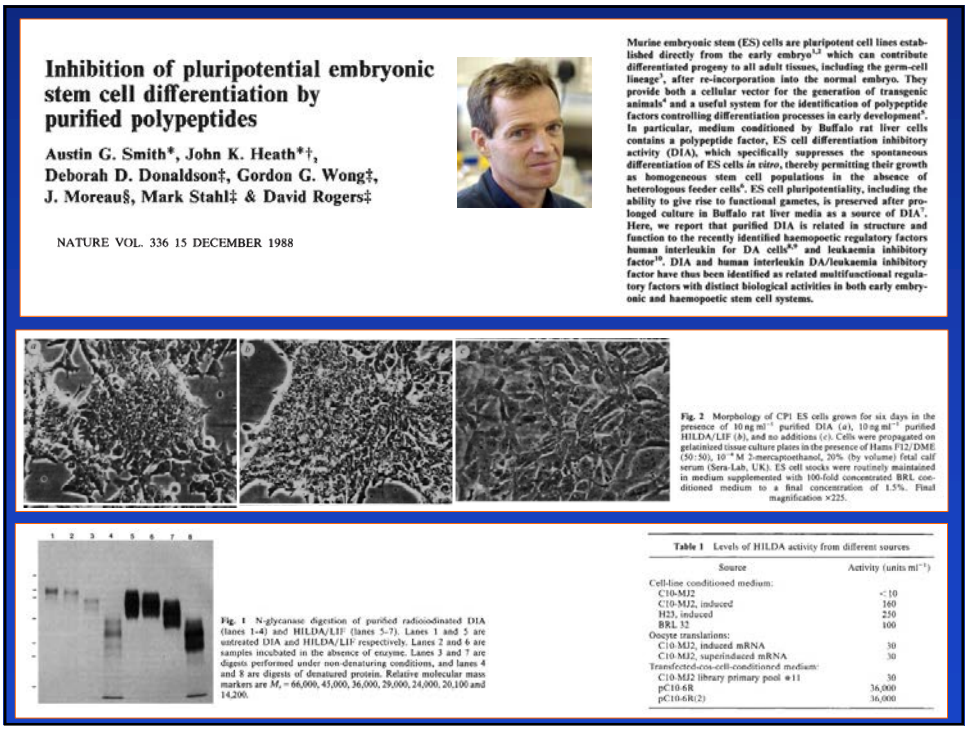


Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides

Austin G. Smith*, John K. Heath*†, Deborah D. Donaldson‡, Gordon G. Wong‡, J. Moreau§, Mark Stahl‡ & David Rogers‡

NATURE VOL. 336 15 DECEMBER 1988

Murine embryonic stem (ES) cells are pluripotent cell lines established directly from the early embryo[1,2] which can contribute differentiated progeny to all adult tissues, including the germ-cell lineage[3], after re-incorporation into the normal embryo. They provide both a cellular vector for the generation of transgenic animals[4] and a useful system for the identification of polypeptide factors controlling differentiation processes in early development[5]. In particular, medium conditioned by Buffalo rat liver cells contains a polypeptide factor, ES cell differentiation inhibitory activity (DIA), which specifically suppresses the spontaneous differentiation of ES cells *in vitro*, thereby permitting their growth as homogeneous stem cell populations in the absence of heterologous feeder cells[6]. ES cell pluripotentiality, including the ability to give rise to functional gametes, is preserved after prolonged culture in Buffalo rat liver media as a source of DIA[7]. Here, we report that purified DIA is related in structure and function to the recently identified haemopoetic regulatory factors human interleukin for DA cells[8,9] and leukaemia inhibitory factor[10]. DIA and human interleukin DA/leukaemia inhibitory factor have thus been identified as related multifunctional regulatory factors with distinct biological activities in both early embryonic and haemopoetic stem cell systems.

Fig. 2 Morphology of CP1 ES cells grown for six days in the presence of 10 ng ml^{-1} purified DIA (*a*), 10 ng ml^{-1} purified HILDA/LIF (*b*), and no additions (*c*). Cells were propagated on gelatinized tissue culture plates in the presence of Hams F12/DME (50:50), 10^{-4} M 2-mercaptoethanol, 20% (by volume) fetal calf serum (Sera-Lab, UK). ES cell stocks were routinely maintained in medium supplemented with 100-fold concentrated BRL conditioned medium to a final concentration of 1.5%. Final magnification ×225.

Fig. 1 N-glycanase digestion of purified radioiodinated DIA (lanes 1–4) and HILDA/LIF (lanes 5–7). Lanes 1 and 5 are untreated DIA and HILDA/LIF respectively. Lanes 2 and 6 are samples incubated in the absence of enzyme. Lanes 3 and 7 are digests performed under non-denaturing conditions, and lanes 4 and 8 are digests of denatured protein. Relative molecular mass markers are M_r = 66,000, 45,000, 36,000, 29,000, 24,000, 20,100 and 14,200.

Table 1 Levels of HILDA activity from different sources

Source	Activity (units ml^{-1})
Cell-line conditioned medium:	
C10-MJ2	< 10
C10-MJ2, induced	160
H23, induced	250
BRL 32	100
Oocyte translations:	
C10-MJ2, induced mRNA	30
C10-MJ2, superinduced mRNA	30
Transfected-cos-cell-conditioned medium:	
C10-MJ2 library primary pool #11	30
pC10-6R	36,000
pC10-6R(2)	36,000

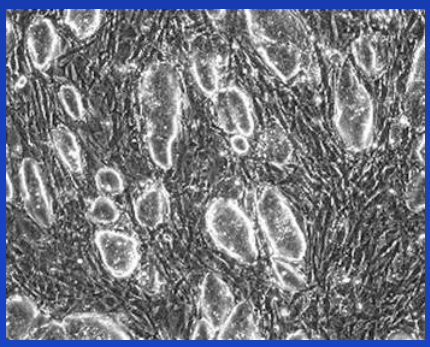
100

Καλλιέργεια ESC ποντικού

Οι καλλιέργειες ESC είναι στην ουσία *πρωτογενείς καλλιέργειες* επομένως οι συνθηκες καλλιέργειας θα πρέπει να διατηρούν αυτές τους ιδιότητες ώστε να μην απομονωθούν παραλαγγές με διαφορετικές ιδιότητες.

Αυτό σημαίνει ότι οι συνθήκες πρέπει να διατηρούνται άριστες για να μη δημιουργείται επιλεκτική πίεση. Δηλαδή:

1) Τα κύτταρα να παραμένουν σε μη διαφοροποιημένη κατάσταση
2) Τα κύτταρα να διατηρούν ακέραιο το αναπτυξιακό τους δυναμικό
3) Τα κύτταρα να διατηρούν φυσιολογικό καρυότυπο.



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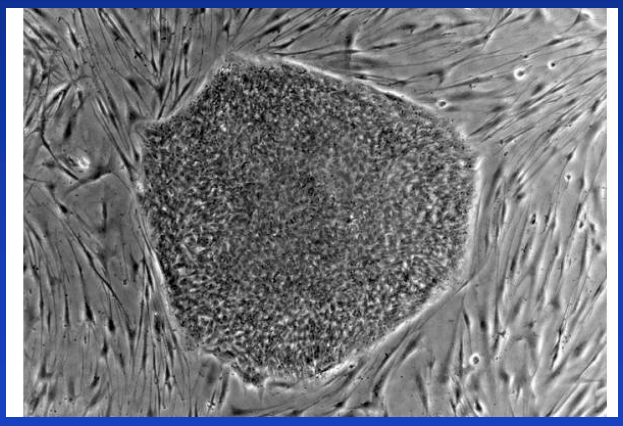
Καλλιέργεια ESC ποντικού



Τα ESC του ποντικού καλλιεργούνται:

1) πάνω σε τάπητα (μονή στοιβάδα) ινοβλαστών οι οποίοι έχουν ακτινοβοληθεί ώστε να μην διαιρούνται ή
2) Παρουσία LIF

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Καλλιέργεια ESC ποντικού

Προκειμένου να διατηρήσουν τις ιδιότητές τους στις συνθήκες καλλιέργειας :

1) Αποφεύγουμε τις ακραίες καταστάσεις δηλ. Δεν απλώνουμε τα κύτταρα ούτε πολύ αραιά ούτε πολύ πυκνά για να μην προκαλέσουμε την ανάπτυξη ανευπλοειδικών κυττάρων.
2) Παρατηρούμε το χρόνο διπλασιασμού τους που πρέπει να είναι 15-20 h
3) Πρακτικά αυτό σημαίνει ότι κάθε τρεις ημέρες χρειάζονται ανακαλλιέργεια.

Οι κατάλληλες συνθήκες καλλιέργειας για κάθε σειρά ελέγχονται με τη χρήση της δοκιμής σχηματισμού κλώνων. Στη δοκιμή αυτή χρησιμοποιεί κανείς έναν πληθυσμό κυττάρων σε διαφορετικά θρεπτικά μέσα. Τα κύτταρα απλώνονται με τέτοιο τρόπο ώστε να σχηματίσουν κλώνους (δηλ πολύ αραιά). Μετά από 6-8 ημέρες οι καλλιέργειες μονιμοποιούνται και μελετάται ο αριθμός των κλώνων που αναπτύχθηκαν σε κάθε μέσο αλλά και η μορφολογία, έκφραση συγκεκριμένων αντιγόνων κλπ.

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Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides

Austin G. Smith*, John K. Heath*†, Deborah D. Donaldson‡, Gordon G. Wong‡, J. Moreau§, Mark Stahl‡ & David Rogers‡

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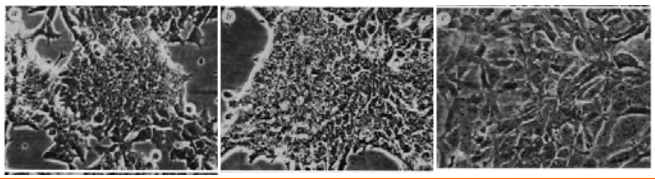

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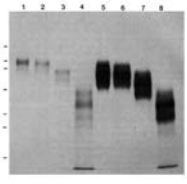

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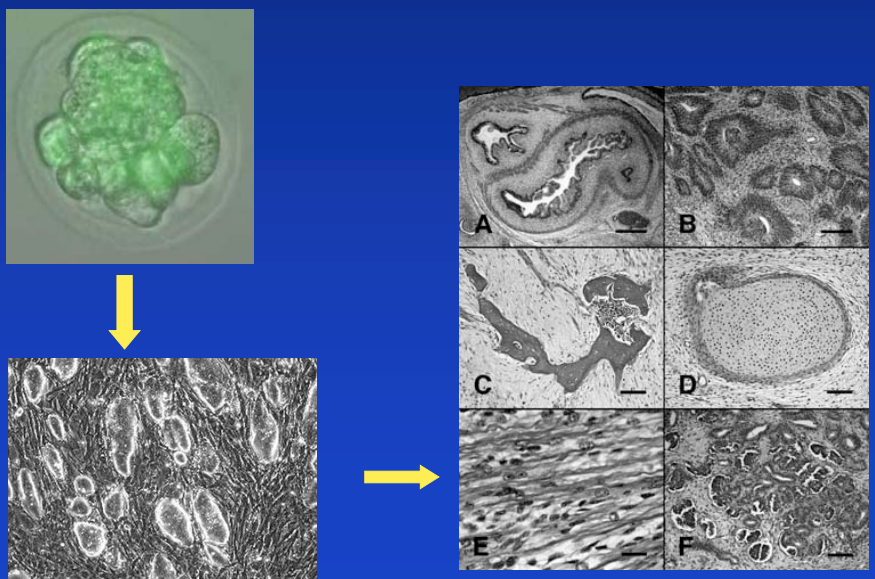
Σηματοδοτικά μονοπάτια & πολυδυναμία ESC



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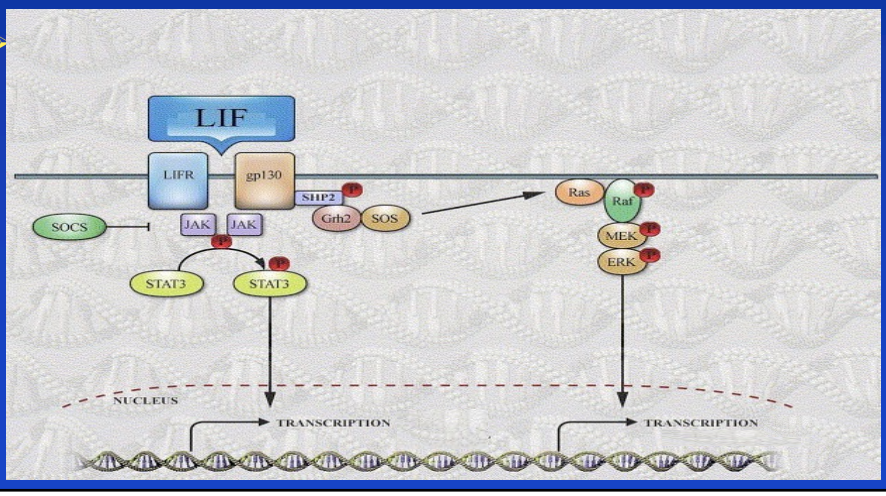
Σηματοδοτικά μονοπάτια & πολυδυναμία ESC

- Η ανακάλυψη ότι τα κύτταρα ES του ποντικού απαιτούν προκειμένου να διατηρήσουν την πολυδυναμία τους την παρουσία LIF (leukemia inhibiting factor- IL-6 family) στο θρεπτικό μέσο, το έναυσμα για τη μελέτη της σηματοδότησης που απαιτείται για τη διατήρηση της πολυδυναμίας.



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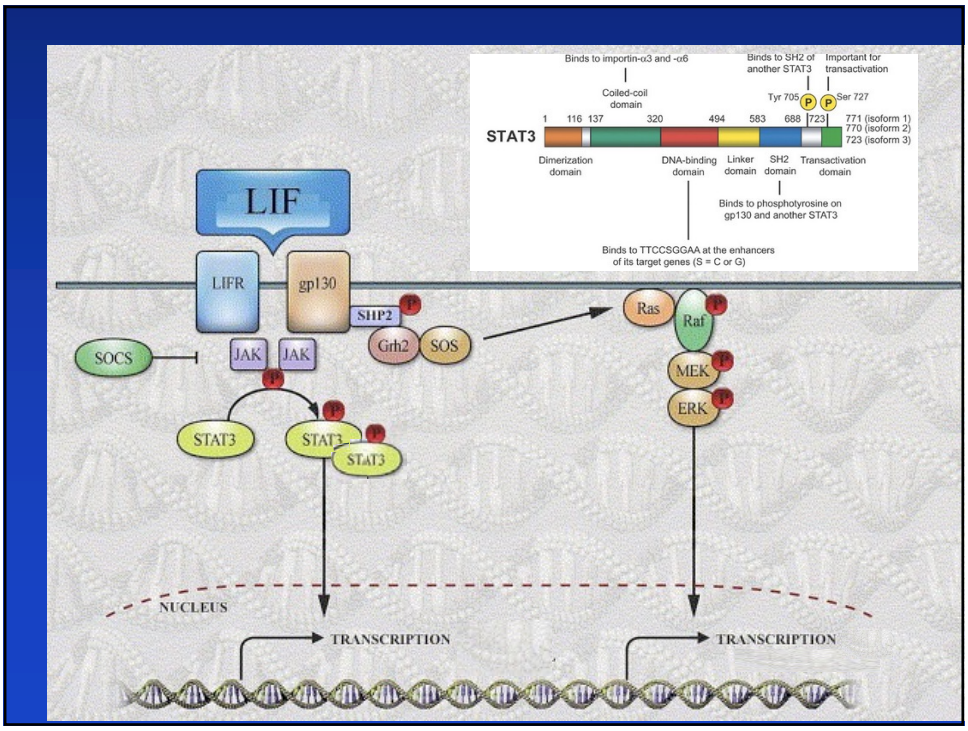
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Coiled-coil domain
Binds to SH2 of another STAT3
Important for transactivation
Tyr 705
Ser 727
1 116 137 320 494 583 688 723
771 (isoform 1)
770 (isoform 2)
723 (isoform 3)
STAT3
Dimerization domain
DNA-binding domain
Linker domain
SH2 domain
Transactivation domain
Binds to phosphotyrosine on gp130 and another STAT3
Binds to TTCCSGGAA at the enhancers of its target genes (S = C or G)
LIF
LIFR
gp130
SHP2
Grb2
SOS
Ras
Raf
MEK
ERK
SOCS
JAK
JAK
STAT3
STAT3
STAT3
NUCLEUS
TRANSCRIPTION
TRANSCRIPTION

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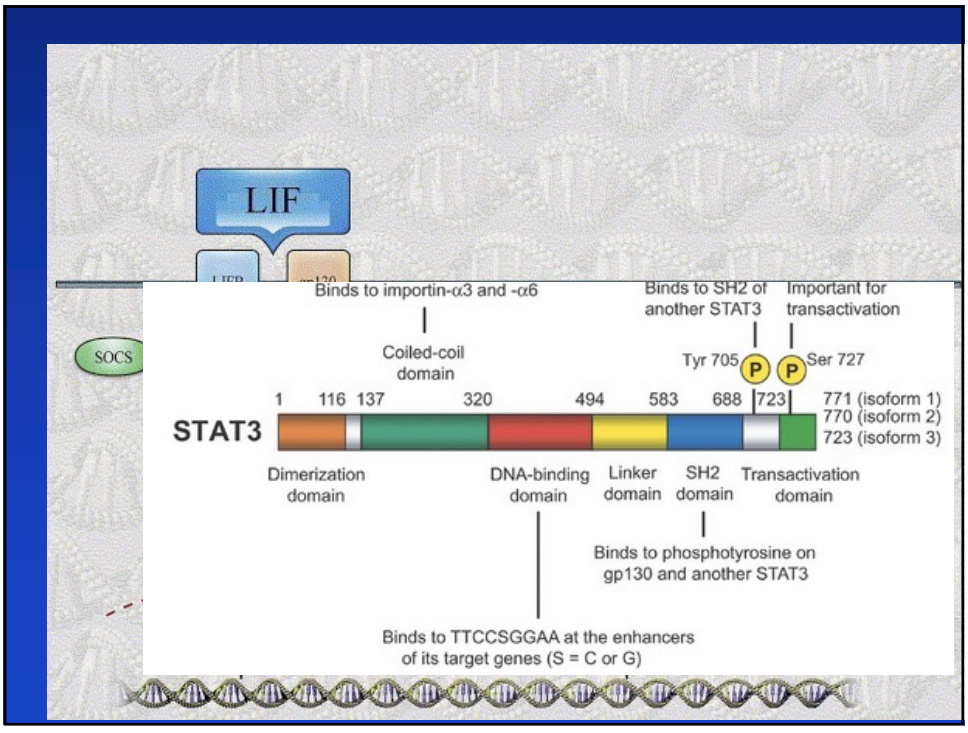
LIF
SOCS
Binds to importin-α3 and -α6
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Tyr 705
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1 116 137 320 494 583 688 723
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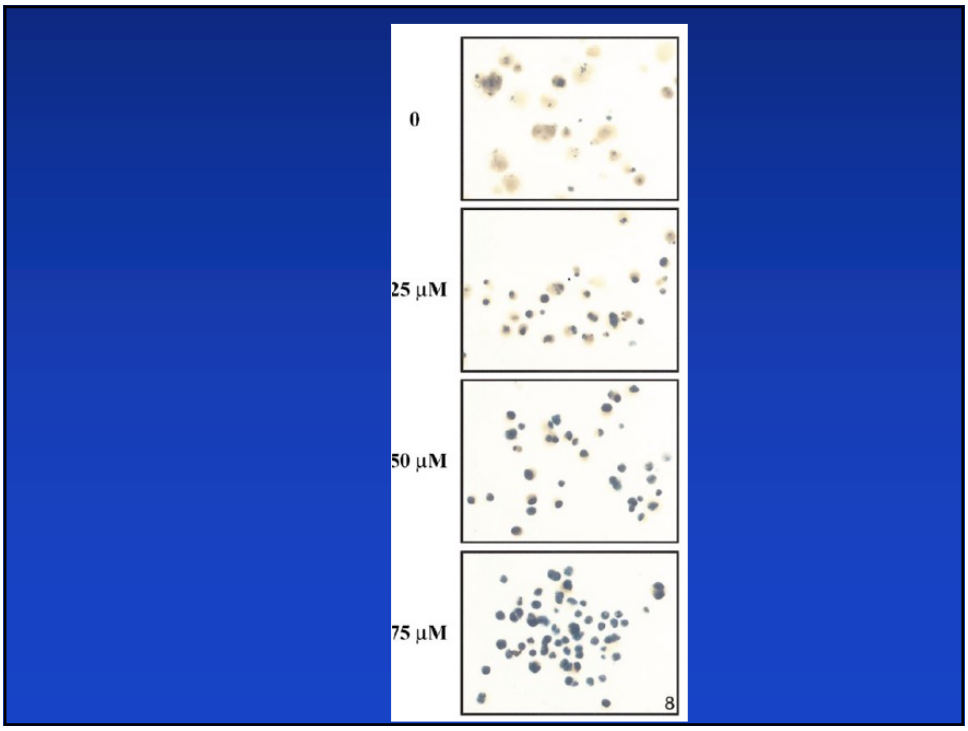

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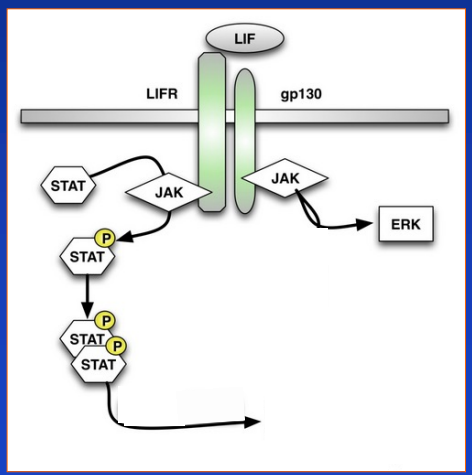
Σηματοδοτικά μονοπάτια & πολυδυναμία ESC

- Η ανακάλυψη ότι τα κύτταρα ES του ποντικού απαιτούν προκειμένου να διατηρήσουν την πολυδυναμία τους την παρουσία LIF (leukemia inhibiting factor-IL-6 family) στο θρεπτικό μέσο, το έναυσμα για τη μελέτη της σηματοδότησης που απαιτείται για τη διατήρηση της πολυδυναμίας.

- Ο LIF παράγεται από τα κύτταρα της τροφοβλάστης και ενεργοποιεί:
  1) Μονοπάτι STAT
  2) Μονοπάτι MAPK



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Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3

Hitoshi Niwa,[1,2] Tom Burdon,[1] Ian Chambers, and Austin Smith[3]

GENES & DEVELOPMENT 12:2048–2060 © 1998

The propagation of embryonic stem (ES) cells in an undifferentiated pluripotent state is dependent on leukemia inhibitory factor (LIF) or related cytokines. These factors act through receptor complexes containing the signal transducer gp130. The downstream mechanisms that lead to ES cell self-renewal have not been delineated, however. In this study, chimeric receptors were introduced into ES cells. Biochemical and functional studies of transfected cells demonstrated a requirement for engagement and activation of the latent transcription factor STAT3. Detailed mutational analyses unexpectedly revealed that the four STAT3 docking sites in gp130 are not functionally equivalent. The role of STAT3 was then investigated using the dominant interfering mutant, STAT3F. ES cells that expressed this molecule constitutively could not be isolated. An episomal supertransfection strategy was therefore used to enable the consequences of STAT3F expression to be examined. In addition, an inducible STAT3F transgene was generated. In both cases, expression of STAT3F in ES cells growing in the presence of LIF specifically abrogated self-renewal and promoted differentiation. These complementary approaches establish that STAT3 plays a central role in the maintenance of the pluripotential stem cell phenotype. This contrasts with the involvement of STAT3 in the induction of differentiation in somatic cell types. Cell type-specific interpretation of STAT3 activation thus appears to be pivotal to the diverse developmental effects of the LIF family of cytokines. Identification of STAT3 as a key transcriptional determinant of ES cell self-renewal represents a first step in the molecular characterization of pluripotency.

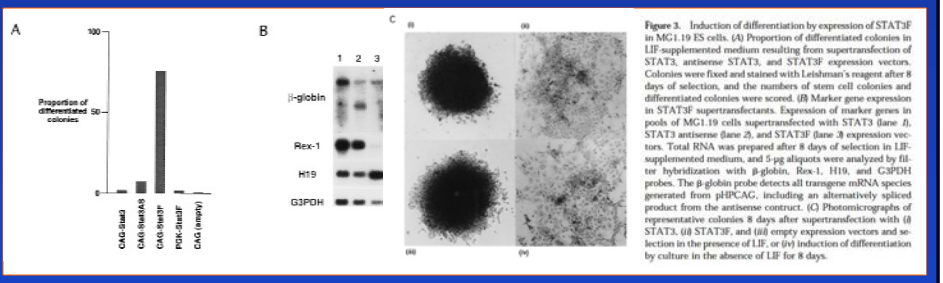

Figure 3. Induction of differentiation by expression of STAT3F in MG1.19 ES cells. (*A*) Proportion of differentiated colonies in LIF-supplemented medium resulting from supertransfection of STAT3, antisense STAT3, and STAT3F expression vectors. Colonies were fixed and stained with Leishman's reagent after 8 days of selection, and the numbers of stem cell colonies and differentiated colonies were scored. (*B*) Marker gene expression in STAT3F supertransfectants. Expression of marker genes in pools of MG1.19 cells supertransfected with STAT3 (lane *1*), STAT3 antisense (lane *2*), and STAT3F (lane *3*) expression vectors. Total RNA was prepared after 8 days of selection in LIF-supplemented medium, and 5-μg aliquots were analyzed by filter hybridization with β-globin, Rex-1, H19, and G3PDH probes. The β-globin probe detects all transgene mRNA species generated from pHPCAG, including an alternatively spliced product from the antisense contruct. (*C*) Photomicrographs of representative colonies 8 days after supertransfection with (*i*) STAT3, (*ii*) STAT3F, and (*iii*) empty expression vectors and selection in the presence of LIF, or (*iv*) induction of differentiation by culture in the absence of LIF for 8 days.

Η έκφραση της STAT-3 είναι απαραίτητη για τη διατήρηση του πολλαπλασιασμού και της πολυδυναμίας. Υπερέκφραση κυρίαρχης αρνητικής μετάλλαξης της STAT-3 (καταργεί διμερισμό και μετατόπιση-ανταγωνίζεται για docking sites gp130) οδηγεί σε διαφοροποίηση.

i: Υπερέκφραση STAT-3, ii: Υπερέκφραση STAT-3K.A., iii: control φορέας, iv: Διαφοροποιημένα ES.

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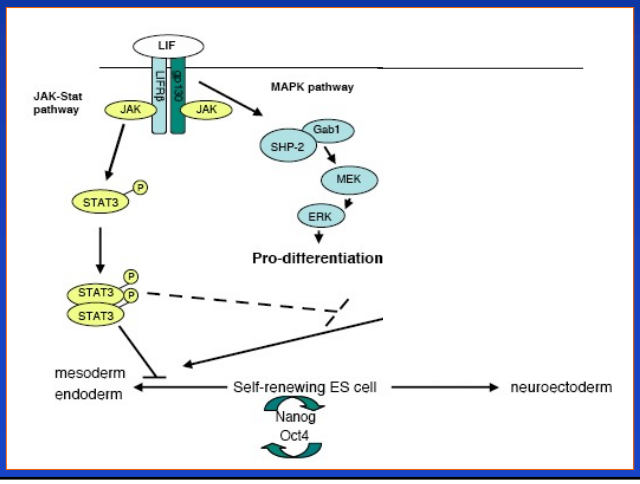
Σηματοδοτικά μονοπάτια & πολυδυναμία ESC

Τα κύτταρα ES του ποντικού απαιτούν προκειμένου να διατηρήσουν την πολυδυναμία τους την παρουσία LIF (leukemia inhibiting factor- IL-6 family) ή άλλων μορίων της ίδιας οικογένειας π.χ. Ογκοστατίνη στο θρεπτικό μέσο. Ο LIF παράγεται από τα κύτταρα της τροφοβλάστης και ενεργοποιεί:

1) Μονοπάτι STAT
2) Μονοπάτι MAPK



112

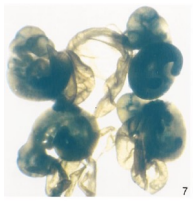
Suppression of SHP-2 and ERK Signalling Promotes Self-Renewal of Mouse Embryonic Stem Cells

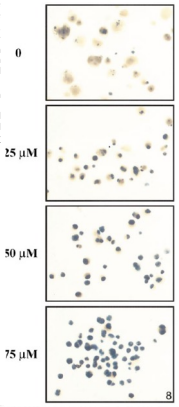
Tom Burdon, Craig Stracey, Ian Chambers, Jennifer Nichols and Austin Smith

Developmental Biology 210, 30–43 (1999)

The propagation of pluripotent mouse embryonic stem (ES) cells depends on signals transduced through the cytokine receptor subunit gp130. Signalling molecules activated downstream of gp130 in ES cells include STAT3, the protein tyrosine phosphatase SHP-2, and the mitogen-activated protein kinases, ERK1 and ERK2. A chimaeric receptor in which tyrosine 118 in the gp130 cytoplasmic domain was mutated did not engage SHP-2 and failed to activate ERKs. However, this receptor did support ES cell self-renewal. In fact, stem cell colonies formed at 100-fold lower concentrations of cytokine than the unmodified receptor. Moreover, altered ES cell morphology and growth were observed at high cytokine concentrations. These indications of deregulated signalling in the absence of tyrosine 118 were substantiated by sustained activation of STAT3. Confirmation that ERK activation is not required for self-renewal was obtained by propagation of pluripotent ES cells in the presence of the MEK inhibitor PD098059. In fact, the growth of undifferentiated ES cells was enhanced by culture in PD098059. Thus activation of ERKs appears actively to impair self-renewal. These data imply that the self-renewal signal from gp130 is a finely tuned balance of positive and negative effectors.

Cells were cultured at low density (1000 cells/cm2) for 48 h in the presence of LIF plus 25 mM PD098059 or in the absence of LIF. before microinjection into mouse blastocysts. ZIN40 cells were used in this experiment, since they carry a nuclearlocalised b-galactosidase marker widely expressed in differentiated cells. Staining of midgestation embryos for b-galactosidase revealed that ES cells treated with PD098059 contributed to chimaeras (Fig. 7). In contrast, cells cultured in the absence of LIF for 48 h were incapable of colonising the embryo (data not shown). Significantly, five-born chimaeras have been obtained from ES cells cultured for 4 days in 25 mM PD098059. Functional contribution of the cells to the germ line has been observed in two chimaeras, as evidenced by transmission of the ES cell-derived coat colour marker. This result confirms that gp130-dependent ERK activity is not required for maintaining the pluripotency of ES cells.





To examine the effect of PD098059 in a more appropriate model of differentiation, ES cells were aggregated to form embryoid bodies and cultured in the presence of the **MEK inhibitor**. The IOUD2 ES cell line used in this experiment carries the *a* chromosomal reporter gene allowing the identification of stem cells in embryoid bodies by staining for b-galactosidase activity. ES cells were aggregated in hanging drops in the absence of LIF for 2 days and then maintained in suspension in bacterial dishes in the presence of 25–100 mM PD098059 for a further 5 days. Embryoid bodies were then plated on gelatin-coated plates overnight and stained with X-gal.

FIG. 7. Effect of PD098059 on ES cell pluripotency. ZIN40 ES cells were treated with 25 μM PD098059 plus 5 U/ml LIF for 48 h, refed with medium containing LIF for a further 24 h, and then microinjected into C57BL/6 blastocysts. Embryos were collected at day 9.5 of pregnancy and stained for β-galactosidase activity. Representative embryos are shown.

FIG. 8. Effect of PD098059 on ES cell differentiation in embryoid bodies. Aggregates of IOUD2 ES cells were formed in hanging drops without LIF for 2 days. They were then transferred to bacterial dishes and cultured for 5 days in medium containing either no inhibitor or 25, 50, or 75 μM PD098059. After attachment overnight on gelatinised plates, the embryoid bodies were stained for β-galactosidase activity.

- Το μονοπάτι MAP που ενεργοποιείται από τον LIF μέσω gp 130 ενέχεται σε γεγονότα που αφορούν την προαγωγη της διαφο-ροποίησης παρά την αναγέννηση/πολυδυνα-μία.
- Αν παρεμποδιστεί το μονοπάτι αυτό (ανα-στολείς μεταλλάξεις gp130 που δεν επιτρέ-πουν την ενεργοποίηση της MAPK) έχουν ως αποτέλεσμα την εμφάνι-ση περισσότερων μη διαφοροποιημένων εμβρυϊκών σωματίων.

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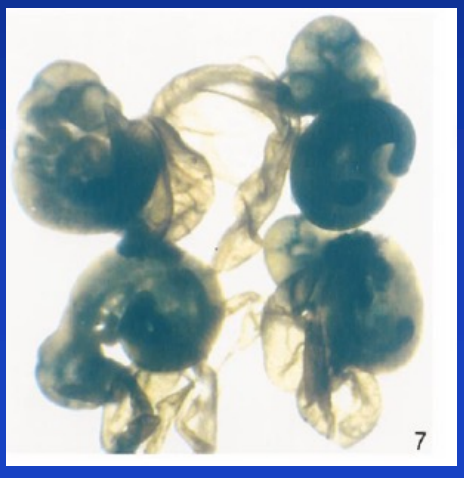

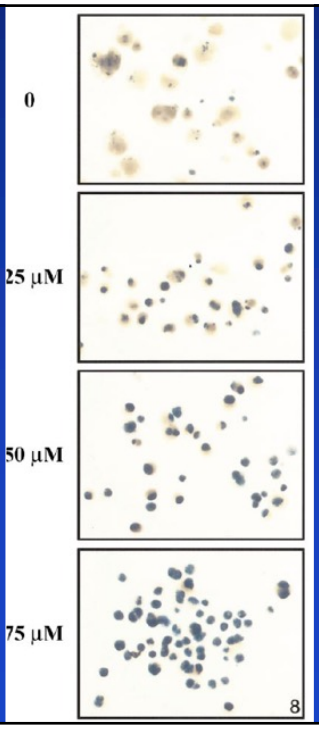

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Leukemia Inhibitory Factor–dependent Transcriptional Activation in Embryonic Stem Cells

Hélène Boeuf, Charlotte Hauss, Fabienne De Graeve, Nathalie Baran, and Claude Kedinger

The Journal of Cell Biology, Volume 138, Number 6, September 22, 1997 1207–1217

Abstract. STAT transcription factors are induced by a number of growth factors and cytokines. Within minutes of induction, the STAT proteins are phosphorylated on tyrosine and serine residues and translocated to the nucleus, where they bind to their DNA targets. The leukemia inhibitory factor (LIF) mediates pleiotropic and sometimes opposite effects both in vivo and in cultured cells. It is known, for example, to prevent differentiation of embryonic stem (ES) cells in vitro. To get insights into LIF-regulated signaling in ES cells, we have analyzed protein-binding and transcriptional properties of STAT recognition sites in ES cells cultivated in the presence and in the absence of LIF. We have detected a specific LIF-regulated DNA-binding activity implicating the STAT3 protein. We show that STAT3 phosphorylation is essential for this LIF-dependent DNA-binding activity. The possibility that ERK2 or a closely related protein kinase, whose activity is modulated in a LIF-dependent manner, contributes to this phosphorylation is discussed. Finally, we show that the multimerized STAT3-binding DNA element confers LIF responsiveness to a minimal thymidine kinase promoter. This, together with our observation that overexpression of STAT3 dominant-negative mutants abrogates this LIF responsiveness, clearly indicates that STAT3 is involved in LIF-regulated transcriptional events in ES cells. Finally, stable expression of such a dominant negative mutant of STAT3 induces morphological differentiation of ES cells despite continuous LIF supply. Our results suggest that STAT3 is a critical target of the LIF signaling pathway, which maintains pluripotent cell proliferation.

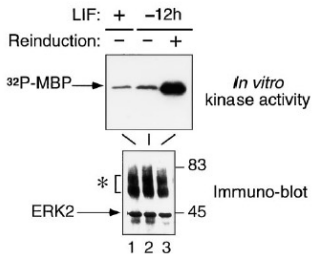

Figure 7. An ERK2-related MAP kinase is activated in ES cells reinduced by LIF. Nuclear extracts from ES cells maintained with LIF or without LIF for 12 h (*−12h*) and reinduced by LIF for 10 min, as indicated, were immunoprecipitated with the anti-ERK2 antibody, as described in Materials and Methods. Two thirds of the reaction were assayed in an in vitro kinase assay using MBP as an exogenous substrate (*top*). The remaining third of the reaction was analyzed by immunoblot with the anti-ERK2 antibody (*bottom*). The asterisk refers to the heavy chains of the Igs. Numbers on the right-hand side indicate the position of protein size markers (kD).

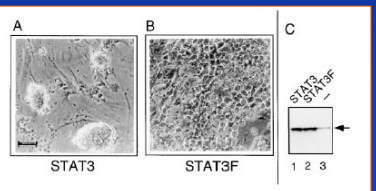

Figure 10. Stable expression of STAT3F dominant negative mutant induces ES cell differentiation. Phase contrast micrographs of Neomycin-resistant ES cell clones stably expressing the wild-type STAT3 (*A*) or the dominant negative STAT3F mutant (*B*). The selected clones were grown on feeder layers of mouse embryo fibroblasts (visible in *A* between the clumps of undifferentiated ES cells) and continuously maintained in the presence of LIF. (*C*) Western blot analysis of equivalent amounts of whole-cell extracts of STAT3- (lane *1*) or STAT3F-transformed cells (lane *2*), or untransformed ES cells (lane *3*). The arrow points to the specific signal obtained with the monoclonal anti-STAT3 antibody. Bar, 100 μm.

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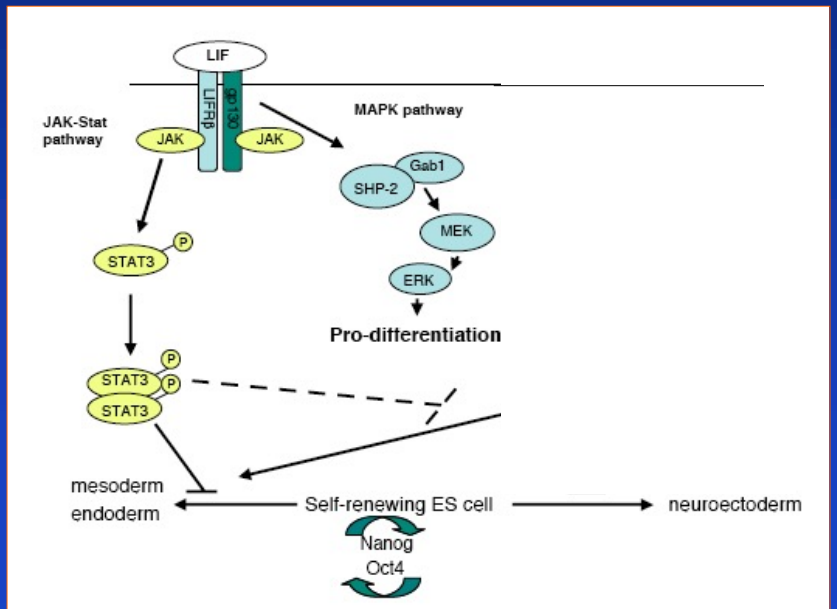
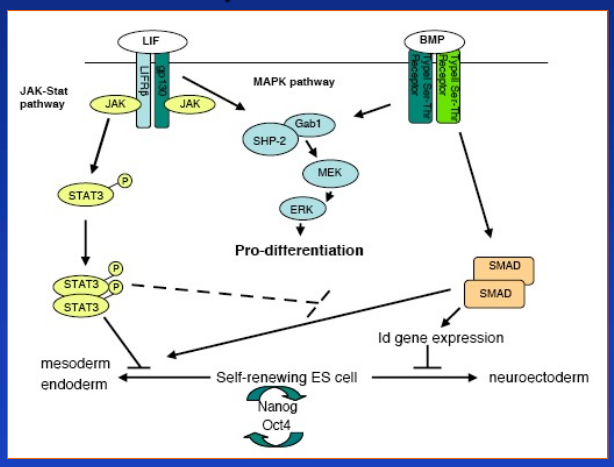


116

Το μονοπάτι BMP



- Τα κύτταρα ES απαιτούν προκειμένου να πολλαπλασιάζονται χωρίς να διαφοροποιούνται εκτός από LIF, και ορό.
- Αν καλλιεργηθούν χωρίς ορό,τότε διαφοροποιούνται σε **πρόδρομα νευρικά κύτταρα**.
- Αν καλλιεργηθούν χωρίς LIF αλλά με BMP τότε διαφοροποιούνται προς **μεσοδερμικά και αιμοποιητικά κύτταρα**.
- **Ο συνδυασμός LIF με BMP διατηρεί την πολυδυναμία των ES.**

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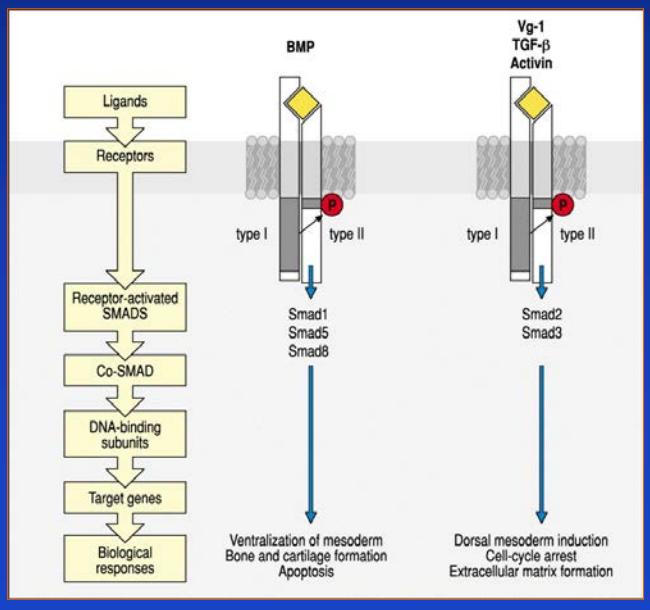
Το μονοπάτι BMP



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BMP Induction of Id Proteins Suppresses Differentiation and Sustains Embryonic Stem Cell Self-Renewal in Collaboration with STAT3

Qi-Long Ying,* Jennifer Nichols, Ian Chambers, and Austin Smith*

The cytokine leukemia inhibitory factor (LIF) drives self-renewal of mouse embryonic stem (ES) cells by activating the transcription factor STAT3. In serum-free cultures, however, LIF is insufficient to block neural differentiation and maintain pluripotency. Here, we report that bone morphogenetic proteins (BMPs) act in combination with LIF to sustain self-renewal and preserve multilineage differentiation, chimera colonization, and germline transmission properties. ES cells can be propagated from single cells and derived de novo without serum or feeders using LIF plus BMP. The critical contribution of BMP is to induce expression of Id genes via the Smad pathway. Forced expression of Id liberates ES cells from BMP or serum dependence and allows self-renewal in LIF alone. Upon LIF withdrawal, Id-expressing ES cells differentiate but do not give rise to neural lineages. We conclude that blockade of lineage-specific transcription factors by Id proteins enables the self-renewal response to LIF/STAT3.

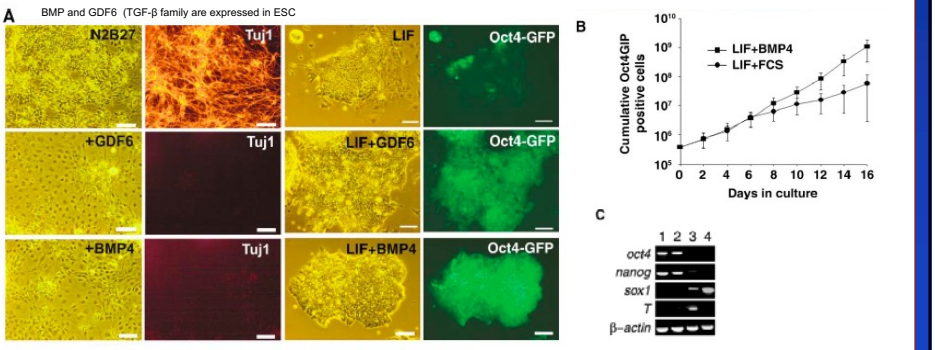

Figure 1. LIF Plus BMP Sustain ES Cell Self-Renewal in Serum-Free Medium

(A) Phase contrast and fluorescent images of *Oct4*-GiP cells cultured in N2B27 with the indicated factors. TuJ1 immunostaining detects neuronal differentiation; green fluorescence reflects activity of the Oct4 promoter in undifferentiated ES cells. Bar: 50 μm.

(B) Plot of cumulative *Oct4*-GFP positive undifferentiated ES cell numbers during progressive passaging in conventional medium with FCS plus LIF or in N2B27 with LIF (10 ng/ml) plus BMP4 (10 ng/ml). Cultures were passaged every 48 hr using cell dissociation buffer and replated at 4×10^6 cells per 10 cm^2 well. The number of GFP-positive cells was determined by FACS analysis at each passage.

(C) RT-PCR analyis of Oct4, Nanog, T (brachyury), and Sox1 mRNAs in (1) ES cells in N2B27 with LIF plus BMP for 6 passages, (2) ES cells cultured in serum with LIF, (3) day 8 embryoid bodies, and (4) day 8 embryoid bodies with retinoic acid treatment.

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The definitive functional attribute of mouse ES cells is their capacity to re-enter embryonic development and contribute to the full repertoire of differentiated tissues in chimeric mice. We injected GFP reporter ES cells into mouse blastocysts after propagation in N2B27 with LIF plus BMP for 3 weeks. Analysis at mid-gestation identified several chimeras with high ES cell contributions to a range of tissues. As a more rigorous test, we used ES cells transfected with *taugfp* and selected and expanded in LIF plus BMP. Liveborn chimeras were obtained and two male animals transmitted the ES cell genome.

We investigated whether the response to BMP may be an adaptation of established ES cells to culture or is manifest during the initial stages of ES cell derivation. We plated blastocysts in N2B27 supplemented with BMP plus LIF. After several days, expanded inner cell masses were dissociated and replated in the same culture conditions.

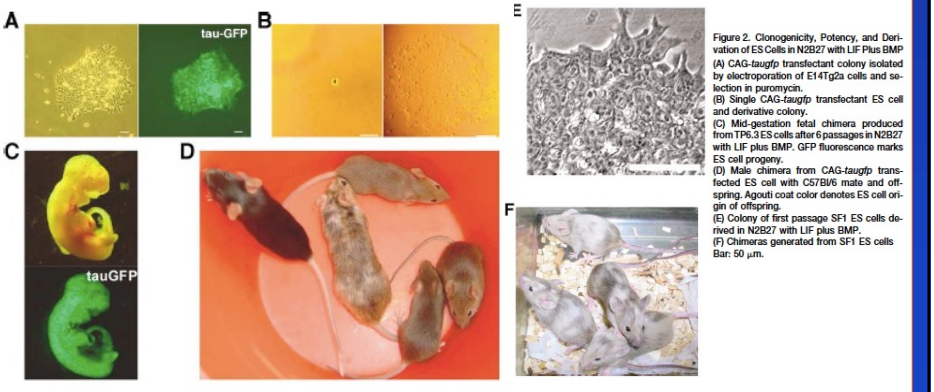

Figure 2. Clonogenicity, Potency, and Derivation of ES Cells in N2B27 with LIF Plus BMP

(A) CAG-*taugfp* transfectant colony isolated by electroporation of E14Tg2a cells and selection in puromycin.

(B) Single CAG-*taugfp* transfectant ES cell and derivative colony.

(C) Mid-gestation fetal chimera produced from TP6.3 ES cells after 6 passages in N2B27 with LIF plus BMP. GFP fluorescence marks ES cell progeny.

(D) Male chimera from CAG-*taugfp* transfected ES cell with C57Bl/6 mate and offspring. Agouti coat color denotes ES cell origin of offspring.

(E) Colony of first passage SF1 ES cells derived in N2B27 with LIF plus BMP.

(F) Chimeras generated from SF1 ES cells Bar: 50 μm.

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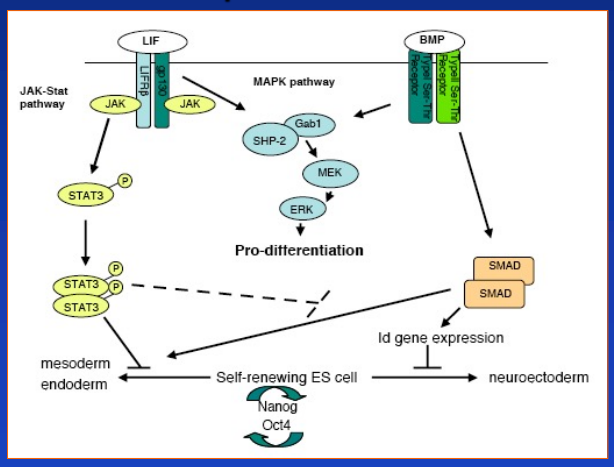
Το μονοπάτι BMP



➢Τα ES του ποντικού καλλιεργούνται συνήθως παρουσία **ορού** και ή τροφικών κυττάρων/LIF .

➢Η απαίτηση για **ορό** μπορεί να ξεπεραστεί αν στο θρεπτικό μέσο προστεθεί **BMP-4**. (ένα σημαντικό ποσοστό των ES που καλλιεργούνται απουσία ορού διαφοροποιείται σε νευρικά κύτταρα)

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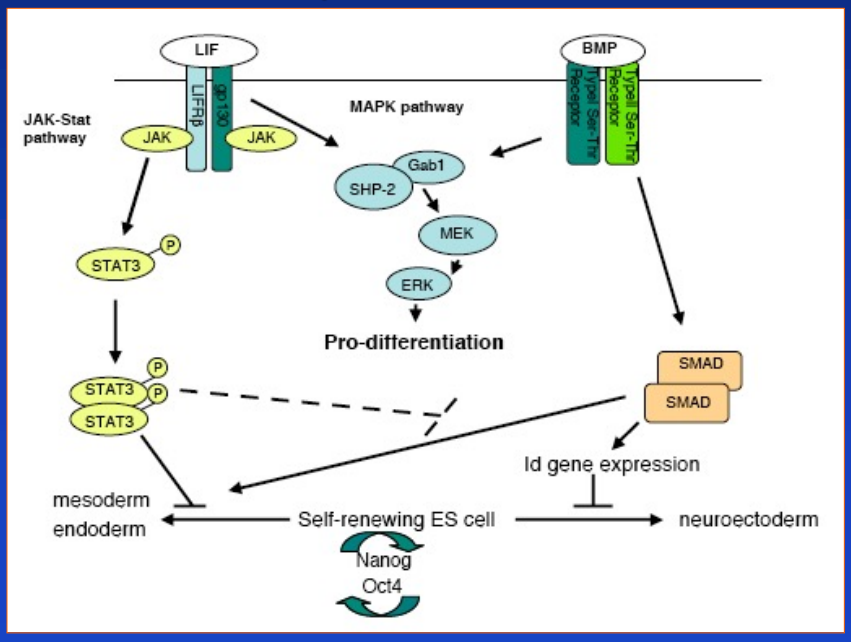
Το μονοπάτι BMP



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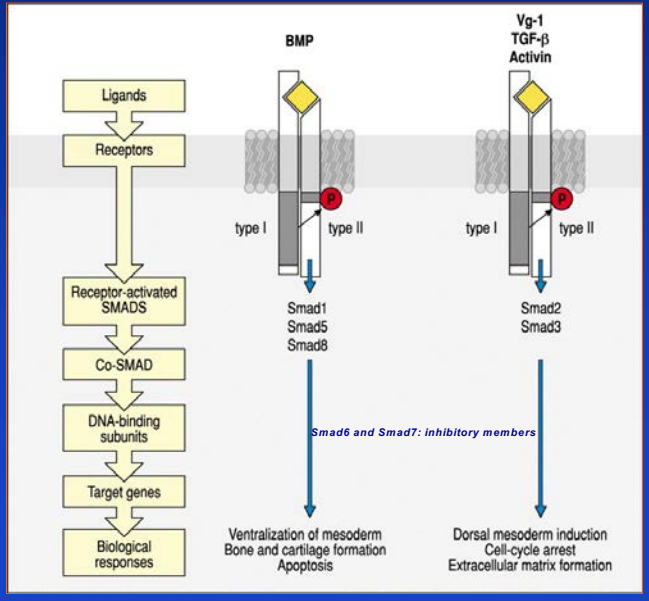

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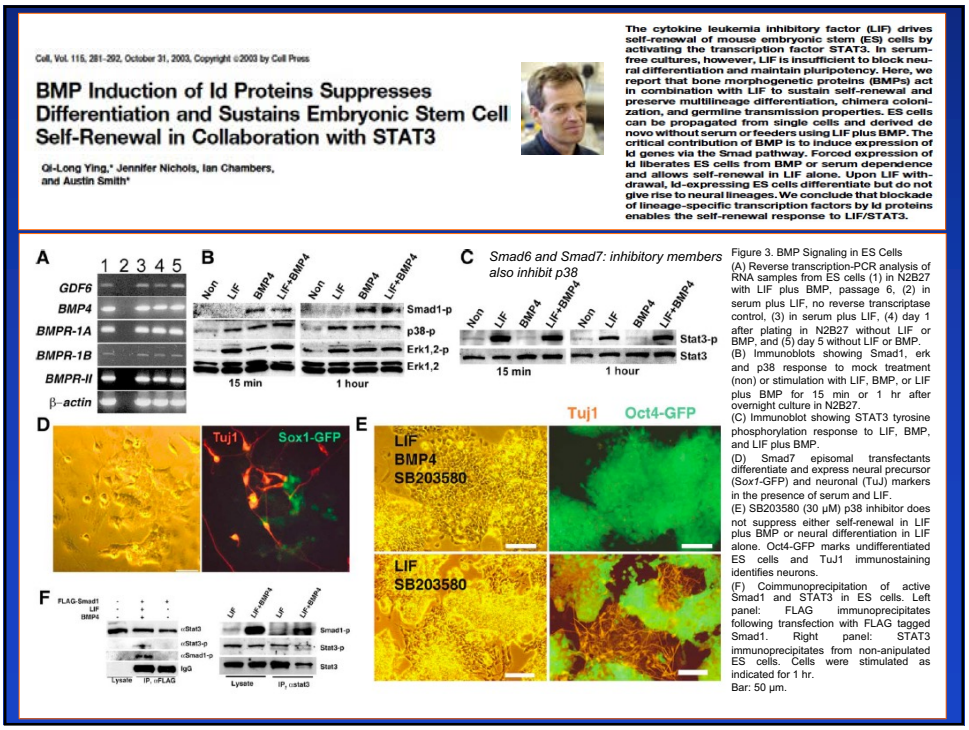

Figure 3. BMP Signaling in ES Cells

(A) Reverse transcription-PCR analysis of RNA samples from ES cells (1) in N2B27 with LIF plus BMP, passage 6, (2) in serum plus LIF, no reverse transcriptase control, (3) in serum plus LIF, (4) day 1 after plating in N2B27 without LIF or BMP, and (5) day 5 without LIF or BMP.

(B) Immunoblots showing Smad1, erk and p38 response to mock treatment (non) or stimulation with LIF, BMP, or LIF plus BMP for 15 min or 1 hr after overnight culture in N2B27.

(C) Immunoblot showing STAT3 tyrosine phosphorylation response to LIF, BMP, and LIF plus BMP.

(D) Smad7 episomal transfectants differentiate and express neural precursor (Sox1-GFP) and neuronal (TuJ) markers in the presence of serum and LIF.

(E) SB203580 (30 μM) p38 inhibitor does not suppress either self-renewal in LIF plus BMP or neural differentiation in LIF alone. Oct4-GFP marks undifferentiated ES cells and TuJ1 immunostaining identifies neurons.

(F) Coimmunoprecipitation of active Smad1 and STAT3 in ES cells. Left panel: FLAG immunoprecipitates following transfection with FLAG tagged Smad1. Right panel: STAT3 immunoprecipitates from non-anipulated ES cells. Cells were stimulated as indicated for 1 hr.

Bar: 50 μm.

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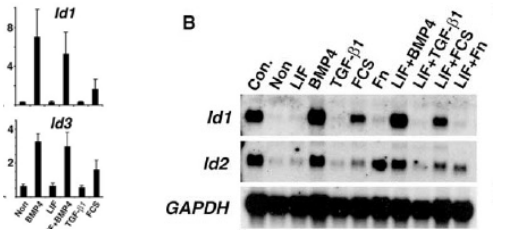

Figure 4. Expression and Function of Ids in ES Cells. (A) LightCycler reverse transcription PCR analyses of gene induction in response to LIF, BMP, or LIF BMP. ES cells were cultured overnight in N2B27 alone, then stimulated for 45 min. (B) Northern hybridization of Id mRNA expressionin Oct4-GiP cells. Con: steady state EScells maintained in serum containing mediumplus LIF. Lanes 2–11: Cells cultured overnight in N2B27 without factors then stimulated as indicated for 45 min. Fn, fibronectin.

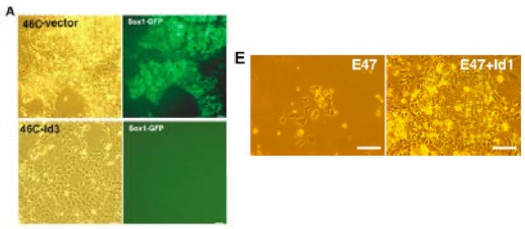

Figure 5. Id Suppresses Neural Differentiation and Is Required for ES Cell Self-Renewal (A) Phase contrast and GFP fluorescence imagesof vector and Id3 stable integrant 46Cclones after 6 days differentiation in N2B27without added factors. Id1 and Id2 transfectantsshowed similar suppression of neural differentiation. (E) Overexpression of E47 blocks ES cell selfrenewal,which can be rescued by increased Id1. 46C/T ES cells were supertransfected with E47 or cosupertransfected with E47 plus Id1 episomal expression vectors and cultured for 6 days under dual puromycin and zeocin selection in serum-containing medium with LIF.

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Σηματοδοτικά μονοπάτια & πολυδυναμία ESC

- Στα κύτταρα ES υπάρχουν υποδοχείς για τον BMP-4.
- Η προσθήκη BMP-4 έχει ως αποτέλεσμα τη φωσφορυλλίωση της Smad1.
- **Ο BMP δεν επηρεάζει τη σηματοδότηση μέσω STAT3 ή Erk.**
- Ο BMP επάγει την έκφραση των Id.
- Οι παράγοντες Id είναι πρωτεΐνες που φέρουν το μοτίβο bHLH και αλληλεπιδρούν με παράγοντες E απομονώνοντάς τους από παράγοντες **bHLH με θετική δράση** (π.χ MASH-1)
- Κύτταρα ES τα οποία υπερεκφράζουν σταθερά Id μπορούν να καλλιεργηθούν παρουσία LIF και απουσία BMP διατηρώντας ταυτοχρόνως την πολυδυναμία τους.

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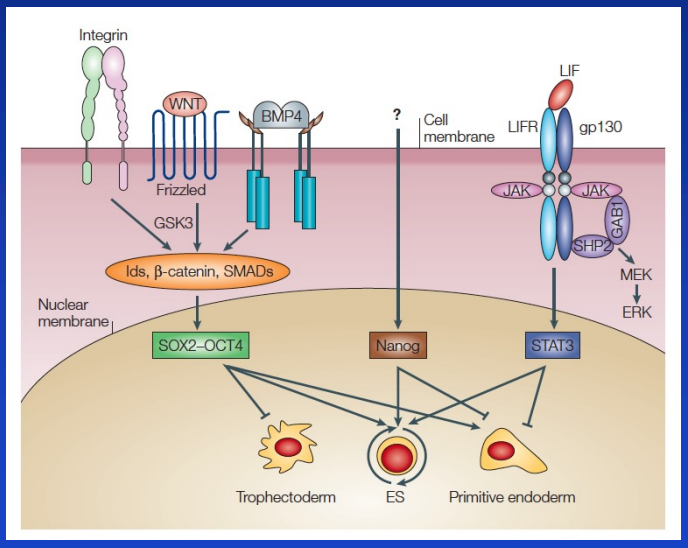
Σηματοδοτικά μονοπάτια & πολυδυναμία ESC



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Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor

Noboru Sato[1], Laurent Meijer[2,3], Leandros Skaltsounis[4], Paul Greengard[2] & Ali H Brivanlou[1]

NATURE MEDICINE VOLUME 10 | NUMBER 1 | JANUARY 2004

Human and mouse embryonic stem cells (HESCs and MESCs, respectively) self-renew indefinitely while maintaining the ability to generate all three germ-layer derivatives. Despite the importance of ESCs in developmental biology and their potential impact on tissue replacement therapy, the molecular mechanism underlying ESC self-renewal is poorly understood. Here we show that activation of the canonical Wnt pathway is sufficient to maintain self-renewal of both HESCs and MESCs. Although Stat-3 signaling is involved in MESC self-renewal, stimulation of this pathway does not support self-renewal of HESCs. Instead we find that Wnt pathway activation by 6-bromoindirubin-3′-oxime (BIO), a specific pharmacological inhibitor of glycogen synthase kinase-3 (GSK-3), maintains the undifferentiated phenotype in both types of ESCs and sustains expression of the pluripotent state-specific transcription factors Oct-3/4, Rex-1 and Nanog. Wnt signaling is endogenously activated in undifferentiated MESCs and is downregulated upon differentiation. In addition, BIO-mediated Wnt activation is functionally reversible, as withdrawal of the compound leads to normal multidifferentiation programs in both HESCs and MESCs. These results suggest that the use of GSK-3-specific inhibitors such as BIO may have practical applications in regenerative medicine.

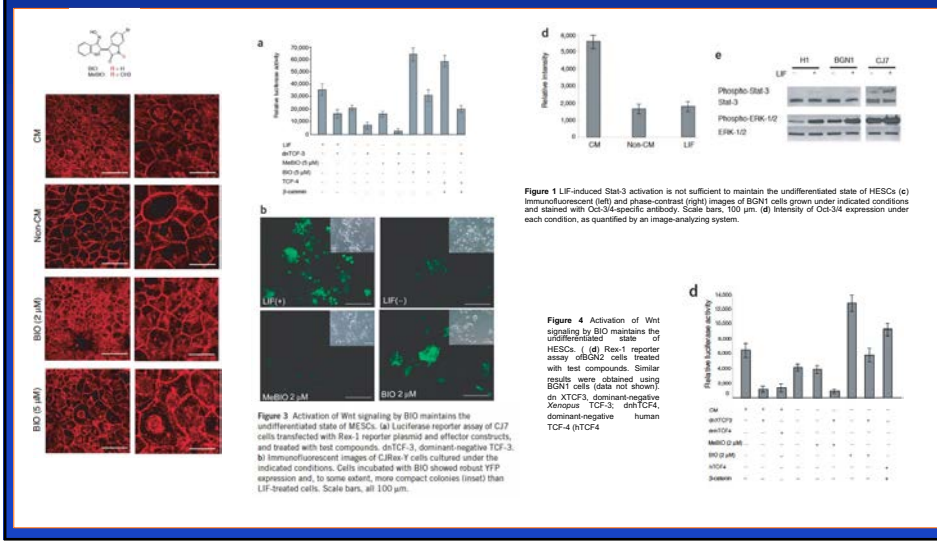

Figure 1 LIF-induced Stat-3 activation is not sufficient to maintain the undifferentiated state of HESCs (**c**) Immunofluorescent (left) and phase-contrast (right) images of BGN1 cells grown under indicated conditions and stained with Oct-3/4-specific antibody. Scale bars, 100 μm. (**d**) Intensity of Oct-3/4 expression under each condition, as quantified by an image-analyzing system.

Figure 3 Activation of Wnt signaling by BIO maintains the undifferentiated state of MESCs. (**a**) Luciferase reporter assay of CJ7 cells transfected with Rex-1 reporter plasmid and effector constructs, and treated with test compounds. dnTCF-3, dominant-negative TCF-3. (**b**) Immunofluorescent images of CJRex-Y cells cultured under the indicated conditions. Cells incubated with BIO showed robust YFP expression and, to some extent, more compact colonies (inset) than LIF-treated cells. Scale bars, all 100 μm.

Figure 4 Activation of Wnt signaling by BIO maintains the undifferentiated state of HESCs. (**d**) Rex-1 reporter assay of BGN2 cells treated with test compounds. Similar results were obtained using BGN1 cells (data not shown). dn XTCF3, dominant-negative *Xenopus* TCF-3; dnhTCF4, dominant-negative human TCF-4 (hTCF4

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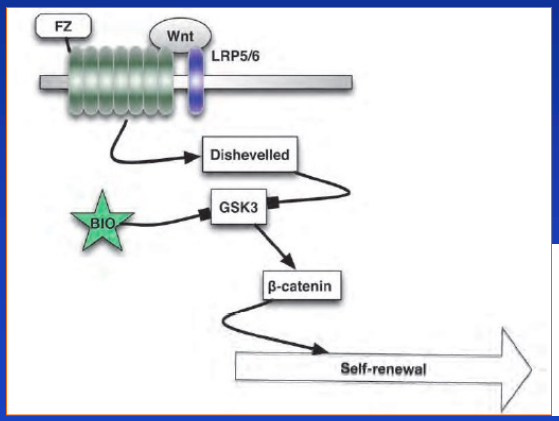
Σηματοδοτικά μονοπάτια & πολυδυναμία ESC



Wnt signal pathway role in self-renewal.
Inhibition of GSK3 prolongs the existence of β-catenin, causing it to accumulate, increasing the pool, which can translocate to the nucleus and activate gene expression. BIO has been shown to be able to maintain pluripotency of mouse and human ES cells in the absence of LIF (Sato et al., 2003). In a similar vain activation of Wnt signaling indirectly by removing the inhibitory effect of APC sustains pluripotency, suggesting the Wnt signaling is required for self-renewal (Kielman et al., 2002). In addition, treatment with Wnt3a was found to stimulate hES cell proliferation (Singla et al., 2006).
L.Quintan (2011). Signaling Pathways in Mouse Embryo Stem Cell Self-Renewal, Embryonic Stem Cells - Basic Biology to Bioengineering, Prof. Michael Kallos (Ed.).

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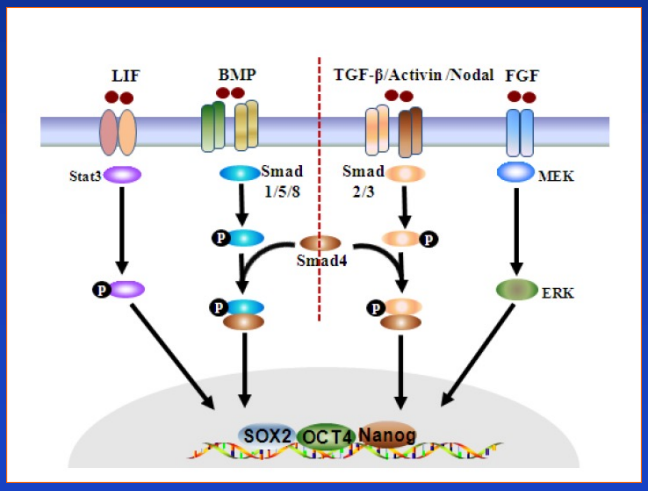
Σηματοδοτικά μονοπάτια & πολυδυναμία hESC



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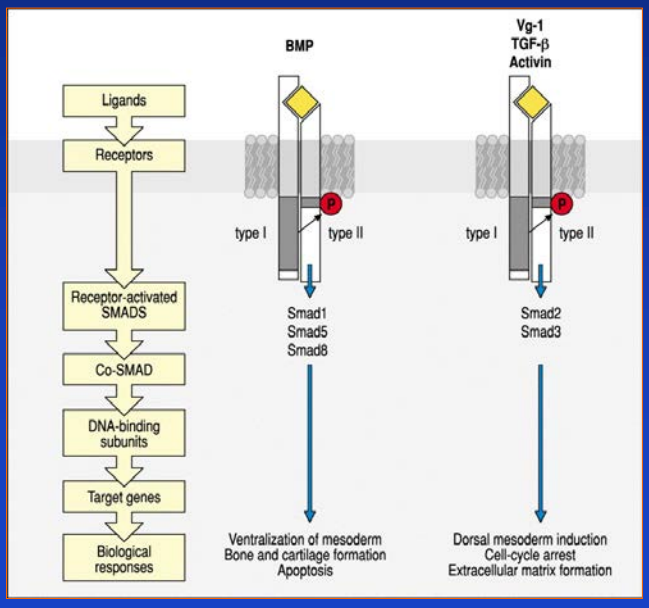
Το μονοπάτι BMP



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Physiological rationale for responsiveness of mouse embryonic stem cells to gp130 cytokines

Jennifer Nichols[1,*], Ian Chambers[1], Tetsuya Taga[2] and Austin Smith[1]

Development 128, 2333-2339 (2001)

Embryonic stem cells are established directly from the pluripotent epiblast of the preimplantation mouse embryo. Their derivation and propagation are dependent upon cytokine-stimulated activation of gp130 signal transduction. Embryonic stem cells maintain a close resemblance to epiblast in developmental potency and gene expression profile. The presumption of equivalence between embryonic stem cells and epiblast is challenged, however, by the finding that early embryogenesis can proceed in the absence of gp130. To explore this issue further, we have examined the capacity of *gp130* mutant embryos to accommodate perturbation of normal developmental progression. Mouse embryos arrest at the late blastocyst stage when implantation is prevented. This process of diapause occurs naturally in lactating females or can be induced experimentally by removal of the ovaries. We report that *gp130*$^{-/-}$ embryos survive unimplanted in the uterus after ovariectomy but, in contrast to wild-type or heterozygous embryos, are subsequently unable to resume development. Inner cell masses explanted from *gp130*$^{-/-}$ delayed blastocysts produce only parietal endoderm, a derivative of the hypoblast. Intact mutant embryos show an absence of epiblast cells, and Hoechst staining and TUNEL analysis reveal a preceding increased incidence of cell death. These findings establish that gp130 signalling is essential for the prolonged maintenance of epiblast in vivo, which is commonly required of mouse embryos in the wild. We propose that the responsiveness of embryonic stem cells to gp130 signalling has its origin in this adaptive physiological function.

Key words: Pluripotency, Blastocyst, Diapause, Epiblast, Self-renewal, Stem cell, Mouse

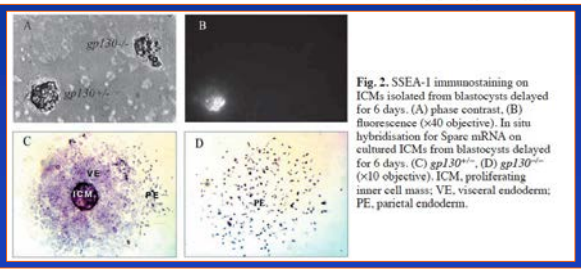


Fig. 2. SSEA-1 immunostaining on ICMs isolated from blastocysts delayed for 6 days. (A) phase contrast, (B) fluorescence (×40 objective). In situ hybridisation for Spare mRNA on cultured ICMs from blastocysts delayed for 6 days. (C) *gp130*$^{+/-}$, (D) *gp130*$^{-/-}$ (×10 objective). ICM, proliferating inner cell mass; VE, visceral endoderm; PE, parietal endoderm.

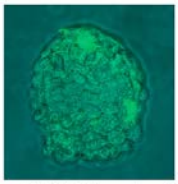
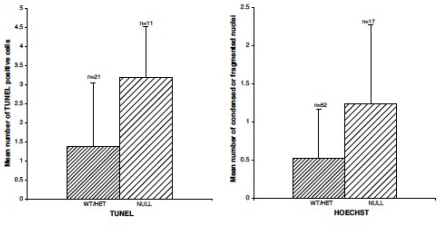


Fig. 4. Composite bright-field and fluorescence image of blastocyst delayed for 3 days and stained for TUNEL. Dead cells fluoresce. Subsequent PCR analysis revealed that this embryo was *gp130*$^{-/-}$. Images were collected using a ×40 objective and Open Lab imaging software.

Fig. 5. Number of dead cells detected by TUNEL analysis and Hoechst staining of blastocysts from *gp130*$^{+/-}$ intercross matings delayed for 3 days. Wt/het, combined *gp130*$^{+/+}$ and *gp1*$^{+/-}$; null, *gp130*$^{-/-}$. Error bars denote standard deviation. Using Student's *t*-test significantly more dead cells per embryo are detected in the *gp130*$^{-/-}$ category: $P<0.01$ using TUNEL analysis; $P<0.002$ by Hoechst staining.



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Σηματοδοτικά μονοπάτια & πολυδυναμία ESC

➢**Έμβρυα στα οποία έχει γίνει knock out είτε το γονίδιο που κωδικοποιεί το LIF είτε το γονίδιο που κωδικοποιεί τη gp130 δεν εμφανίζουν προβλήματα μέχρι το μέσον της εγκυμοσύνης.**

➢**Ο ρόλος του μονοπατιού LIF είναι η διατήρηση της πολυδυναμίας κατά τη διάπαυση.**

➢**Τα έμβρυα στα οποία έχει γίνει knock out είτε το γονίδιο που κωδικοποιεί το LIF είτε το γονίδιο που κωδικοποιεί το gp130 δεν μπορούν να αναπτυχθούν φυσιολογικά σε περίπτωση διάπαυσης.**

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LIF και διάπαυση

✓**Εμβρυϊκή διάπαυση:** η παροδική διακοπή της ανάπτυξης του εμβρύου.
✓Πολύ συνηθισμένη στα αρθρόποδα.
✓Παρατηρείται σε πολλά είδη θηλαστικών περίπου 100 έχουν περιγραφεί
✓Ο ρυθμός πολλαπλασιασμού των κυττάρων της βλαστοκύστης επιβραδύνεται (σχεδόν σταματά)
✓Μερικές μέρες μέχρι και μερικούς μήνες
✓Σε υψηλή ενεργειακή κατάσταση ATP/ADP
✓Χαμηλή μεταβολική και συνθετική ενεργότητα
✓Υποχρεωτική σε μερικά είδη (νυχτερίδες, εντομοφάγα)- εξασφάλιση γέννησης του μικρού την άνοιξη
✓Περιστασιακή σε άλλα (τρωκτικά, μαρσιποφόρα) (διάφοροι περιβαλλοντικοί λόγοι)
✓Επανέναρξη της ανάπτυξης μετά από αλλαγή στο ορμονικό προφίλ της μητέρας.

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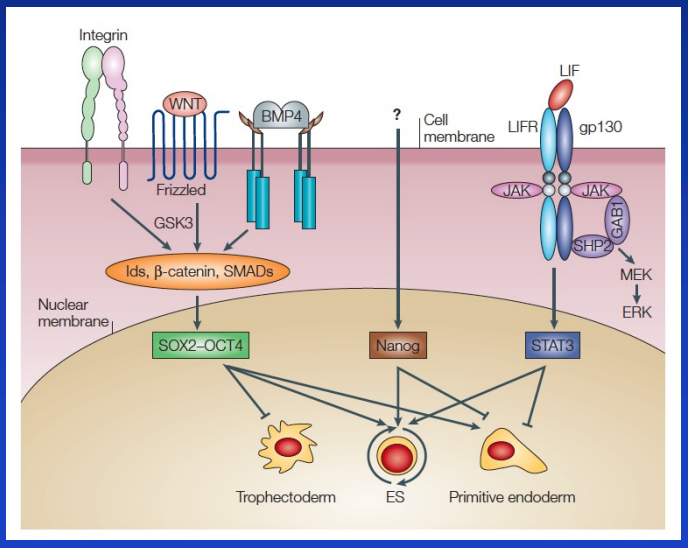
Σηματοδοτικά μονοπάτια & πολυδυναμία ESC



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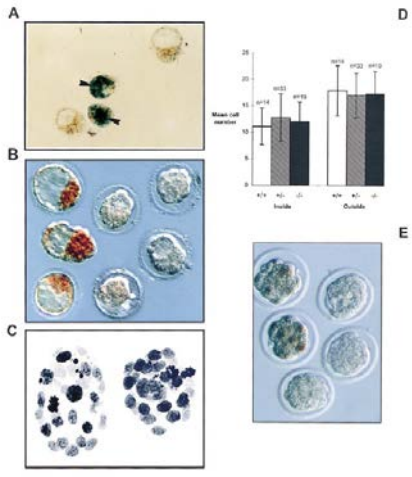
Cell, Vol. 95, 379–391, October 30, 1998,

Formation of Pluripotent Stem Cells in the Mammalian Embryo Depends on the POU Transcription Factor Oct4

Jennifer Nichols,* Branko Zevnik,*§ Konstantinos Anastassiadis,† Hitoshi Niwa,*‖ Daniela Klewe-Nebenius,* Ian Chambers,* Hans Scholer,† and Austin Smith*‖

Oct4 is a mammalian POU transcription factor expressed by early embryo cells and germ cells. We report that the activity of Oct4 is essential for the identity of the pluripotential founder cell population in the mammalian embryo. Oct4-deficient embryos develop to the blastocyst stage, but the inner cell mass cells are not pluripotent. Instead, they are restricted to differentiation along the extraembryonic trophoblast lineage. Furthermore, in the absence of a true inner cell mass, trophoblast proliferation is not maintained in *Oct4*$^{-/-}$ embryos. Expansion of trophoblast precursors is restored, however, by an Oct4 target gene product, fibroblast growth factor-4. Therefore, Oct4 also determines paracrine growth factor signaling from stem cells to the trophectoderm.

Figure 1. Morphology, Staining, and Cell Numbers of Embryos from Intercross Matings of *Oct4* Mutants (A) b-galactosidase staining of 3.5-day blastocysts. Two embryos show light staining characteristic of heterozygotes and two show the darker staining associated with homozygous mutants. Note the staining of the inner cells in the latter (arrow). (B) Immunostaining of 3.5-day blastocysts for *Oct4*. The panel shows three embryos with immunoreactive ICMs and four nonstaining embryos. Seven out of 32 intercross embryos examined at this stage failed to stain, whereas the inner cell mass was strongly immunoreactive in all of more than 50 blastocysts examined from matings of wild-type mice. (C) Confocal images after propidium iodide staining of two of the embryos shown in (B), *Oct4*-positive (left) and *Oct4*-deficient (right). (D) Inside and outside cell numbers determined after differential labeling of freshly isolated 3.5-day embryos. Individual specimens were recovered from the slides for genotype determination by PCR. Data are means 6 SEM. There are no significant differences within the groups (t test, P>0.75). (E) Immunostaining of early morulae (2.5-day) for *Oct4*. Note the nuclear localization in the two positively staining embryos. Three out of 15 intercross morulae failed to stain, whereas 21/21 control embryos gave specific nuclear staining



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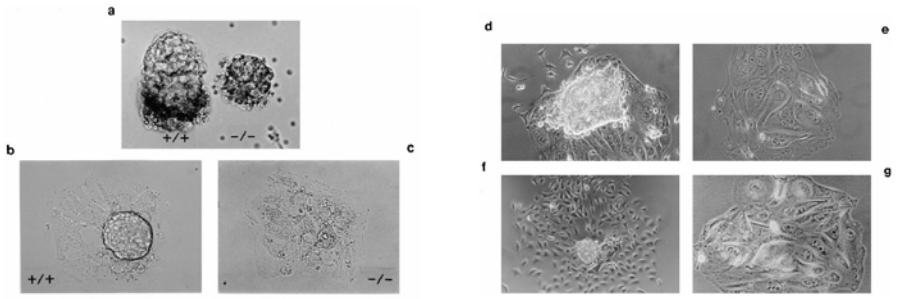

Figure 2. Outgrowth Cultures of Intercross Embryos (A–C) Peri-implantation stage (5.25 days) wild-type (1/1) and homozygous *Oct4* mutant embryos (2/2) freshly dissected from nascent implantation sites (A) after overnight culture (B and C). (D and E) Whole 3.5-day blastocyst cultures after 4 days. (F and G) Cultures of immunosurgically isolated internal cells after 4 days. (D and F), Wild type; (E and G), homozygous *Oct4* mutant. Objective magnification: (A) 34; (B–G) 310.

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Table 2. Second Round Targeting of the *Oct4* Gene

(A) Introduction of *Oct4*ireshph construct into ES cells heterozygous for *Oct4*iresβgeo allele

			Integration Event		
Selection	Colonies	X-Gal − ve	Random	Retargeting	2nd allele
Hyg	12	9	3	9	0
Hyg + G418	4	0	4	0	0

(B) Introduction of *Oct4*iresβgeo construct into ES cells heterozygous for *Oct4*ireshph allele

			Integration Event		
Selection	Colonies	Xgal + ve	Random	Retargeting	2nd allele
G418	8	6	2	6	0
G418 + Hyg	21	ND	21	0	0
G418 + Hyg[a]	96	8	8	0	0

(C) Introduction of *Oct4*ireshph construct into ES cells heterozygous for *Oct4*ireszeo allele and vice versa

			Integration Event		
Second vector	Selection	Colonies	Random	Retargeting	2nd allele
hph	zeo + hyg	20	20	0	0
zeo	zeo	3	0	3	0
zeo	zeo + hyg	21	21	0	0

Oct4 targeting constructs were introduced into parental CGR8 ES cells or previously targeted heterozygous derivatives by electroporation and grown up under selection in G418, hygromycin B (hyg), or zeocin (zeo) as indicated. Clones were analyzed as appropriate by X-Gal staining for retention of the *Oct4*iresβgeo allele and by DNA hybridization with *Oct4* genomic probes as described (Mountford et al., 1994).
[a] Only clones showing stem cell restricted X-Gal staining were analyzed by DNA hybridization.

"..We conclude that a functional Oct4 indispensable for main-tenance of the self-renewing undifferentiated ES cell phenotype..."

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Cell, Vol. 113, 643–655, May 30, 2003, (

Functional Expression Cloning of Nanog, a Pluripotency Sustaining Factor in Embryonic Stem Cells

Ian Chambers,* Douglas Colby, Morag Robertson, Jennifer Nichols, Sonia Lee, Susan Tweedie, and Austin Smith

Embryonic stem (ES) cells undergo extended proliferation while remaining poised for multilineage differentiation. A unique network of transcription factors may characterize self-renewal and simultaneously suppress differentiation. We applied expression cloning in mouse ES cells to isolate a self-renewal determinant, Nanog. Nanog is a divergent homeodomain protein that directs propagation of undifferentiated ES cells. Nanog mRNA is present in pluripotent mouse and human cell lines, and absent from differentiated cells. In preimplantation embryos, Nanog is restricted to founder cells from which ES cells can be derived. Endogenous Nanog acts in parallel with cytokine stimulation of Stat3 to drive ES cell self-renewal. Elevated Nanog expression from transgene constructs is sufficient for clonal expansion of ES cells, bypassing Stat3 and maintaining Oct4 levels. Cytokine dependence, multilineage differentiation, and embryo colonization capacity are fully restored upon transgene excision. These findings establish a central role for Nanog in the transcription factor hierarchy that defines ES cell identity.

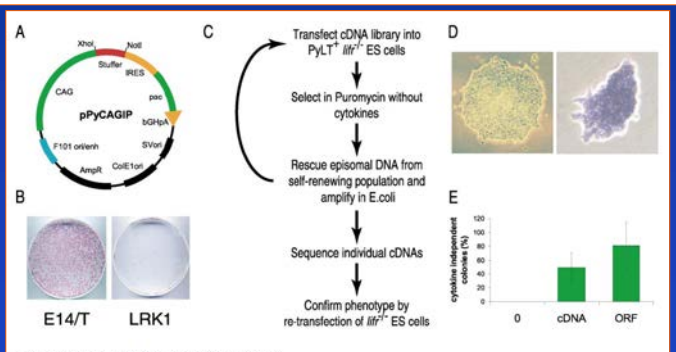

Figure 1. Components of the Expression Cloning Strategy

(A) pPyCAGIP episomal expression vector. The plasmid carries a polyoma origin with the F101 mutation allowing episomal replication in ES cells. cDNA is cloned directionally in place of the stuffer fragment within a transcription unit linked to the puromycin resistance gene (*pac*) through an IRES.

(B) Reduced background of self-renewal in ES cells deleted for the *lifr* gene. E14/T or the *lifr* targeted subclone, LRK1, were transfected with pPyCAGIP and plated at 10^6 per 9 cm petri dish. Selection was applied 30 hr later and plates stained for alkaline phosphatase after 12 days.

(C) Logic of the expression strategy. Plasmid directing ES cell self-renewal amplifies during ES cell propagation and can be recovered and enriched by further rounds of selection in ES cells.

(D) Colonies of LRK1 cells expressing Nanog cDNA from the pPyCAGIP episome in the absence of cytokine; left, colony morphology; right, in situ hybridization for Oct4 mRNA.

(E) Quantitation of stem cell colonies formed following transfection of LRK1 cells with pPyCAGIP derivatives carrying no insert (0), Nanog cDNA, or Nanog ORF. Data are the average of at least three independent experiments; bars indicate standard deviations.

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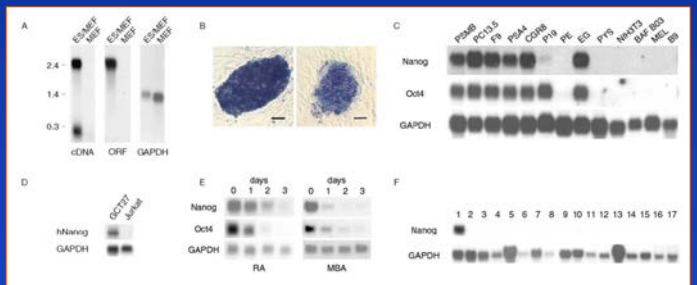

Figure 3. Expression of Nanog in Pluripotent Cell Lines

(A) Hybridization of RNA from MEFs and from MEF/ES cell cocultures used for library construction. 1 μg pA^+ RNA was loaded per lane and hybridized with probes for Nanog cDNA (left), GAPDH (right), and Nanog ORF (middle). Positions of RNA markers (kb) are shown to the left.

(B) Nanog in situ hybridization of a MEF/ES cell coculture (left) and a feeder-free culture in which an undifferentiated cluster of ES cells is surrounded by differentiated cells (right); bars are 50 μm.

(C) Nanog expression in cell lines. RNAs were from PSMB, PC13.5, F9, PSA4 and P19 EC cells; CGR8, ES cells; PE, D7-A3 parietal endoderm-like; EG, embryonic germ cells; PYS, parietal yolk sac; NIH3T3, fibroblasts; BAFB03, pro-B cells; MEL, erythroleukaemia; B9, plasmacytoma.

(D) Human *nanog* RNA is expressed in EC cells. RNAs were from embryonal carcinoma (GCT27) (Pera et al., 1989) and lymphoid (Jurkat) cells.

(E) Nanog is downregulated during ES cell differentiation. E14Tg2a cells were induced to differentiate by application of retinoic acid (RA) or 3-methoxybenzamide (MBA) for the number of days shown.

(F) Lack of detectable Nanog mRNA in adult tissues. RNAs were: 1, CGR8 ES cells; 2, adipose; 3, kidney; 4, liver; 5, heart; 6, spleen; 7, brain; 8, bone marrow; 9, tongue; 10, eye; 11, oviduct; 12, thymus; 13, skeletal muscle; 14, skin; 15, ovary; 16, seminiferous vesicle; 17, lung.

Northern analysis was performed by sequential hybridization with probes for *nanog*, *GAPDH*, and *oct4* (C and E), *hnanog* and *GAPDH* (D), and *nanog* and *GAPDH* (F).

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Figure 4. Expression of Nanog In Vivo

(A) Preimplantation embryos. Top: embryos of 1, 2, and 6 cells. Middle: 8-cell embryo, late morula and early blastocyst. Bottom: blastocysts at expanded, hatched, and implanting stages. Embryos were hybridized in the same reaction and stained for the same time. All panels are shown at equal magnification.

(B) E11.5 genital ridges from female (top) and male (bottom) embryos. Hybridization appears localized to the primordial germ cells overlying the somatic tissue.

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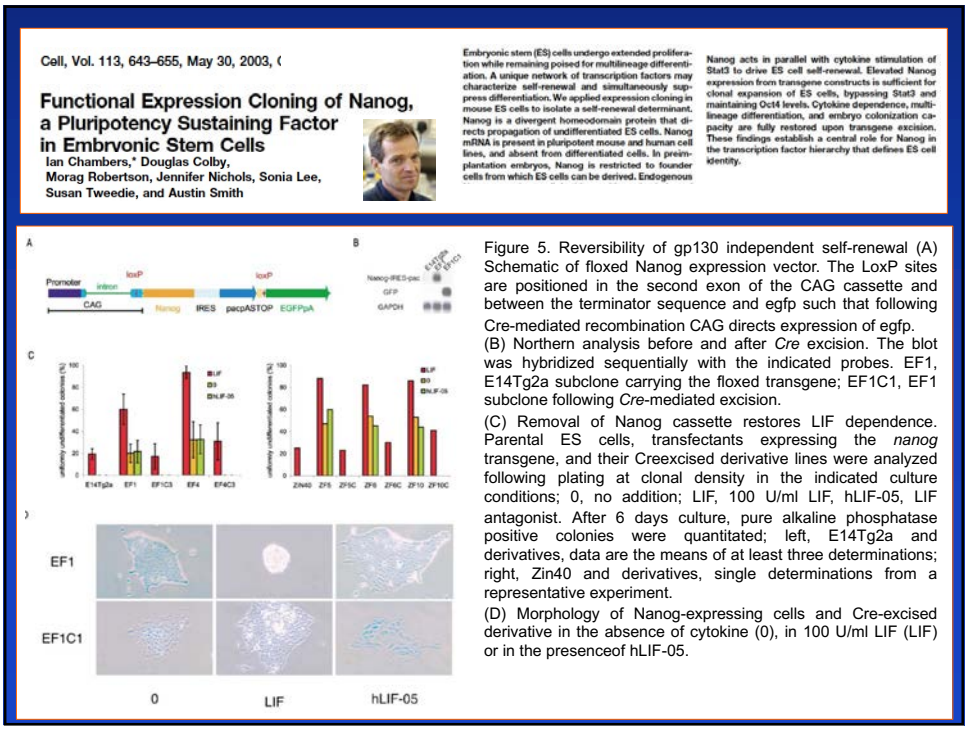

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Figure 5. Reversibility of gp130 independent self-renewal (A) Schematic of floxed Nanog expression vector. The LoxP sites are positioned in the second exon of the CAG cassette and between the terminator sequence and egfp such that following Cre-mediated recombination CAG directs expression of egfp.

(B) Northern analysis before and after *Cre* excision. The blot was hybridized sequentially with the indicated probes. EF1, E14Tg2a subclone carrying the floxed transgene; EF1C1, EF1 subclone following *Cre*-mediated excision.

(C) Removal of Nanog cassette restores LIF dependence. Parental ES cells, transfectants expressing the *nanog* transgene, and their Creexcised derivative lines were analyzed following plating at clonal density in the indicated culture conditions; 0, no addition; LIF, 100 U/ml LIF, hLIF-05, LIF antagonist. After 6 days culture, pure alkaline phosphatase positive colonies were quantitated; left, E14Tg2a and derivatives, data are the means of at least three determinations; right, Zin40 and derivatives, single determinations from a representative experiment.

(D) Morphology of Nanog-expressing cells and Cre-excised derivative in the absence of cytokine (0), in 100 U/ml LIF (LIF) or in the presenceof hLIF-05.

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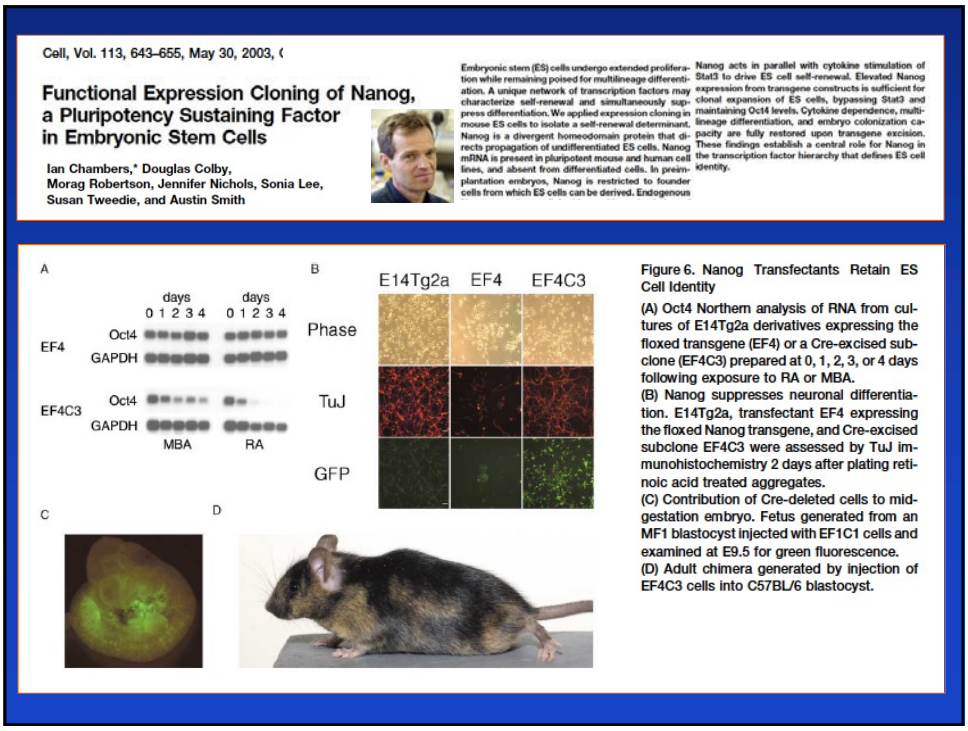

Cell, Vol. 113, 643–655, May 30, 2003, (

Functional Expression Cloning of Nanog, a Pluripotency Sustaining Factor in Embryonic Stem Cells

Ian Chambers,* Douglas Colby, Morag Robertson, Jennifer Nichols, Sonia Lee, Susan Tweedie, and Austin Smith

Embryonic stem (ES) cells undergo extended proliferation while remaining poised for multilineage differentiation. A unique network of transcription factors may characterize self-renewal and simultaneously suppress differentiation. We applied expression cloning in mouse ES cells to isolate a self-renewal determinant. Nanog is a divergent homeodomain protein that directs propagation of undifferentiated ES cells. Nanog mRNA is present in pluripotent mouse and human cell lines, and absent from differentiated cells. In preimplantation embryos, Nanog is restricted to founder cells from which ES cells can be derived. Endogenous Nanog acts in parallel with cytokine stimulation of Stat3 to drive ES cell self-renewal. Elevated Nanog expression from transgene constructs is sufficient for clonal expansion of ES cells, bypassing Stat3 and maintaining Oct4 levels. Cytokine dependence, multilineage differentiation, and embryo colonization capacity are fully restored upon transgene excision. These findings establish a central role for Nanog in the transcription factor hierarchy that defines ES cell identity.

Figure 6. Nanog Transfectants Retain ES Cell Identity

(A) Oct4 Northern analysis of RNA from cultures of E14Tg2a derivatives expressing the floxed transgene (EF4) or a Cre-excised subclone (EF4C3) prepared at 0, 1, 2, 3, or 4 days following exposure to RA or MBA.

(B) Nanog suppresses neuronal differentiation. E14Tg2a, transfectant EF4 expressing the floxed Nanog transgene, and Cre-excised subclone EF4C3 were assessed by TuJ immunohistochemistry 2 days after plating retinoic acid treated aggregates.

(C) Contribution of Cre-deleted cells to midgestation embryo. Fetus generated from an MF1 blastocyst injected with EF1C1 cells and examined at E9.5 for green fluorescence.

(D) Adult chimera generated by injection of EF4C3 cells into C57BL/6 blastocyst.

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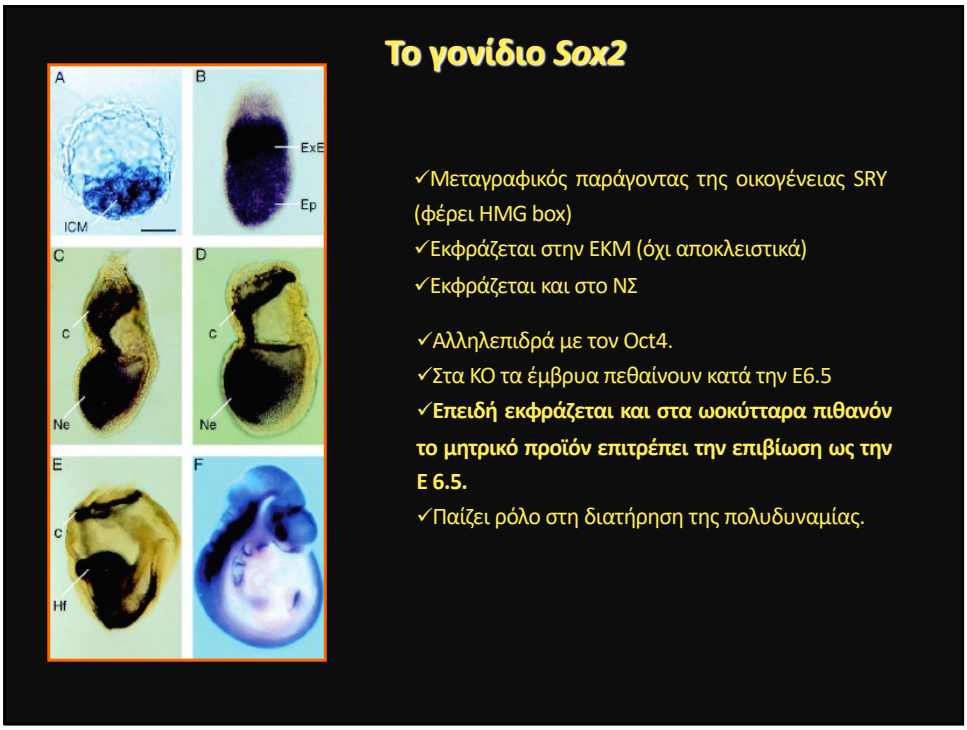

Το γονίδιο *Sox2*

- ✓Μεταγραφικός παράγοντας της οικογένειας SRY (φέρει HMG box)
- ✓Εκφράζεται στην ΕΚΜ (όχι αποκλειστικά)
- ✓Εκφράζεται και στο ΝΣ

- ✓Αλληλεπιδρά με τον Oct4.
- ✓Στα ΚΟ τα έμβρυα πεθαίνουν κατά την Ε6.5
- ✓**Επειδή εκφράζεται και στα ωοκύτταρα πιθανόν το μητρικό προϊόν επιτρέπει την επιβίωση ως την Ε 6.5.**
- ✓Παίζει ρόλο στη διατήρηση της πολυδυναμίας.

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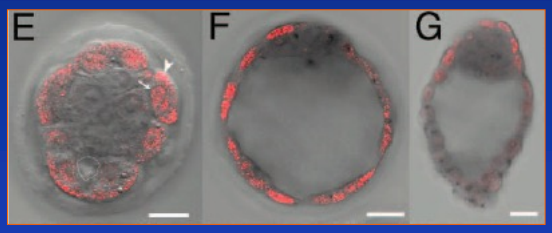
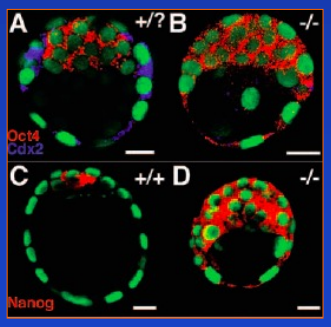
Το γονίδιο *Cdx2*..



✓Ομόλογο του *caudal*
✓Φέρει ομοιοεπικράτεια
✓Εκφράζεται στα κύτταρα του τροφοεξωδέρματος (TE)

✓Καταστέλλει την έκφραση των *Oct4* και *nanog* στα κύτταρα του τροφοεξωδέρματος.
✓Ο Oct4 καταστέλλει την έκφραση του *cdx2* στην ΕΚΜ.
✓Οι Oct4 και *cdx2* ρυθμίζουν την έκφραση του μεταγραφικού παράγοντα *eomesodermin* που ενέχεται στη διαφοροποίηση του TE



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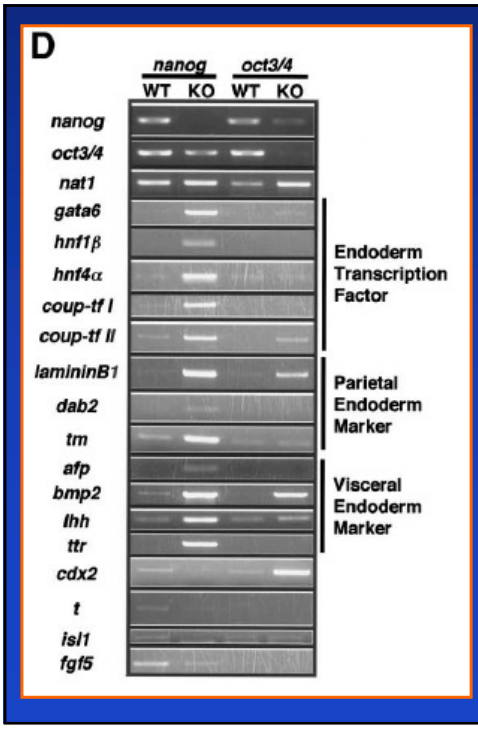

Απουσία παραγόντων πολυδυναμίας εκφράζονται γονίδια που επάγουν διαφοροποίηση

➢Απουσία nanog επάγονται γονίδια που ενέχονται στη διαφοροποίηση των ES.

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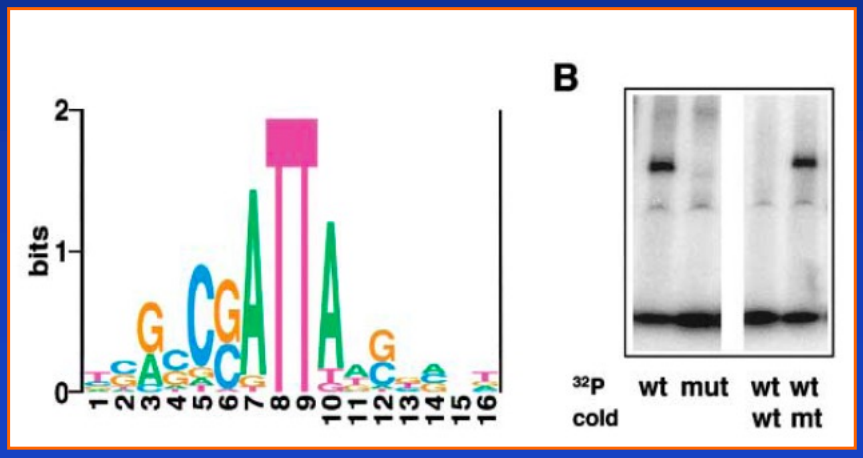
Προσδιορισμός θέσεων πρόσδεσης των μεταγραφικών παραγόντων πολυδυναμίας



➢SELEX για την ταυτοποοίηση της αλληλουχίας αναγνώρισης της nanog.

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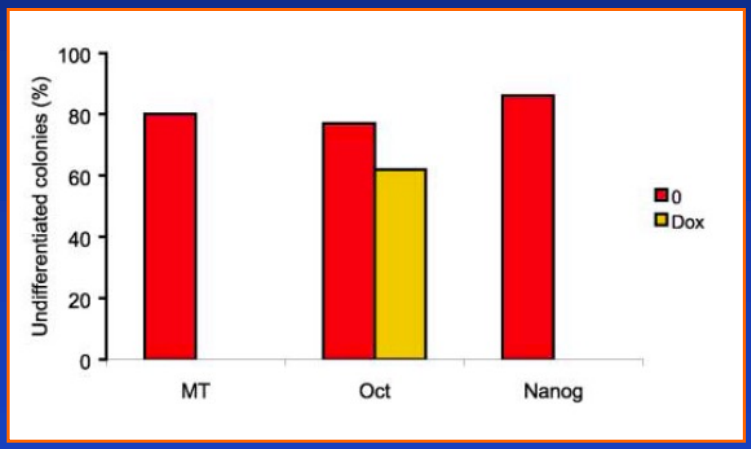
nanog



➢Η δράση της nanog είναι **ανεξάρτητη** από τα μονοπάτια JAK, MEK.

➢**Για τη δράση της nanog απαιτείται η Oct-4**

➢Η nanog δεν αποτελεί στόχο της Oct-4 (η έκφρασή της σε Oct-/- είναι φυσιολογική)

➢Καταστέλλει τα GATA4,GATA6 που έχει δειχθεί ότι προάγουν τη διαφοροποίηση σε πρωτογενές ενδόδεμα

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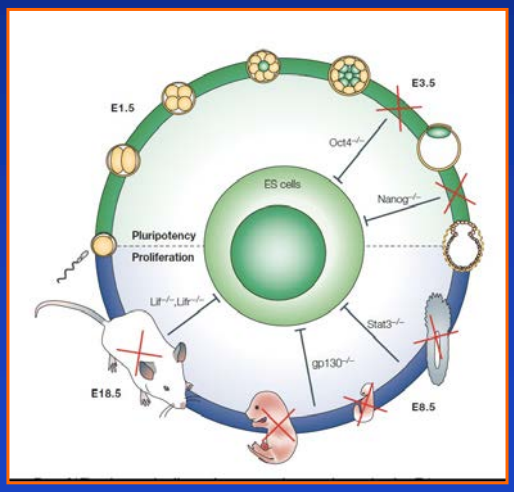

NATURE REVIEWS | MOLECULAR CELL BIOLOGY, vol. 6, 872 2006

The phenotypic effects of gene-targeting experiments in mice. This figure depicts the phenotypic effects of gene-targeting experiments in mice that provided insights into the pluripotency of embryos and embryonic stem cells. *Oct4–/–* (octamer-binding transcription factor-4), *Nanog–/–*, *Stat3–/–* (signal transducer and activator of transcription-3), *gp130–/–* (glycoprotein-130), *Lif –/–* (leukaemia inhibitory factor) and *Lifr –/–* (LIF receptor) embryos can develop to various stages *in vivo*— as highlighted by the outer ring in the figure, but they all fail (truncated line from the ring to the centre) to yield ESCs (embryonic stem cells) *in vitro* (see centre of the figure). So, ESCs need factors that neither the ICM (inner cell mass) nor the primitive ectoderm require. It is important to make clear that ICM and ESCs might be equivalent but are not equal. ESCs might only represent some aspects of the natural embryo, and studying cells and phenomena *in vivo* is indispensable. E1.5, E3.5, E8.5 and E18.5 represent the number of days of embryonic (E) development, although developmental stages following E10.5 should properly be referred to as fetal.

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Table 1

Gene expression analyses of transcription factors in ES cell pluripotency and embryonic development

Transcription factor	Protein family	Expression pattern	Loss-of-function phenotype		Gain-of-function phenotype in ES cells
			Embryonic development	ES cells	
Oct4	Pit–Oct–Unc protein family	Oocytes, fertilized embryo, ICM, epiblast, ES cells, embryonic carcinoma cells, germ cells	Embryonic lethal (blastocyst stage), differentiation of epiblast into trophectoderm lineage	Loss of pluripotency, differentiation into trophectoderm lineage	Differentiation into primitive endoderm and mesoderm
Nanog	Novel homeodomain protein	Morula, ICM, epiblast, ES cells, embryonic carcinoma cells, germ cells	Embryonic lethal (E5.5), lack of epiblast, differentiation of ICM into primitive endoderm	Loss of pluripotency, differentiation into primitive endoderm	LIF–Stat3-independent self-renewal, resistance to retinoic acid-induced differentiation
Sox2	SRY-related HMG box protein	Oocytes, ICM, epiblast, germ cells, multipotent cells of extraembryonic ectoderm, cells of neural lineage, brachial arches, gut endoderm	Embryonic lethal (E6.5), failure to maintain epiblast	Unknown	Unknown
Stat3	Signal transducer and activator of transcription family protein	Wide ranges of cell types	Embryonic lethal (E6.5–7.5)	Differentiation into primitive endoderm and mesoderm (Stat3 signaling is dispensable in human ES cells)	LIF-independent self renewal
Cdx2	Caudal-type homeodomain protein	Outer morula cells, trophectoderm cell lineages	Embryonic lethal due to implantation failure (lack of functional trophectoderm)	Normal contribution to all cell lineages except trophectoderm and intestinal cells	Differentiation into trophoblast
Gata6	GATA-binding protein	Extraembryonic endoderm lineages	Embryonic lethal (E5.5–7.5), defects in visceral endoderm formation	Unknown	Differentiation into primitive endoderm
Gata4	GATA-binding protein	Extraembryonic endoderm lineages	Embryonic lethal (E8–9), defects in heart morphogenesis	Can generate cardiac myocytes, inability to generate visceral endoderm and definitive endoderm of foregut	Differentiation into primitive endoderm

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Οκταμερή και στοιχεία Sox στον υποκινητή του *nanog*

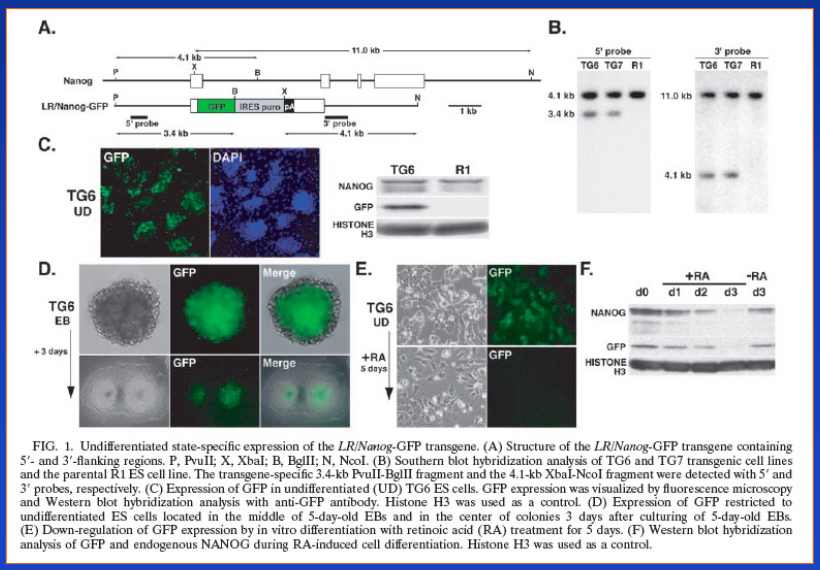

FIG. 1. Undifferentiated state-specific expression of the *LR/Nanog*-GFP transgene. (A) Structure of the *LR/Nanog*-GFP transgene containing 5′- and 3′-flanking regions. P, PvuII; X, XbaI; B, BglII; N, NcoI. (B) Southern blot hybridization analysis of TG6 and TG7 transgenic cell lines and the parental R1 ES cell line. The transgene-specific 3.4-kb PvuII-BglII fragment and the 4.1-kb XbaI-NcoI fragment were detected with 5′ and 3′ probes, respectively. (C) Expression of GFP in undifferentiated (UD) TG6 ES cells. GFP expression was visualized by fluorescence microscopy and Western blot hybridization analysis with anti-GFP antibody. Histone H3 was used as a control. (D) Expression of GFP restricted to undifferentiated ES cells located in the middle of 5-day-old EBs and in the center of colonies 3 days after culturing of 5-day-old EBs. (E) Down-regulation of GFP expression by in vitro differentiation with retinoic acid (RA) treatment for 5 days. (F) Western blot hybridization analysis of GFP and endogenous NANOG during RA-induced cell differentiation. Histone H3 was used as a control.

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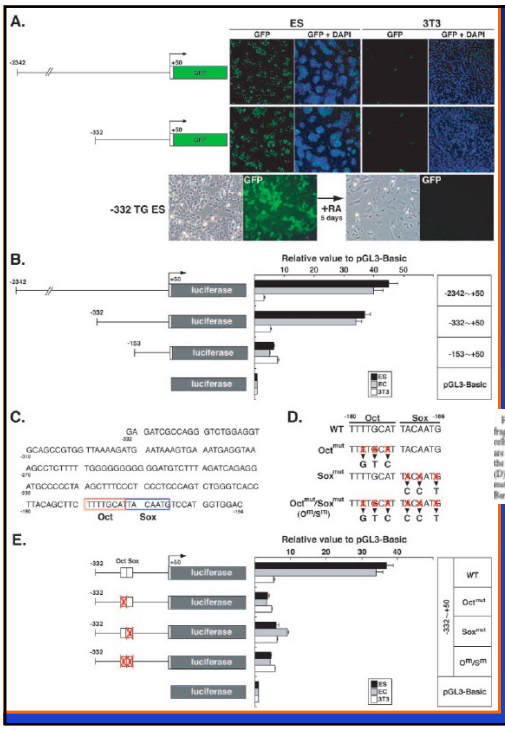

Οκταμερή και στοιχεία Sox στον υποκινητή του *nanog*

FIG. 2. Octamer and Sox elements are required for *Nanog* expression. (A) Transient expression of GFP transgenes with −2342 or −332 5′ end fragments in R1 ES and NIH 3T3 cells. Transcriptional down-regulation of GFP was detected in differentiated −332-GFP TG ES (−332 TG ES) cells by treatment with retinoic acid (RA). (B) Luciferase assays with deletion constructs in R1 ES, F9 EC, and NIH 3T3 cells. Luciferase activities are shown relative to those of pGL3-Basic. Bars represent the means ± standard errors of three independent experiments. (C) DNA sequence of the mouse 5′-flanking region between positions −332 and −154. Octamer (Oct) and Sox elements are outlined in red and blue, respectively. (D) Sequence mutations introduced into Octamer and/or Sox elements. (E) Luciferase assays with the −332 5′-end fragment with or without mutations in Octamer and/or Sox elements in R1 ES, F9 EC, and NIH 3T3 cells. Luciferase activities are shown relative to those of pGL3-Basic. Bars represent the means ± standard errors of three independent experiments.

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Οκταμερή και στοιχεία Sox στον υποκινητή του *nanog*

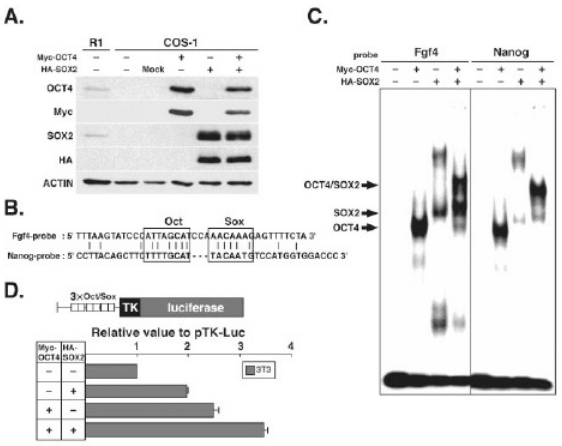

FIG. 4. Binding of exogenous OCT4 and SOX2 to Octamer and Sox elements in COS-1 cells. (A) Western blot hybridization analysis of exogenous Myc-tagged OCT4 and HA-tagged SOX2 expression with anti-OCT4, anti-SOX2, anti-Myc, and anti-HA antibodies. Actin was used as a control. (B) DNA sequences of Nanog and Fgf4 probes. Octamer (Oct) and Sox elements are outlined. (C) EMSA with Nanog and Fgf4 probes and COS-1 cells. Bands of the OCT4-DNA, SOX2-DNA, and OCT4/SOX2-DNA complexes are indicated. (D) Cotransfection reporter assays with Oct4 and Sox2 expression constructs in NIH 3T3 cells. Bars represent the means ± standard errors of three independent experiments.

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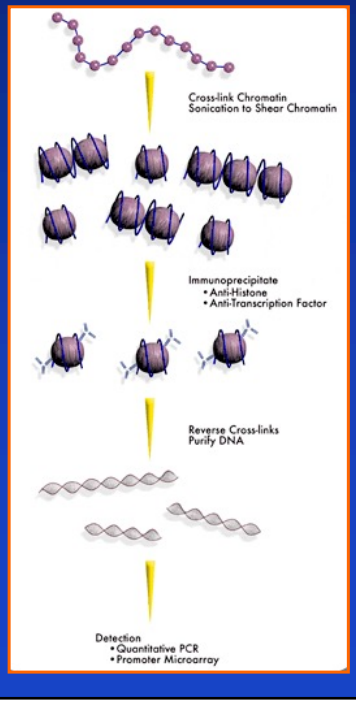

Στόχοι των Oct4, nanog και Sox2

Πειράματα ChIP σε επίπεδο γονιδιώματος και επακόλουθη αλληλούχιση.

OCT4, Sox-2 NANOG στον ποντικό και στον άνθρωπο..

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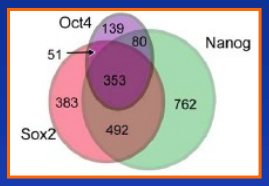
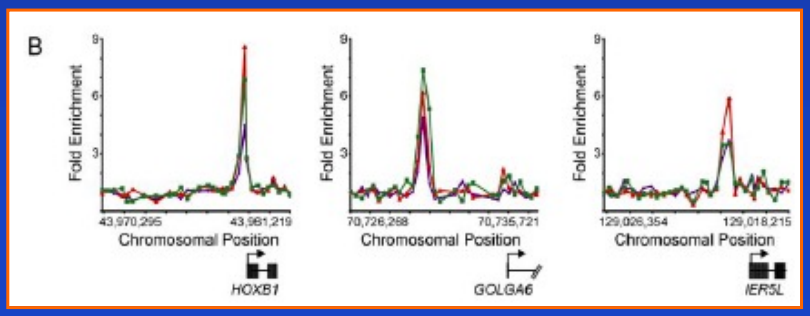
Στόχοι των Oct4, nanog και Sox2

60 μερή από -8 έως + 2 kb 17.917 γονιδίων (για το 98% των γονιδίων τα ρυθμιστικά στοιχεία εντοπίζονται σε αυτές τις 10 kb.)
Oct4 - 623 γονίδια (3%)
Nanog - 1687 γονίδια
Sox2 - 1271 γονίδια
Ενεργά ή σε καταστολή



353 γονίδια αναγνωρίζονται και από τους τρεις παράγοντες



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Στόχοι των Oct4, nanog και Sox2

Table 2. Examples of Inactive Homeodomain Genes Co-occupied by OCT4, SOX2, and NANOG

Gene Symbol	Entrez Gene ID	Gene Name
ATBF1	463	AT binding transcription factor 1
DLX1	1745	distal-less homeobox 1
DLX4	1748	distal-less homeobox 4
DLX5	1749	distal-less homeobox 5
EN1	2019	engrailed homolog 1
ESX1L	80712	extraembryonic, spermatogenesis, homeobox 1-like
GBX2	2637	gastrulation brain homeobox 2
GSC	145258	goosecoid
HOP	84525	homeodomain-only protein
HOXB1	3211	homeobox B1
HOXB3	3213	homeobox B3
HOXC4	3221	homeobox C4
IPF2	3651	insulin promoter factor 2
ISL1	3670	ISL1 transcription factor, LIM/homeodomain (islet-1)
LBX1	10660	transcription factor similar to *D. melanogaster* homeodomain protein lady bird late
LHX2	9355	LIM homeobox 2
LHX5	64211	LIM homeobox 5
MEIS1	4211	myeloid ecotropic viral integration site 1 homolog (mouse)
NKX2-2	4821	NK2 transcription factor related, locus 2 (*Drosophila*)
NKX2-3	159296	NK2 transcription factor related, locus 3 (*Drosophila*)
ONECUT1	3175	one cut domain, family member 1
OTP	23440	orthopedia homolog (*Drosophila*)
OTX1	5013	orthodenticle homolog 1 (*Drosophila*)
PAX6	5080	paired box gene 6
TITF1	7080	thyroid transcription factor 1

Στα κατεσταλμένα γονίδια περιλαμβάνονται πολλοί μεταγραφικοί παράγοντες που ενέχονται στη διαφοροποίηση.

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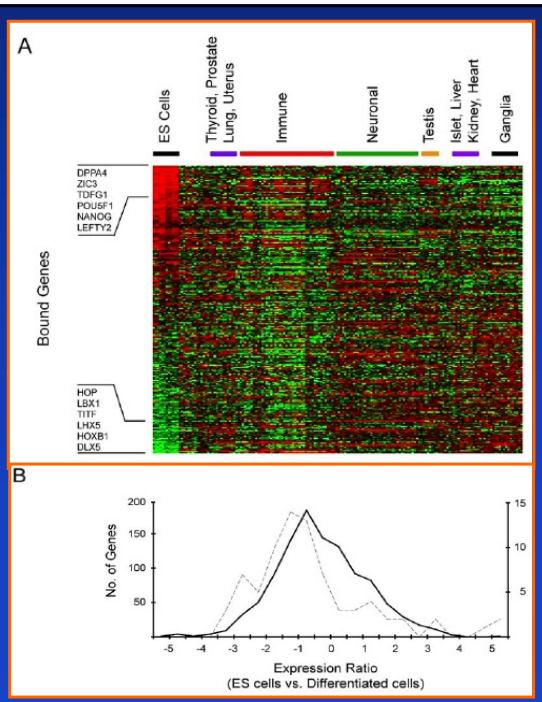

Σύγκριση των προτύπων έκφρασης των 353 γονιδίων στα οποία προσδένονται οι Oct4 Sox2 και nanog μεταξύ των ES και 79 διαφοροποι-ημένων κυτταρικών τύπων. Εμφαση στην ανάλυση της έκφρασης μεταγραφικών πα-ραγόντων.

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Ρυθμιστικά κυκλώματα στα ES

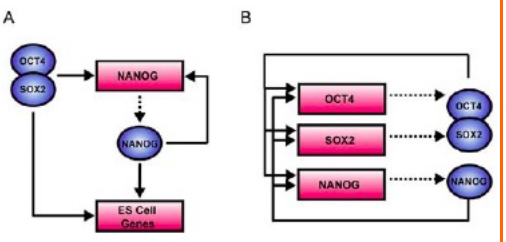

Figure 4. Transcriptional Regulatory Motifs in Human ES Cells
(A) An example of feedforward transcriptional regulatory circuitry in human ES cells. Regulators are represented by blue circles; gene promoters are represented by red rectangles. Binding of a regulator to a promoter is indicated by a solid arrow. Genes encoding regulators are linked to their respective regulators by dashed arrows.
(B) The interconnected autoregulatory loop formed by OCT4, SOX2, and NANOG.

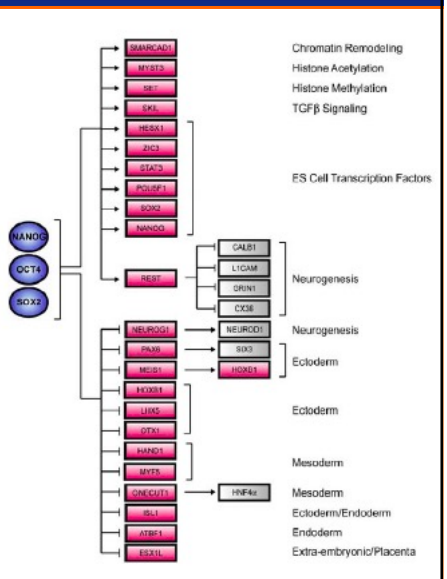

Figure 5. Core Transcriptional Regulatory Network in Human ES Cells

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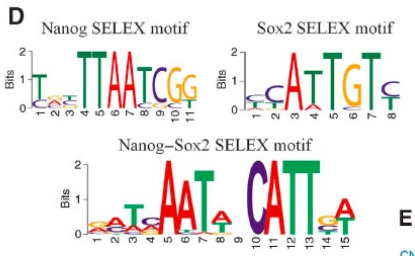
Ρυθμιστικά κυκλώματα στα ES … πολυπλοκότητα



(D) Top panel, SELEX motifs generated for Nanog and Sox2 expressed individually from a total of 19 (Nanog) and 15 (Sox2) sequences submitted to MEME; Middle panel, SELEX motif generated for Nanog/Sox2 complex from 38 sequences submitted to MEME

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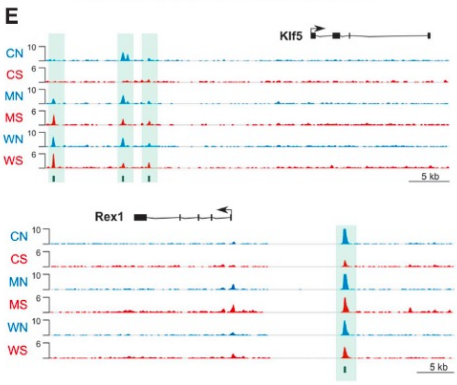

(E) Nanog and Sox2 ChIP-seq peaks located near the transcriptional start sites of Zfp42 and Rex-1. The peaks that contain the Nanog/Sox2 motif are highlighted in the shaded box; Nanog (N) and Sox2 (S) peaks

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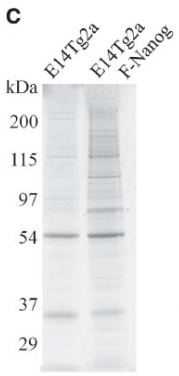
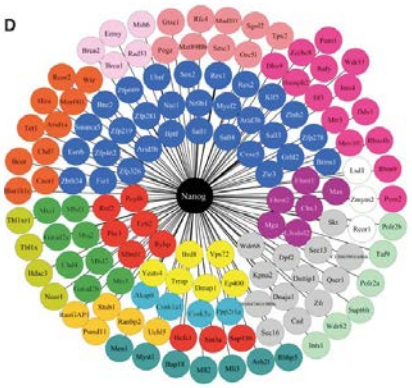
Ρυθμιστικά κυκλώματα στα ES … πολυπλοκότητα





Περίπου το 1/3 των πρωτεϊνών που αλληλεπιδρούν με τη Nanog εμπλέκονται σε επιγενετικούς μηχανισμούς....

(C) Coomassie-stained SDS–polyacrylamide gel of the FLAG immunoprecipitation from E14Tg2a F-Nanog and control E14Tg2a cells. (D) Proteins detected by mass spectrometry analysis are grouped in classes. Transcription factors are shown in blue circles, NuRD components are in green, Trrap/p400 complex is in yellow, PcG components are in red, E2F6 complex is in purple, Sin3a complex is in burgundy, N-CoR complex is in khaki, LSD1 complex is white, MII complex is in blue green, chromatin remodelling transcriptional regulation proteins are in dark orange, transcriptional machinery proteins are in pale green, proteins involved in phosphorylation are in pale blue, proteins involved in ubiquitination are in amber, proteins involved in RNA processing are in fuschia, proteins involved in cell cycle or DNA replication are in coral, proteins involved in DNA repair are in pink and other proteins are in grey.

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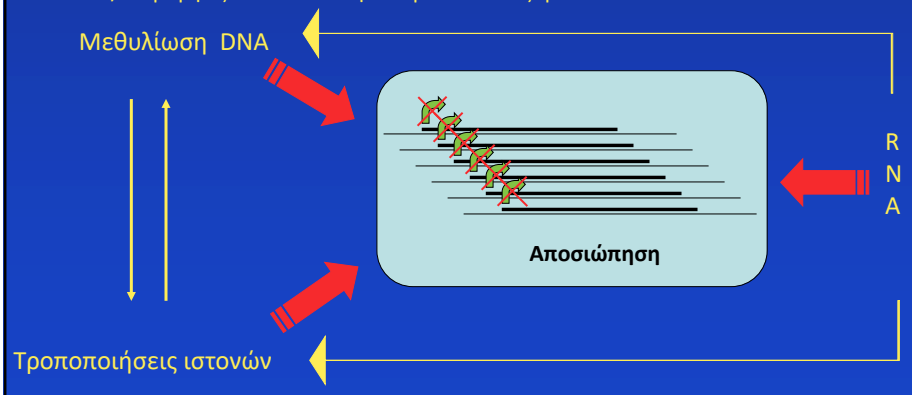
Επιγενετικοί μηχανισμοί

↗ Στα εμβρυϊκά βλαστικά κύτταρα εκφράζεται το 30-60% των γονιδίων (ενώ στα διαφοροποιημένα μόλις 10-20%)

↗ Επομένως ένα μεγάλο ποσοστό της χρωματίνης έχει ανοιχτή διαμόρφωση.

↗ Οι επιγενετικές τροποποιήσεις αφορούν συνήθως αποσιωπήσεις και πραγματοποιούνται με 3 μηχανισμούς: μεθυλίωση του DNA, τροποποιήσεις των ιστονών/ισομορφές ιστονών και φαινόμενα που εξαρτώνται από το RNA.



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Επιγενετικοί μηχανισμοί-Μεθυλίωση

↗ Η μεθυλίωση αφορά **κατάλοιπα C στη αλληλουχία CpG** και καταλύεται από ειδικές μεθυλοτρανσφεράσες.

↗Σε πολλούς υποκινητές (25-40000) εντοπίζονται νησίδες CpG >500bp με CG content πάνω από 50%). Οι νησίδες συνήθως δεν μεθυλιώνονται.
↗ Η μεθυλίωση των νησίδων έχει ως αποτέλεσμα την αποσιώπηση.

1. De Novo methylation -Dnmt 3A and 3B
2. Maintenance Methylation-αντιγραφή -Dnmt 1
3. Demethylation of DNA (?)
4. Passive demethylation

↗ Τα knock out των Dnmt 1 και 3B είναι θνησιγόνα κατά την ανάπτυξη ή αμέσως μετά (3A στην 4η εβδομάδα ζωής). Για άλλες Dnmt άγνωστος ρόλος (Dnmt2 ή Dnmt3L).

↗Οι Dnmt αλληλεπιδρούν με:
1. Απακετυλάσες ιστονών
2. Καταστολείς της μεταγραφής (π.χ pRb).
3. Ογκογονίδια (PML-RAR).
4. methyl CpG binding proteins (Mecp2/1, mbd2, mbd3) που αλληλεπιδρούν με πρωτεΐνες που τροποποιούν τη χρωματίνη.

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Επιγενετικοί μηχανισμοί - ιστόνες

↗ Τροποποιήσεις των ιστονών

↗Ισομορφές ιστονών

↗Ένζυμα που τροποποιούν τις ιστόνες.

↗Συγχρονισμός τροποποιήσεων κατά την ανάπτυξη

↗Πρωτεΐνες που αλληλεπιδρούν με τροποποιημένες ιστόνες

↗Ο κώδικας των ιστονών.

«Different combinations of histone modifications, especially located near or within a gene's promoter, may be **VERY SPECIFIC** to the transcriptional state of the gene.»

Peterson CL et al, Current Biology, 2004

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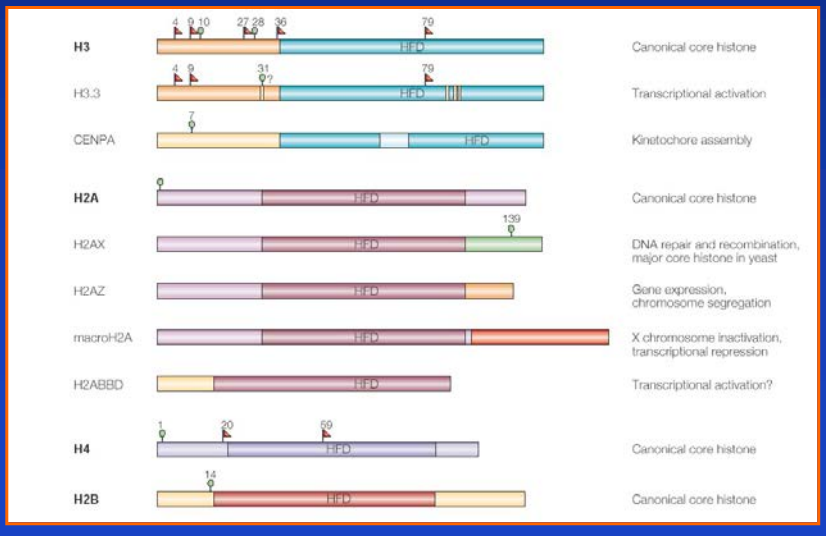
Επιγενετικοί μηχανισμοί - ιστόνες



Οι νουκλεοσωμικές ιστόνες και οι ισομορφές τους

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Επιγενετικοί μηχανισμοί - ιστόνες

Ακετυλίωση: HATs - CBP,p300, GCN5, ATF2, Tip 60…

Απακετυλίωση: HDACs- class I and II

Μεθυλίωση σε κατάλοιπα:

Λυσίνης: SET-domain HMTase και non-SET domain HMTase (Dot1)

Αργινίνης: PRMT family, CARM1

Απομεθυλίωση: LSD1

Ουβικουιτινιλίωση: ubiquitin conjugase Rad6/ligase Bre1for H2B

Απο-Ουβικουιτινιλίωση: SAGA-associated Ubp10

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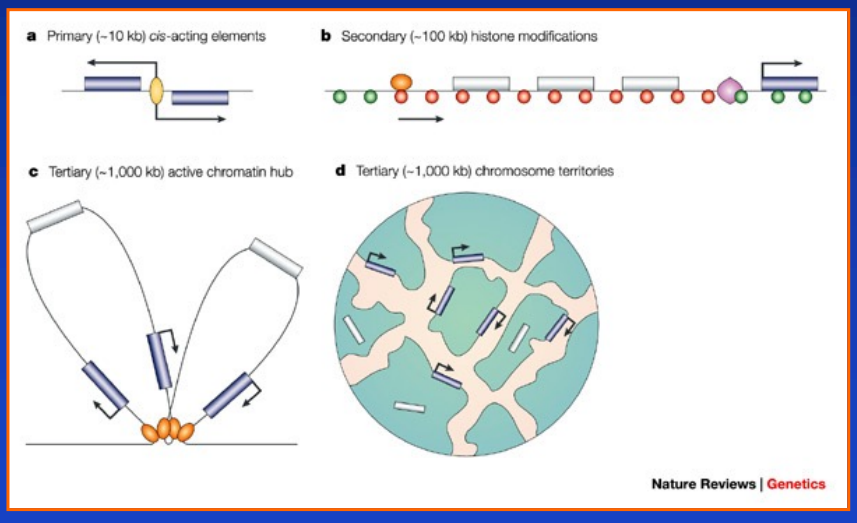
Επιγενετικοί μηχανισμοί - ιστόνες



Nuclear architecture and histone code
(Heterochromatin vs. Euchromatin)

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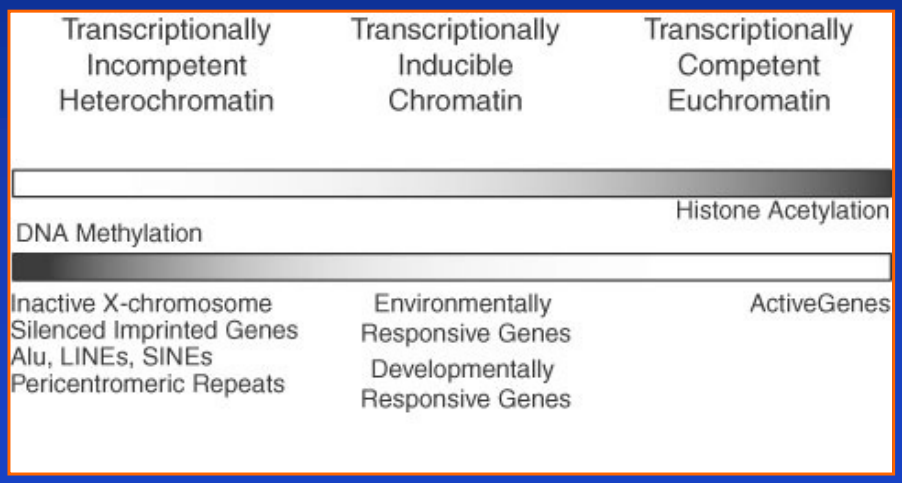
Επιγενετικοί μηχανισμοί - Μεθυλίωση DNA/ιστόνες



Οι νουκλεοσωμικές ιστόνες και οι ισομορφές τους

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Επιγενετικοί μηχανισμοί - RNA?

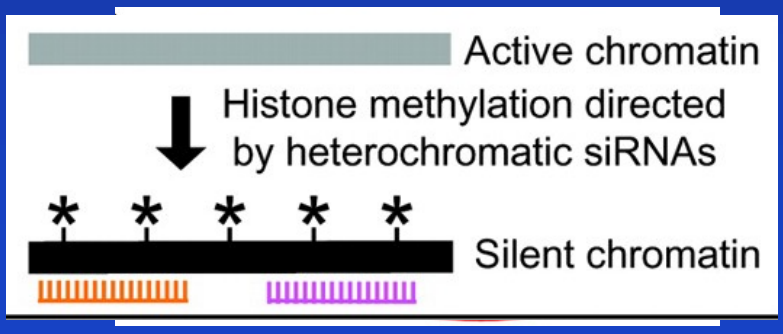
Short interfering RNA (siRNA)

Micro RNA (miRNA)

Double-stranded RNA (ds RNA)

Short heterochromatic RNA (sh RNA)

transcripts from repeated sequences (ALU, LTR)



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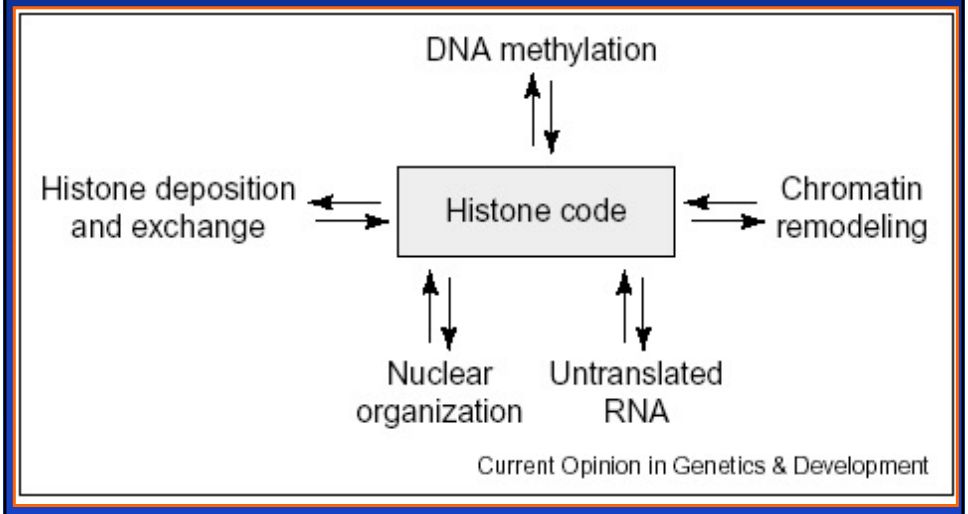
Επιγενετικοί μηχανισμοί



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Επιγενετικοί μηχανισμοί στα ESC

↗ Στα πολυδύναμα ESC στα οποία μεταγράφεται το 30-60% των γονιδίων θεωρείται ότιη δομή της χρωματίνης είναι γενικά «επιτρεπτική» για τη μεταγραφική μηχανή.

↗ Οι ετεροχρωματινικές περιοχές εμφανίζονται κατά τη διαφοροποίηση. Η HP1 διάχυτη στα πολυδύναμα- εντοπισμένη στις ετεροχρωματινικές εστίες κατά τη διαφοροποίηση.

↗ Κατά τη διαφοροποίηση αυξάνονται τα επίπεδα της H3K9me3 και ενσωμάτωση της macroH2A.

↗ Μελέτες της δυναμικής της χρωματίνης έχουν δείξει ότι η δομή είναι εξαιρετικά δυναμική - αντικαταστάσεις στοιχείων σε πολύ μικρά χρονικά διαστήματα.

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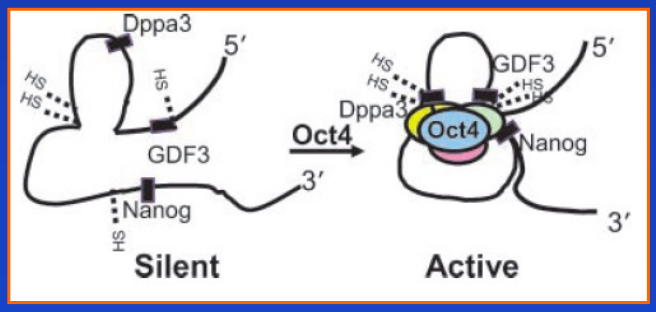
Επιγενετικοί μηχανισμοί στα ESC

↗Η δομή της χρωματίνης παίζει σημαντικό ρόλο στην έκφραση των γονιδίων που εκφράζονται στα ESC.

↗ Η έκφραση του Nanog ελέγχεται από στοιχεία που απαντούν ανοδικά σπό αυτό (160kb).

↗Τα στοιχεία αυτά (Dnase I hypersensitive sites) αναγνωρίζονται από μια σειρά παραγόντων πολυδυναμίας που αλληλεπιδρούν επηρεάζοντας τη διαμόρφωση της χρωματίνης σχηματίζοντας μια δομή που ενεργοποιεί τη μεταγραφή. Το σύμπλοκο αποσυγκροτείται απουσία Oct4

J. Cell. Physiol. 219: 1–7, 2009.



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Επιγενετικοί μηχανισμοί στα ESC

↗ Στα πολυδύναμα ESC πολλά γονίδια φέρουν "διττή σήμανση" (bivalent mark)

Στους υποκινητές πολλών αναπτυξιακά ελεγχόμενων γονιδίων (Sox, Hox, Pax, και Pou) απαντά ταυτοχρόνως σήμανση με θετική δράση (H3K4me3) και σήμανση με αρνητική δράση (H3K27me3).

↗Κατά τη διαφοροποίηση απαλοιφεται είτε η αρνητική σήμανση (αν στη γενεαλογία εκφράζεται το συγκεκριμένο γονίδιο) είτε η θετική σήμανση (αν στην εν λόγω κυτταρική γενεαλογία το συγκεκριμένο γονίδιο δεν εκφράζεται)

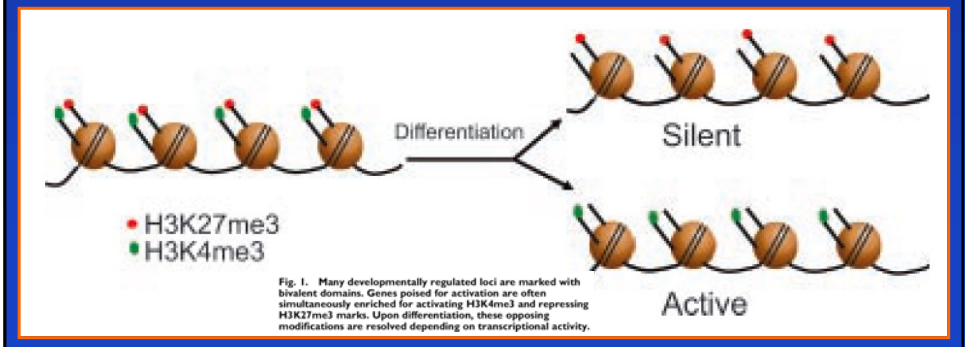

Fig. 1. Many developmentally regulated loci are marked with bivalent domains. Genes poised for activation are often simultaneously enriched for activating H3K4me3 and repressing H3K27me3 marks. Upon differentiation, these opposing modifications are resolved depending on transcriptional activity.

J. Cell. Physiol. 219: 1–7, 2009.

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Επιγενετικοί μηχανισμοί στα ESC

↗Στα πολυδύναμα ESC πολλά γονίδια των οποίων η έκφραση οδηγεί σε διαφοροποίηση **καταστέλλονται μέσω της δράσης των κατασταλτικών συμπλόκων Polycomb (PRC1, PRC2)**.

↗Το PRC1 συγκροτείται από 10 υπομονάδες μεταξύ των οποίων και οι Ring1 A και 1B, Bmi1 και Cbx8. Ουβικουϊτινίωση της λυσίνης 119 της ιστόνης H2A

↗Το PRC2 συγκροτείται (core) από τις Ezh2, Suz12, and Ee D. Το PRC2 καταλύει τη δι και τρι- μεθυλίωση της H3 στην λυσίνη 27.

↗ Αδρανοποίηση πολλών από τα γονίδια των PRC έχει ως αποτέλεσμα προβλήματα στην ανάπτυξη.

1. Αν απουσιάζει το Ezh2 δεν είναι δυνατόν να απομονωθούν από βλαστοκύστεις ESC
2. Αν απουσιάζει το Eed 2 απομονώνονται ESC αλλά δεν είναι δυνατόν να διατηρηθούν μή διαφοροποιημένα.
3. Σε ESC από έμβρυα που στερούνται της Suz12 παρατηρείται απορρύθμιση της έκφρασης πολλών γονιδίων.

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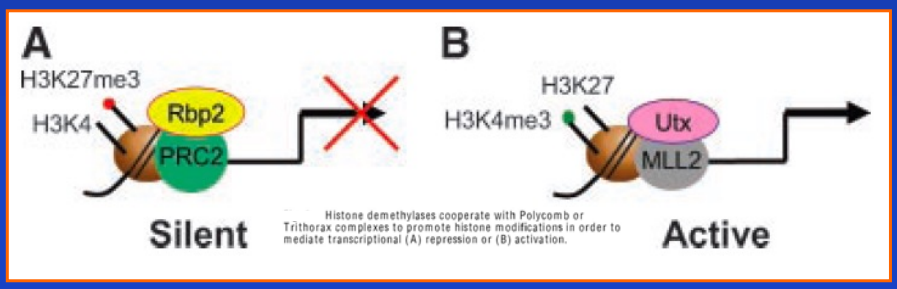
Επιγενετικοί μηχανισμοί στα ESC

↗Αναλύσεις σε επίπεδο γονιδιώματος σε ESC έχουν δείξει ότι τα σύμπλοκα αυτά εντοπίζονται σε νουκλεοσώματα στα οποία απαντά επίσης τριμεθυλιωμένη H3K27.

↗Τα γονίδια που ελέγχονται από τα PRC1 και PRC2 είναι γονίδια τα οποία ενεργοποιούνται κατά τη διαφοροποίηση.

↗Με τα σύμπλοκα αλληλεπιδρούν και πρωτεΐνες που τροποποιούν ιστόνες (πχ Rbp2 απομεθυλάση λυσίνης)- εδώ η απομεθυλίωση ενισχύει την καταστολή της έκφρασης.

↗Αντίστροφη δράση εμφανίζουν τα συμπλέγματα στα οποία συμμετέχουν πρωτεΐνες Trithorax.



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Επιγενετικοί μηχανισμοί στα ESC

↗**Οι παράγοντες πολυδυναμίας ελέγχουν την έκφραση μορίων που διεκπεραιώνουν επιγενετικούς μηχανισμούς.**

↗ Η Oct4 ελέγχει την έκφραση γονιδίων που τροποποιούν ιστόνες.

↗Οι απομεθυλάσες των ιστονών Jmjd1a και Jmjd2c είαναι στόχοι της Oct4 Στα ESC η απουσία της Jmjd1a έχει ως αποτέλεσμα την απουσία πολλών γονιδίων που συνδέονται με την πολυδυναμία. Επιπλέον τα κύτταρα διαφοροποιούνται.

↗Αλλά η Jmjd2c απαιτείται για την έκφραση της Nanog

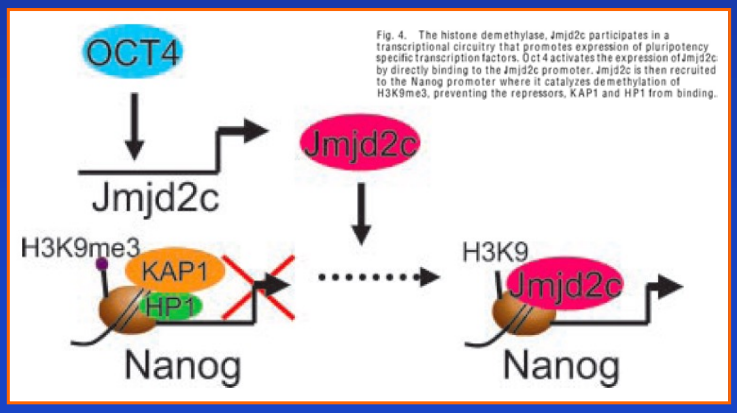

Fig. 4. The histone demethylase, Jmjd2c participates in a transcriptional circuitry that promotes expression of pluripotency specific transcription factors. Oct 4 activates the expression of Jmjd2c by directly binding to the Jmjd2c promoter. Jmjd2c is then recruited to the Nanog promoter where it catalyzes demethylation of H3K9me3, preventing the repressors, KAP1 and HP1 from binding.

J. Cell. Physiol. 219: 1–7, 2009.

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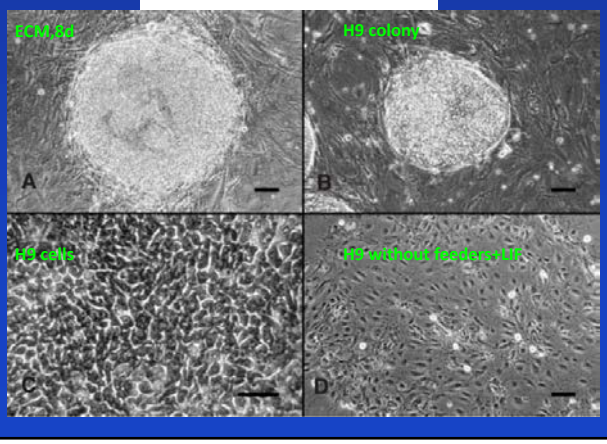
Embryonic Stem Cell Lines Derived from Human Blastocysts

James A. Thomson,* Joseph Itskovitz-Eldor, Sander S. Shapiro, Michelle A. Waknitz, Jennifer J. Swiergiel, Vivienne S. Marshall, Jeffrey M. Jones

SCIENCE VOL 282 6 NOVEMBER 1998



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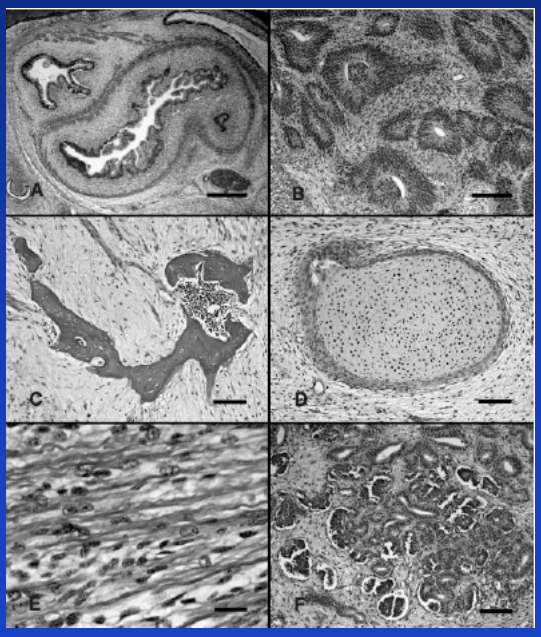
Ανθρώπινα ES



➢Ανθρώπινα ES μπορούν να δημιουργήσουν τερατώματα σε ποντίκια SCID. Στα τερατώματα απαντώνται διάφορες δομές:

A: δομή που μοιαζει με έντερο

B:νευροεπιθηλιακές ροζέτες

C: οστίτης ιστός

D: χόνδρος

E:γραμμωτός μυς

F: σπειράματα.

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Ανθρώπινα κύτταρα ES

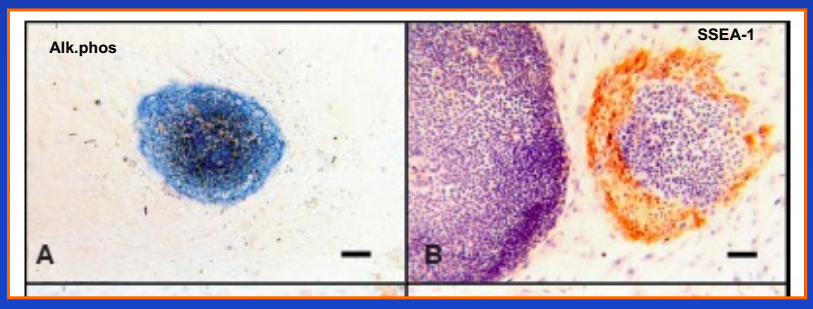
Τα ανθρώπινα εμβρυϊκά βλαστοκύτταρα διαφέρουν από τα εμβρυϊκά βλαστοκύτταρα του ποντικού:

➢Καλλιεργούνται δυσκολώτερα από αυτά του ποντικού.

➢Παρότι εκφράζουν *Oct-4* και *nanog* και *Sox2* δεν εκφράζουν άλλους χαρακτηριστικούς δείκτες που είναι παρόντες στα ES του ποντικού.

➢Δεν εξαρτώνται από LIF.

➢Χρειάζονται τροφικά κύτταρα και bFGF.



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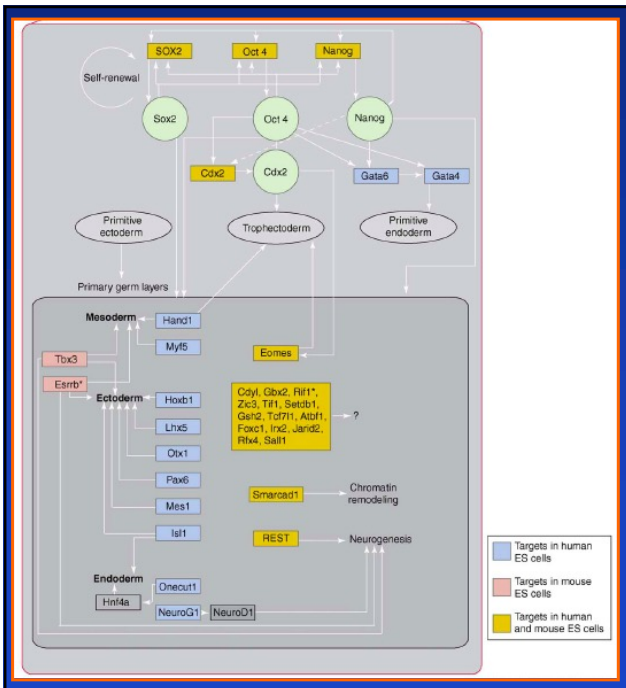

ChIP combined with genome wide methodologies to map the binding sites of OCT4 and NANOG in mouse and human ES genomes

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Ρυθμιστικά κυκλώματα στα ES

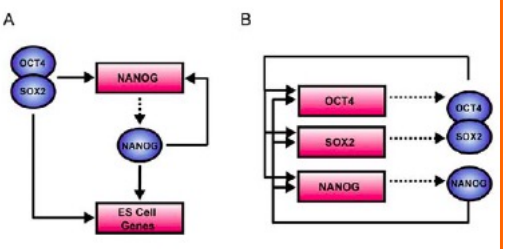

Figure 4. Transcriptional Regulatory Motifs in Human ES Cells
(A) An example of feedforward transcriptional regulatory circuitry in human ES cells. Regulators are represented by blue circles; gene promoters are represented by red rectangles. Binding of a regulator to a promoter is indicated by a solid arrow. Genes encoding regulators are linked to their respective regulators by dashed arrows.
(B) The interconnected autoregulatory loop formed by OCT4, SOX2, and NANOG.

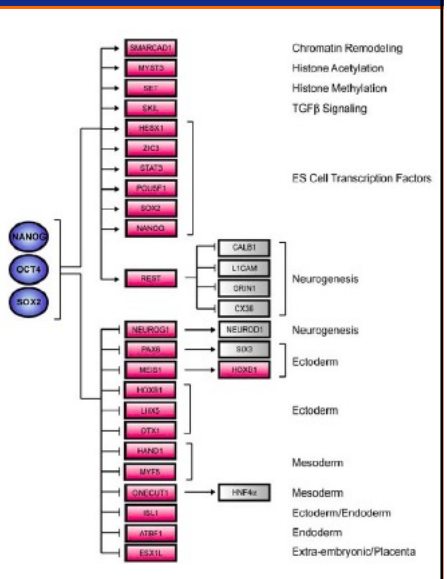

Figure 5. Core Transcriptional Regulatory Network in Human ES Cells

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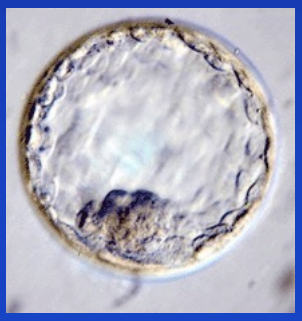
Απομόνωση ESC ποντικού

Τα εμβρυϊκά βλαστοκύτταρα απομονώθηκαν αρχικά από βλαστοκύστεις :

1) Μετά από ανατομή και απομόνωση της εσωτερικής κυτταρικής μάζας
2) Από ολόκληρη τη βλαστοκύστη χωρίς απομόνωση της ΕΚΤ.

ΟΜΩΣ .....



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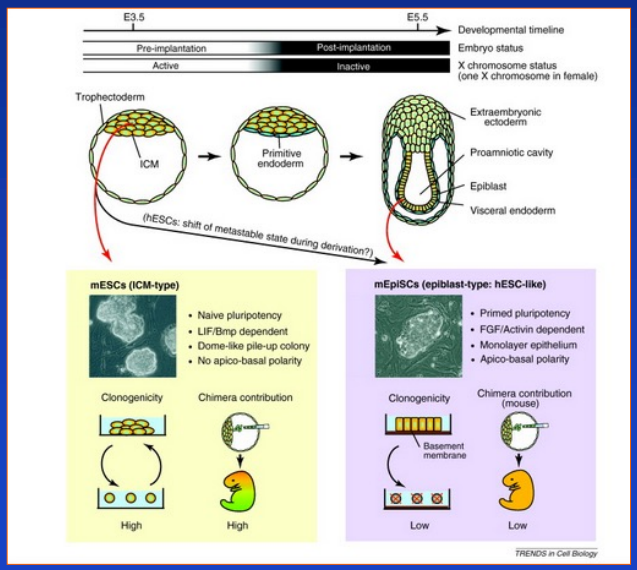
Απομόνωση ESC ποντικού



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Απομόνωση ESC ποντικού

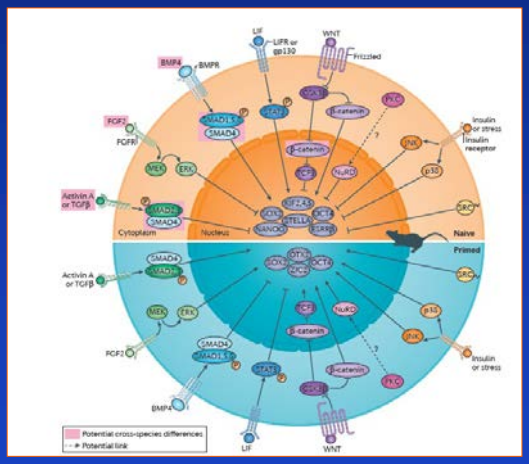

Figure 2 | **Signalling pathways and their influence on naive and primed pluripotent states.** Different signalling pathways can positively or negatively regulate naive and primed murine pluripotent stem cells. Note that the majority of the signalling pathways shown have opposing effects on the naive and primed pluripotent states in mice (for example, the leukaemia inhibitory factor (LIF)–signal transducer and activator of transcription 3 (STAT3) and fibroblast growth factor 2 (FGF2)–ERK signalling pathways). It is important to highlight that other pathways not included in this scheme are likely to also be involved in such regulation and will probably be further characterized in the future. Such pathways may include HIPPO, RHO, NOTCH and nuclear factor-κB signalling. Pink boxes highlight signalling pathways that may function differently in the regulation of mouse and human pluripotent cells. More specifically, it remains to be fully understood whether signalling induced by low doses of transforming growth factor-β (TGFβ), activin–NODAL, nuclear β-catenin or FGF2 (MEK–ERK independent) influences human naive pluripotency in a different manner to that previously observed in rodent naive embryonic stem cells. Dashed arrows indicate potential links that remain to be established. BMP, bone morphogenetic protein; ESRRβ, oestrogen-related receptor-β; GSK3β, glycogen synthase kinase 3β; JNK, Jun-like kinase; NuRD, nucleosome remodelling and deacetylases; OCT4, octamer-binding protein 4; PKC, protein kinase C; TCF3, transcription factor 3. Adapted from Poster http://www.nature.com/nrm/posters/pluripotency/index.html, Nature Publishing Group.

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ESC και Epi ESC

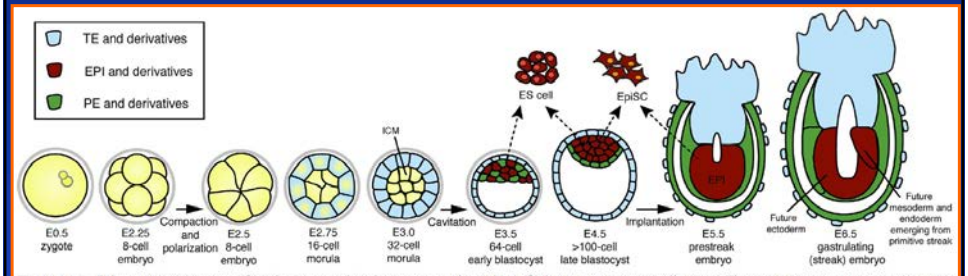

Figure 1.1 Schematic overview of early mouse development and origins of pluripotent stem cell types. The totipotent zygote undergoes three rounds of cleavage, producing an 8-cell embryo, which then undergoes compaction and polarization. From the 8-cell stage onward, cell divisions produce two populations of cells: outside cells, which will become the trophectoderm (TE), and inside cells, which will form the inner cell mass (ICM). The ICM further segregates into the primitive endoderm (PE) and the pluripotent epiblast (EPI) by the blastocyst stage. The EPI matures after implantation and gradually loses pluripotency when gastrulation commences at E6.5 (E, embryonic day). During gastrulation, the three germ layers are formed: mesoderm and endoderm precursors migrate through the primitive streak, while nonmigrating EPI cells form ectodermal tissues. Two types of pluripotent stem cells can be derived from the EPI at indicated stages: embryonic stem (ES) cells and epiblast stem cells (EpiSCs).

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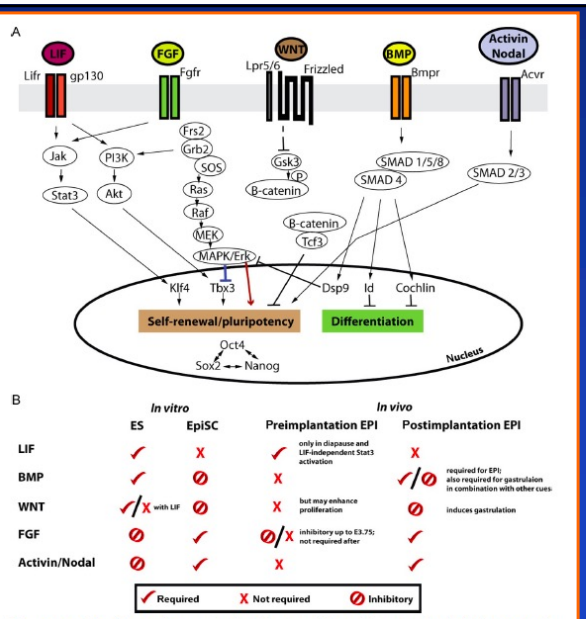

Figure 1.4 Signaling pathways involved in regulating self-renewal and pluripotency in the mouse. (A) Cross talk among intracellular signaling cascades involved in regulating self-renewal and pluripotency. Red line ES specific, blue line EpiSC specific. (B) Signaling pathways utilized in the maintenance of self-renewal and pluripotency *in vitro* (ES and EpiSCs) and *in vivo* (pre- and postimplantation EPI).

ESC και Epi ESC

↗ Τα ESC έχουν πολλές διαφορές με τα EpiSCs, αν και με τα κλασσικά τεστ (κλωνικότητα, καρκινο-γένεση, χίμαιρες) φαίνο-νται ίδια.

↗ Αποκρίνονται διαφορε-τικά σε σηματοδοτικά μόρια.

↗Τα ανθρώπινα μοιάζουν με τα EpiSCs.

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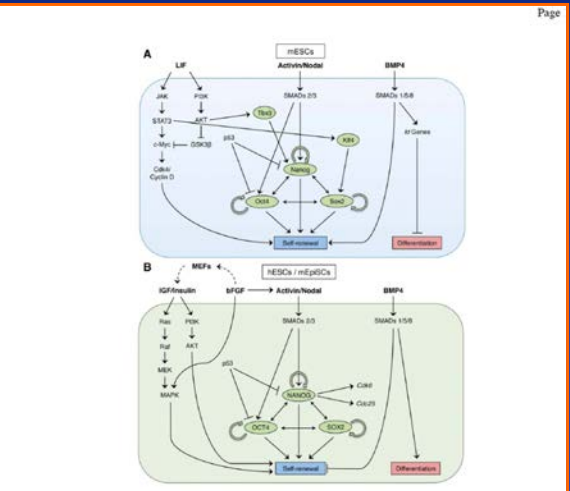

Fig. 1.
Mouse and human ESC survival pathways. (A) Mouse ESCs require LIF and BMP4 for maintenance. (B) Human ESCs and mouse EpiSCs require IGF/insulin and bFGF for maintenance. Human ESC-derived fibroblast-like cells and MEFs are also stimulated by bFGF in culture to secrete IGF (dashed arrows). In both cell types, Nanog, Oct4, and Sox2 form a positive auto-regulatory loop.
Stem Cells. 2013 July ; 31(7): 1227–1236. doi:10.1002/stem.1384.

ESC και Epi ESC

↗Τα εμβρυϊκά βλαστοκύτ-ταρα είναι ένας πληθυσμός που δεν απαντά *in vivo* ή απαντά για πολύ μικρό διάστημα καθώς το δυ-ναμικό του άμεσα περιορί-ζεται επομένως αποκρίνο-νται διαφορετικά σε πολλά σήματα.

↗Αυτό ισχύει και για τα EpiSCs

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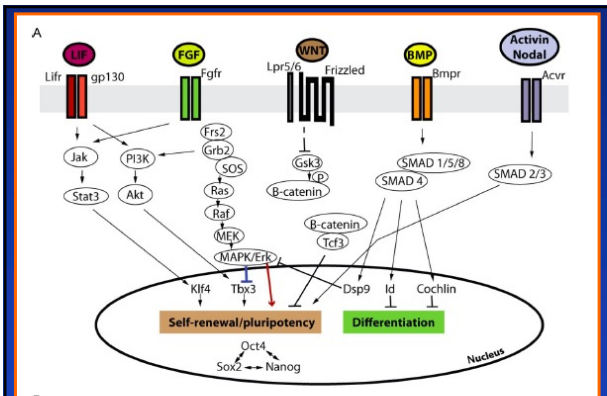

Figure 1.4 Signaling pathways involved in regulating self-renewal and pluripotency in the mouse. (A) Cross talk among intracellular signaling cascades involved in regulating self-renewal and pluripotency. Red line ES specific, blue line EpiSC specific. (B) Signaling pathways utilized in the maintenance of self-renewal and pluripotency *in vitro* (ES and EpiSCs) and *in vivo* (pre- and postimplantation EPI).

ESC και Epi ESC

↗Τα εμβρυϊκά βλαστοκύτ-ταρα είναι ένας πληθυσμός που δεν απαντά *in vivo* ή απαντά για πολύ μικρό διάστημα καθώς το δυ-ναμικό του άμεσα περιορί-ζεται επομένως αποκρίνο-νται διαφορετικά σε πολλά σήματα.

↗Αυτό ισχύει και για τα EpiSCs

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Naive Pluripotent Stem Cells Derived Directly from Isolated Cells of the Human Inner Cell Mass

Ge Guo,[1] Ferdinand von Meyenn,[4] Fatima Santos,[4] Yaoyao Chen,[1] Wolf Reik,[4,5] Paul Bertone,[1] Austin Smith,[1,3,*] and Jennifer Nichols[1,2,*]

Stem Cell Reports | Vol. 6 | 437–446 | April 12, 2016 |

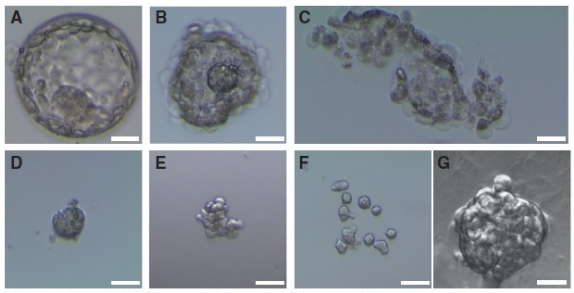

Figure 1. Cell Line Derivation from Dissociated Human Inner Cell Mass Cells
(A) Day-6 blastocyst.
(B) Trophoblast lysis.
(C) Discarded trophoblast.
(D) Isolated inner cell mass.
(E) Decompacted ICM.
(F) Dissociated ICM.
(G) Primary stem cell clone grown from a single ICM cell.
(H) Colony at passage 8.

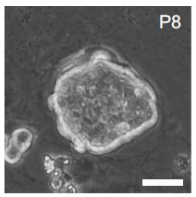

Table 1. Derivation of Naive Epiblast Stem Cell Lines

Experiment	Embryos Surviving Thaw	Blastocysts[a]	Dissociated ICMs	Cell Lines	Cumulative Passages
1	24	4	1	HNES1	P30
2	9	4	2	HNES2	P22
				HNES3	P29
3	20	4	4	HNES4[b]	P21
4	5	2	1	[c]	
Total	58	14	8	4	

[a]Embryos cavitated by day 6.
[b]Primary colonies lost in three cases associated with incubator humidity failure.
[c]Primary colonies emerged but failed to expand after five passages.

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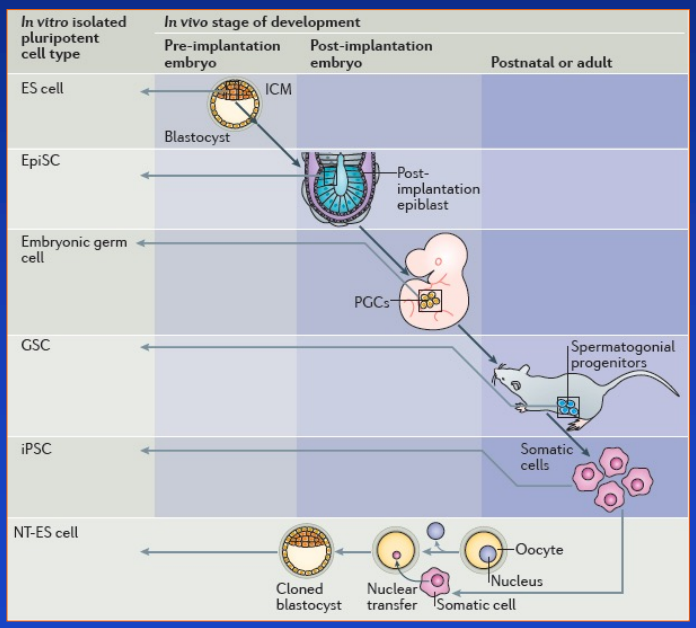
ES από το πρώιμο έμβρυο...αλλά και από .....



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