Charakterisierung und Manipulation der Immunogenität embryonaler Stammzellen des Neuweltaffen *Callithrix jacchus*

Von der Naturwissenschaftlichen Fakultät
der Gottfried Wilhelm Leibniz Universität Hannover
zur Erlangung des Grades einer
Doktorin der Naturwissenschaften
Dr. rer. nat.
genehmigte Dissertation
von
Dipl.Biol. Gesine Fleischmann
geboren am 27.April 1981 in Goslar

2009

Referent:	Prof. Dr. Peter A. Horn, Institut für Transfusionsmedizin, Medizinische Hochschule Hannover
1. Koreferent:	Prof. Dr. Anaclet Ngezahayo, Institut für Biophysik, Leibniz Universität Hannover
2. Koreferent:	Prof. Dr. Ulrich Martin, Leibniz Forschungslaboratorien für Biotechnologie und künstliche Organe, Medizinische Hochschule Hannover

Tag der Promotion: 09.02.2009

Diese Arbeit wurde am Institut für Transfusionsmedizin der Medizinischen Hochschule Hannover unter der Anleitung von Prof. Dr. Peter A. Horn angefertigt.

-meinen Eltern Brigitte und Wilfried Fleischmann-

Danksagung

Mein besonderer Dank gilt Prof. Dr. Peter A. Horn. Ohne seine Betreuung, die zahlreichen Diskussionen, Ratschläge und Denkanstöße wäre das Gelingen dieser Arbeit nicht möglich gewesen. Ich möchte mich auch auf diesem Wege für das Vertrauen und die Verantwortung, die ich übernehmen durfte bedanken. Vielen Dank auch für die Unterstützung meiner Auslandsaufenthalte und das Ermöglichen der Teilnahme an zahlreichen Kongressen.

Ein weiterer Dank gilt Prof Dr. A. Ngezahayo und Prof. Dr. Ulrich Martin für die Übernahme der Begutachtung der Arbeit. Ebenso bedanken möchte ich mich bei Prof. Dr. Rainer Blasczyk für die Möglichkeit diese Arbeit im Institut für Transfusionsmedizin der Medizinischen Hochschule Hannover durchführen zu können. Ein großer Dank gilt Prof. Dr. Erika Sasaki, die unserer Arbeitsgruppe die Zelllinie cjes001 zur Verfügung gestellt hat.

Ein besonderer Dank gilt Kirsten Elger für die Unterstützung während der gesamten Zeit. Vielen Dank für Deine Geduld, das Zuhören, das Gegenlesen und dass Du mir oft den Rücken freigehalten hast.

Ebenso gebührt dieser Dank Dr. Melanie Wurm. Vielen Dank für die Zeit in Japan, für die nette Zusammenarbeit, viele informative Gespräche und Diskussionen und für eine Menge Kaffeepausen um den Kopf wieder frei zu kriegen.

Allen Mitarbeitern der Transfusionsmedizin, vor allem denen aus dem Medical Park sowie Claudia van Deventer möchte ich für die Unterstützung danken. Ebenso bedanke ich mich bei den Mitarbeitern von Imusyn für die nette Zusammenarbeit.

Weiterhin gilt mein Dank allen, die zu dem Gelingen dieser Arbeit beigetragen haben. Der `Stem Cell Research Group` des DPZ, insbesondere Dr. Thomas Müller und Dr. Rüdiger Behr für die Kooperation und die gute Zusammenarbeit. Vielen Dank an die AG Martin des Lebao, insbesondere an Dr. Kristin Schwanke für die Unterstützung bei der Etablierung der Kulturbedingungen für die ESC.

Der größte Dank gilt meiner Familie ohne die ich diesen Weg nicht hätte gehen können.

Außerdem bedanken möchte ich mich bei Anja Schöner, Helgrid Grünhagen, Cordula Hallmann und Thimo Holze, die die Aufs und Abs, die eine Promotion mit sich bringt mit mir mitgemacht haben und mir oft Probleme privater Natur abgenommen haben, damit ich mich auf die Arbeit konzentrieren konnte.

DANKE!

Zusammenfassung

Embryonale Stammzellen (ESC) bieten viele Möglichkeiten im Hinblick auf therapeutische Anwendungen, insbesondere in der regenerativen Medizin. Auf Grund ethischer Konflikte und gesetzlicher Vorgaben ist es in Deutschland problematisch, Versuche an humanen ESC durchzuführen. Eine Alternative bietet die Arbeit an murinen ESC. Eine andere Möglichkeit ist die Arbeit an ESC nicht humaner Primaten, die phylogenetisch gesehen mit dem Menschen enger verwandt sind. In dieser Arbeit wurde als Modell der Neuweltaffe Marmoset *Callithrix jacchus* gewählt.
Die Marmoset Stammzellline cjes001 wurde etabliert und mit spezifischen Markern sowohl in immunhistochemischen Färbungen als auch mit spezifischen Primern in einer Polymerasekettenreaktion (PCR) charakterisiert, um ihre Stammzelleigenschaften nachzuweisen. Dazu wurden gängige Marker wie Oct3/4, SSEA-3, SSEA-4, Tra-1-60, Tra-181, Sox-2 und Alkalische Phosphatase nachgewiesen. Außerdem wurde ein Pluripotenznachweis in Form einer Teratominduktion in immundefizienten NOD/SCID Mäusen erfolgreich durchgeführt.

ESC wachsen üblicherweise auf einem Feeder-Zellrasen aus Fibroblasten von 13,5 Tage alten Mausembryonen (MEF), dessen Proliferation entweder chemisch durch Mitomycin C oder durch Bestrahlung mit γ-Strahlen gehemmt ist. Es ist unklar, welche Auswirkungen die Behandlung der MEF auf das Wachstum und die Pluripotenz bzw. die Differenzierung der ESC hat. In dieser Studie wurden beide Methoden der Inaktivierung gegenüber gestellt und analysiert. Zum Einen wurde eine quantitative Abschätzung der sezernierten Faktoren mit Hilfe eines *bead-based multiplex arrays* (Rules Based Medicine, Austin, Texas, USA) vergleichend an mit Mitomycin C behandelten MEF und bestrahlten MEF durchgeführt. Bestrahlte MEF sezernieren höhere Mengen verschiedener Faktoren wie IP10, Insulin and Eotaxin als MEF, die chemisch durch Mitomycin C inaktiviert wurden. Außerdem wurde ein MTT-Test (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid) durchgeführt, um die metabolische Aktivität der Zellen beurteilen zu können. Bestrahlte MEF weisen eine signifikant höhere metabolische Aktivität auf als chemisch inaktivierte MEF. Ferner wurde die Proliferationsrate der ESC sowie deren Morphologie beurteilt. Embryonale Stammzellen, die auf bestrahlten MEF kultiviert werden, zeigen eine geringere Tendenz zu differenzieren als ESC, die auf Mitomycin C behandelten MEF wuchsen.

Die Manipulation embryonaler Stammzellen bietet einen vielversprechenden Ansatz therapeutische Anwendungen zu vereinfachen. Insbesondere im Hinblick auf die Immunogenität und die damit verbundenen Probleme bei Transplantationen könnte die Manipulation von ESC weiterhelfen. Eines der größten Probleme in der regenerativen Medizin und den zell-basierten Therapien ist der Haupthistokompatibilitätskomplex (Abk. MHC von engl. Major Histocompatibility Complex) transplantierter Zellen. Die Herunterregulierung des MHC mittels shRNA durch RNA Interferenz wurde bereits erfolgreich an anderen Zelltypen durchgeführt. Diese Methode steht in der Diskussion, auch die Immunogenität von Marmoset ESC kontrollieren zu können. Der Silencing-Effekt wurde durchflusszytometrisch sowie durch Real Time PCR bestimmt und zeigte eine Reduzierung der MHC-Expression um bis zu 85%.

Stichworte: Embryonale Stammzellen, *Callithrix jacchus*, Maus embryonale Fibroblasten (MEF), Mitomycin C, γ-Bestrahlung, Hauptkompatibilitätskomplex, shRNA

Abstract

Embryonic stem cells (ESC) hold tremendous potential for therapeutic applications, including regenerative medicine. Many experiments cannot be conducted in human ESC in Germany because of ethical or practical limitations. An alternative is the work with murine ESC. Another possibility is the work with non human primate ESC. They are phylogenetic closer to humans. In this study the new world monkey marmoset *Callithrix jacchus* was chosen as a model.

The marmoset embryonic stem cell line cjes001 was established. To show the stem cell characteristics of this cell line it was stained with specific makers in immunohistochemistry and characterized by PCR with specific primers. Current markers like Oct3/4, SSEA-3, SSEA-4, Tra-1-60, Tra-1-81, Sox-2 and alkaline phosphatise were detected. Furthermore a teratoma induction in immune deficient NOD/SCID mice was performed.

ESC are typically grown on mouse embryonic fibroblasts (MEF) of 13,5 day old mouse embryos as feeder cells whose proliferation is arrested either by treatment with Mitomycin C or by γ-irradiation. Until now it is unclear which effect treatment of MEF has on proliferation and differentiation of ESC.

In this study both methods of inactivation were compared and analysed. To assess the impact of these treatments on the ability of MEF to support growth of undifferentiated ESC, we quantified cytokines and growth factors in the supernatant of both Mitomycin-treated and γ-irradiated MEF by *bead-based multiplex array* (Rules Based Medicine, Austin, Texas, USA). Comparing γ-irradiated and Mitomycin-treated MEF suggested higher amounts of some cytokines including IP10, Insulin and Eotaxin by the former.

We also assessed whether the method of inactivation had an effect on growth kinetics and differentiation of primate ESC. First, we used an MTT assay to evaluate the cellular metabolic activity of growth arrested feeder cells. There was a significant ($p<0.02$) difference between the different ways of inactivation with γ-irradiated cells displaying a higher metabolic activity. There appeared to be a trend to a lower number of differentiated ESC colonies on the γ-irradiated feeder cells, suggesting that this may be a preferable method of growth arrest.

Genetic modification of embryonic stem cells or adult stem cells is expected to significantly advance the use of stem cell based regenerative treatments. Especially in terms of immunogenicity and the risks in transplantation manipulation of ESC could help to avoid the immune barrier. The expression of the highly polymorphic major histocompatiblility complex (MHC) on transplanted cells is a huge problem in this field of work. Silencing of MHC by RNA interference using shRNA was shown successful in human cells. In marmoset cells this method should work too. The achievement of the expression of MHC class I was followed by RT-PCR and flow cytometry. The transduction of RNAi cassettes containing the sequences for shRNAs targeting β2m suppressed MHC class I protein expression by up to 85%.

Keywords: Embryonic stem cells, *Callithrix jacchus*, mouse embryonic fibroblasts (MEF), Mitomycin C, γ-irradiation, major histocompatibility complex, shRNA

Inhaltsverzeichnis

Abkürzungen

AP	Alkalische Phosphatase
bFGF	basic fibroblast growth factor
ß2m	beta-2-microglobulin
dsRNA	doppelsträngige RNA
EB	Embryoid bodies
ESC	Embryonale Stammzellen
ICM	Innere Zell Masse (von engl. Inner Cell Mass)
LIF	Leukemia inhibitory Factor
MEF	embryonale Maus Fibroblasten (von engl. Mouse embryonic fibroblasts)
MHC	Hauptkompabilitätskomplex (von engl. Major Histocompatibility Complex)
Oct3/4	Octamer 3/4
PCR	Polymerasekettenreaktion
shRNA	short hairpin RNA
siRNA	small interference RNA
SSEA-3	Stage-Specific Embryonic Antigen-3
SSEA-4	Stage-Specific Embryonic Antigen-4

Vorwort

In Absprache mit der Leibniz Universität Hannover und denen in der *Gemeinsamen Ordnung der naturwissenschaftlichen Fachbereiche für die Promotion zum Doktor der Naturwissenschaften* festgelegten Bestimmungen besteht diese kumulative Dissertation aus wissenschaftlichen Artikeln, die publiziert worden sind oder für die Publikation in etablierten wissenschaftlichen Fachzeitschriften vorbereitet sind.

Die Dissertation befasst sich mit der Charakterisierung und Manipulation der Immunogenität embryonaler Stammzellen des Neuweltaffen Marmoset *Callithrix jacchus*.

Zu den veröffentlichten Artikeln habe ich außerdem den von mir publizierten “Meeting Report, Symposium in Stem Cell Repair and Regeneration 2006”, der 2007 in Cloning and Stem Cells erschienen ist, beigefügt. Der Artikel gibt einen Überblick über die auf dem Kongress vorgestellten Forschungsfelder und erzielten Ergebnisse der Teilnehmer und somit auch einen Überblick über den Stand der Wissenschaft im Feld der Stammzellforschung zu dem angegebenen Zeitpunkt, welcher der Beginn meiner Doktorarbeit war.

Die Ergebnisse der Arbeiten werden hier zusammengefasst und ausführlich im Hinblick auf die Einordnung in den Stand der Wissenschaft und die Zukunftsperspektiven diskutiert.

Im Folgenden sind die beigefügten Artikel aufgelistet:

1. **Fleischmann G** (2007): Symposium in Stem Cell Repair and Regeneration, Cloning and Stem Cells 9(2): 141-143

2. Müller T, **Fleischmann G**, Eildermann K, Mätz-Rensing K, Horn PA, Sasaki E, Behr R (2008) A novel stem cell line derived from the common marmoset monkey (*Callithrix jacchus*) exhibiting germ cell-like characteristics, Human Reproduction (in review)

3. **Fleischmann G**, Müller T, Behr R, Blasczyk R, Sasaki E, Horn PA (2008) Growth characteristics of the non-human primate embryonic stem cell line cjes001 depending on feeder cell treatment, Cloning and stem cells (submitted)

4. **Fleischmann G**, Figueiredo C, Seltsam A, Blasczyk R, Horn PA (2008) Embryonic Stem Cells: MHC Expression and Immunogenicity of Stem Cell-Derived Cellular Therapeutics, Stem Cell Applications in Diseases, Nova Science Publishers, Inc. (Editor F. Columbus) (Preliminary accepted)

2. Kumulative Auswertung

2.1. Einleitung

Die hier vorgestellte Arbeit beschäftigt sich mit der Etablierung und Charakterisierung, der Optimierung von Kulturbedingungen und Manipulation der Immunogenität embryonaler Stammzellen des Neuweltaffen Marmoset (Weißbüschelaffe) *Callithrix jacchus*.

Embryonale Stammzellen (ESC) bieten großes Potential im Hinblick auf therapeutische Anwendungen, insbesondere in der regenerativen Medizin, aber auch für das Verständnis von Grundlagen in der Stammzellforschung. Insbesondere ihre Fähigkeit in verschiedene Zelltypen differenzieren zu können bietet große Möglichkeiten (Mountford 2008, Wobus 2001). Aufgrund ethischer und gesetzlicher Limitationen ist es in Deutschland häufig nicht möglich Versuche an humanen ESC durchzuführen. Eine Alternative bietet die Arbeit an ESC nicht humaner Primaten. In dieser Arbeit wurde als Modellorganismus der Neuweltaffe *Callithrix jacchus* gewählt.

2.1.1. Stammzellen

Stammzellen können aufgrund ihres Differenzierungspotentials in vier Gruppen unterschieden werden: totipotent, pluripotent, multipotent oder unipotentent (Gage 2000).

Als totipotent werden Stammzellen bezeichnet, die in der Lage sind wieder einen vollständigen Organismus auszubilden. Dies ist jedoch nur im frühen Embryonalstadium, beim Menschen bis zum 8-Zell-Stadium, möglich. Beim Durchlaufen des Morulastadiums verlieren die Zellen ihre Totipotenz und gehen in das Stadium der Pluripotenz über (Donovan and Gearhart 2001). Pluripotente Stammzellen sind in der Lage in über 200 verschiedene Zelltypen zu differenzieren, sind jedoch nicht fähig einen vollständigen Organismus auszubilden. Sie können aus der Blastozyste in den Tagen 5 bis 14 entnommen werden. Weiter differenzierte Zellen werden als multipotent bezeichnet. Dazu gehören Zellen, die in der Lage sind in zwei bis drei Zelltypen zu differenzieren, wie z.B. hämatopoetische Stammzellen. Die letzte Gruppe bilden die unipotenten Zellen, die lediglich in der Lage sind, gleiche Zellen hervorzubringen und nicht weiter zu differenzieren, wie z.B. Fibroblasten.

2.1.2. Embryonale Stammzellen (ESC)

Embryonale Stammzellen werden im Allgemeinen als pluripotent bezeichnet. Sie werden aus der inneren Zellmasse (ICM) der Blastozyste an Tag 5 bis 6 gewonnen (Evans and Kaufman 1981, Martin 1981). Sie sind in der Lage in alle drei Keimblätter (Entoderm, Mesoderm, Ektoderm) zu differenzieren und können somit alle verschiedenen Zelltypen bilden.

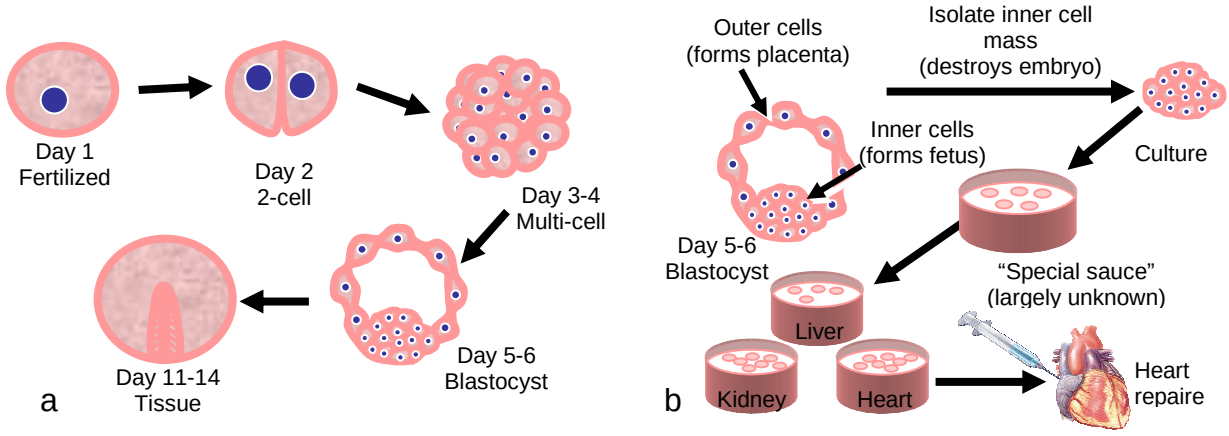

Abb.1: a Entwicklung der befruchteten Oozyte bis Tag 14. Tag 2 zeigt das Zwei-Zell-Stadium, an den Tagen 3 und 4 ist bereits ein Vielzellstadium zu sehen, das sogenannte Morulastadium. Einen Tag später an den Tagen 5 und 6 ist die Blastozyste mit der ICM zu sehen aus der die ESC entnommen werden. b Aus der Blastozyste wird die ICM isoliert. Unter speziellen Kulturbedingungen lassen sich undifferenzierte ESC kultivieren, die in verschiedene Zelltypen wie Herz, Leber oder Niere differenziert werden können. Diese Zellen können für die regenerative Medizin genutzt werden.

Embryonale Stammzellen wurden erstmals 1981 aus der Blastozyste einer Maus isoliert (Evans and Kaufman 1981). Im Folgenden wurden ES-Zellen von verschiedenen weiteren Spezies gewonnen, so dass heute ES Zelllinien von Nagern (Martin 1981), Kaninchen (Graves and Moreadith 1993) und Primaten (Thomson et al. 1995) etabliert sind. 1998 wurden die ersten humanen ESC isoliert und es gelang sie als Zelllinie zu etablieren (Thomson et al. 1998).

Durch ihre Fähigkeit in alle Zelltypen differenzieren zu können, bieten ESC ein enormes Potential für die regenerative Medizin und für eine therapeutische Nutzung. Die Fähigkeit der Pluripotenz ist nicht nur in vivo sondern auch in vitro gegeben,

sofern die Kulturbedingungen optimal und konstant gehalten werden. Die Differenzierung in verschiedene Zelltypen lässt sich durch unterschiedliche Kulturbedingungen induzieren (Baylink 1983, Jaiswal et al. 1997, Pittenger et al. 1999, Soukas et al. 2001). So lässt sich eine kardiale Differenzierung z.B. durch Kokultivierung von aus ESC entstandenen Embryoid bodies (EB) mit END-2 Zellen oder OP9 Zellen und den entsprechenden Differenzierungsmedien induzieren (Boheler et al. 2002, Schwanke et al. 2006, Wobus et al. 1991). Auch wenn dies derzeit noch nicht möglich und eine klinische Anwendung noch weit entfernt ist, ist es vorstellbar, dass auf lange Sicht aus ESC differenzierte Zellen in großer Menge für therapeutische Zwecke hergestellt werden können (Passier 2003). Dies ist ein großer Vorteil gegenüber adulten Stammzellen, da deren Expansion sich in der Regel als deutlich schwieriger darstellt. Mit embryonalen Stammzellen wäre es vorstellbar, diese unbegrenzt zu expandieren und gezielt zu differenzieren.

Je nach Spezies unterscheiden sich die Kultivierungsbedingungen für embryonale Stammzellen (Ginis et al. 2004). Um die Pluripotenz der Zelllinien erhalten zu können müssen die spezifischen Kulturbedingungen eingehalten werden. So muss z.B. bei der Kultivierung muriner embryonaler Stammzellen Leukemia inhibitory factor (LIF) zugesetzt werden, während dies bei der Kultivierung von ESC von Menschen und von Marmosets nicht notwendig ist (Sasaki et al. 2005, Skottman et al. 2006). Außerdem ist für humane und murine ESC beschrieben, dass eine Feeder-freie Kultivierung möglich ist, für ESC nicht humaner Primaten ist dies bislang nicht in Langzeitkultur gelungen (Amit and Itskovitz-Eldor 2006a, Amit and Itskovitz-Eldor 2006b, Beattie et al. 2005, Bigdeli et al. 2008, Xu et al. 2001).

2.1.3. Therapeutisches Potential und Limitationen

Für die Weiterführung der Forschung an embryonalen Stammzellen in den klinischen Bereich, müssen die Versuche in nicht-murinen Modellen, die phylogenetisch näher am Menschen sind durchgeführt werden.

Um die kontrovers diskutierte Forschung an human ESC zu umgehen, bietet der nicht humane Primat *Callithrix jacchus* durch die nahe Verwandtschaft zum Menschen eine gute Alternative zu der Arbeit mit humanen ESC (Fischbach and

Fischbach 2004, Horn et al. 2006, Mountford 2008, Nakatsuji and Suemori 2002, Nikol'skii et al. 2007, Suemori and Nakatsuji 2006). Aber auch der Rhesusaffe weist mit einer fast 90%igen Genhomologie zu humanen Stammzellen eine gute Alternative zu humanen ESC auf (Clark et al. 2003).

Ein Problem zeigt sich in der Generierung maligner Tumore bei der Injektion embryonaler Stammzellen in den Organismus (Teramoto et al. 2005, Wakitani et al. 2003) als auch in ihrer Immunogenität, wenn diese auch weitaus geringer scheint als bisher angenommen. Der Vorteil embryonaler Stammzellen gegenüber adulten Stammzellen liegt dabei in der verhältnismäßig geringen Expression von MHC Klasse I sowie in der Abwesenheit von MHC Klasse II (s. 2.1.7. MHC) (Drukker et al. 2002) . Somit ist die Gefahr der Abstoßung deutlich minimiert.

2.1.4. *Callithrix jacchus*

Der Neuweltaffe Marmoset *Callithrix jacchus*, das Weißbüscheläffchen, gehört zur Familie der Krallenaffen. Er ist aufgrund seiner geringen Größe (18cm-30cm), seines geringen Gewichts (300g-500g) und seiner nahen Verwandtschaft zum Menschen ein sehr gut geeignetes Tierversuchs-Modell. Ein weiterer Vorteil ist die kurze Generationszeit und zumeist werden Zwillinge geboren. Im Vergleich zum Rhesus-Affen ist die Haltung von Marmoset durch ihre geringe Größe wesentlich kostengünstiger. Marmosets sind außerdem unkompliziert im Umgang. Ihre Lebenserwartung beträgt in freier Wildbahn ~10 Jahre und in menschlicher Obhut ~20 Jahre (Wurm 2007).

2.1.5. Die embryonale Stammzelllinie cjes001

Die embryonale Stammzelllinie cjes001 wurde uns für unsere Arbeiten von PhD Erika Sasaki, Central Institute for *Experimental Animals (CIEA), Laboratory of Applied Developmental Biology, Department of Marmoset Research, Kawasaki, JAPAN* zur Verfügung gestellt (Sasaki et al. 2005). Die Linie wurde in Kooperation mit der Forschergruppe „Stammzelle“ des Deutschen Primatenzentrums (DPZ) in Göttingen charakterisiert.

Der Stammzellcharakter und somit die Pluripotenz embryonaler Stammzellen wird durch die Expression verschiedener charakteristischer Marker wie Oct 3/4, Sox-2, Nanog, SSEA-3, SSEA-4, Tra-1-60, Tra-1-81 oder Alkalische Phosphatase

nachgewiesen (Avilion et al. 2003, Berstine et al. 1973, Cai et al. 2002, Henderson et al. 2002, Scholer et al. 1989). Diese werden durch immunhistochemische Färbungen, durchflusszytometrisch oder per PCR nachgewiesen. Mit diesen Markern wird die Expression der Gene, die für die Pluripotenz charakteristisch sind auf der Oberfläche der Zellen bzw. intrazellulär nachgewiesen. Eine weitere Bestätigung der Pluripotenz ist die Induktion von Teratomen in immundefizienten Mäusen. Es werden ESC subcutan in NOD/SCID Mäuse injiziert. Durch ihre Eigenschaft in alle drei Keimblätter zu differenzieren, bilden sich bei der Injektion in immundefiziente Mäuse Teratome. Außerdem wird zur vollständigen Charakterisierung neu etablierter ESC-Linien eine Karyotypisierung vorgenommen.

Eine weitere Eigenschaft embryonaler Stammzellen ist die spontane Differenzierung in diverse Zelltypen über Embryoid bodies. Über verschiedene Differenzierungsmarker wie VASA, SCP3 oder GCNF als Beispiel für Keimzellmarker lässt sich die Differenzierung nachweisen (Castrillon et al. 2000).

2.1.6. Feeder-Zellen

Embryonale Stammzellen werden üblicherweise auf Fibroblasten kultiviert, die aus 13,5 Tage alten Maus-Embryonen gewonnen werden. Diese müssen in ihrer Proliferation arretiert werden, um ein Überwachsen der Stammzellkolonien zu verhindern. Zur Inaktivierung der MEF wird üblicherweise eine der folgenden zwei Methoden angewendet: Zum einen eine chemische Inaktivierung mittels Mitomycin C oder zum anderen Bestrahlung mit einer γ-Strahlenquelle. Dass die MEF einen Einfluss auf die ESC ausüben ist zu erwarten, wenn man bedenkt, dass die Feeder-Zellen verschiedene Faktoren sezernieren und die ESC direkten Kontakt zu ihnen haben. Der genaue Einfluss den die MEF auf die ESC ausüben scheint bisher jedoch unklar.

Standardmäßig werden in verschiedenen Laboren unterschiedliche Mausstämme für die Gewinnung von MEF genutzt. Die meistgenutzten Stämme sind CF1 und NMRI. Ob es Unterschiede in der Qualität der MEF bezüglich der verschiedenen Mausstämme gibt ist bis jetzt auch noch unklar.

Für verschiedene andere Zelltypen wurde bereits verglichen, welchen Einfluss MEF haben (Roy et al. 2001). Es ist jedoch bisher nicht untersucht worden, welchen Einfluss die Art der Inaktivierung auf das Wachstum und den Erhalt des Stammzellcharakters hat.

Außerdem gibt es Ansätze einer feeder-freien Kultivierung, da eine therapeutische Anwendung nur möglich ist, wenn standardisierte Bedingungen herrschen (Amit and Itskovitz-Eldor 2006a, Amit and Itskovitz-Eldor 2006b, Beattie et al. 2005, Bigdeli et al. 2008, Hong-mei and Gui-an 2006, Xu et al. 2001, Zhang et al. 2006). Eine feeder-freie Kultivierung ist bisher für murine und humane ESC gelungen, konnte jedoch nicht in Langzeitkultur für Primaten gezeigt werden.

2.1.7. Hauptkompatibilitätskomplex (MHC)

Der Hauptkompatibilitätskomplex (Abk. MHC von engl. Major Histocompatibility Complex) umfasst eine Gruppe von Genen, die Proteine für die Immunerkennung kodieren. Im humanen Genom finden sich diese Gene auf Chromosom 6. Die Proteinkomplexe des MHC sind Antigene, die auf der Oberfläche jeder Körperzelle zu finden sind. Die Zellen sind somit als eigen gekennzeichnet und können von den Leukozyten als körpereigen erkannt werden . Werden körperfremde Zellen transplantiert, werden über den MHC diese Zellen als solche erkannt und eliminiert. Eine Möglichkeit diesen Mechanismus zu umgehen bietet die Behandlung des Patienten mit Immunsuppressiva. Eine andere Möglichkeit mit einem geringeren Risiko stellt die Manipulation embryonaler Stammzellen dar. Denkbar wäre eine Herunterregulierung des MHC in embryonalen Stammzellen, die dann gezielt in die benötigte Richtung differenziert werden können. Dieser Ansatz bietet gute Möglichkeiten die therapeutische Behandlung mit ESC zu vereinfachen. Die Herunterregulierung des MHC mittels shRNA durch RNA Interferenz wurde bereits erfolgreich an anderen Zelltypen durchgeführt. (Figueiredo et al. 2006). Der Silencing-Effekt kann durchflusszytometrisch sowie durch Real Time PCR bestimmt werden.

2.1.8. RNA-Interferenz

Die RNA-Interferenz (RNAi) ist ein natürliches Phänomen einer posttranskriptionellen Hemmung der Genexpression. Sie wurde ursprünglich in Pflanzen als *post transcriptional gene silencing* beschrieben (Al-Kaff, 1998). Ausgelöst wird dieser Vorgang durch doppelsträngige RNA-Moleküle (dsRNA), die über small hairpin RNA (shRNA) zu sogenannten small interfering RNA (siRNA) abgebaut werden. Die

Fragmente sind ~20 Basenpaare lang. Die dsRNA wird durch den Proteinkomplex Dicer in siRNA geteilt (Bernstein et al. 2001, Hunter 2000). RISC (RNA induced silencing complex) ist ein weitere Proteinkomplex, der eine wichtige Rolle in der RNAi spielt. RISC enthält ein 20-23 Basenpaare langes Nukleomer. Er wirkt als Einzelstrang-RNase und bindet spezifisch an die Ziel-mRNA. Die Spezifität ist bedingt durch die sequenzidentische siRNA. Der komplementäre Antisense-Strang liegt an der Außenseite des RISC, so dass dieser an die Ziel-mRNA binden und diese degradieren kann.

Experimentell gelang dieser sogenannte Knock-Down erstmals in *C.elegans* (Fire et al. 1998).

Die Inhibition der Gene kann durch quantitative Messung der mRNA mittels Real Time PCR nachgewiesen werden. Außerdem kann das Einbringen der siRNA durchflusszytometrisch bestimmt werden, wenn die verwendeten Vektoren GFP markiert sind.

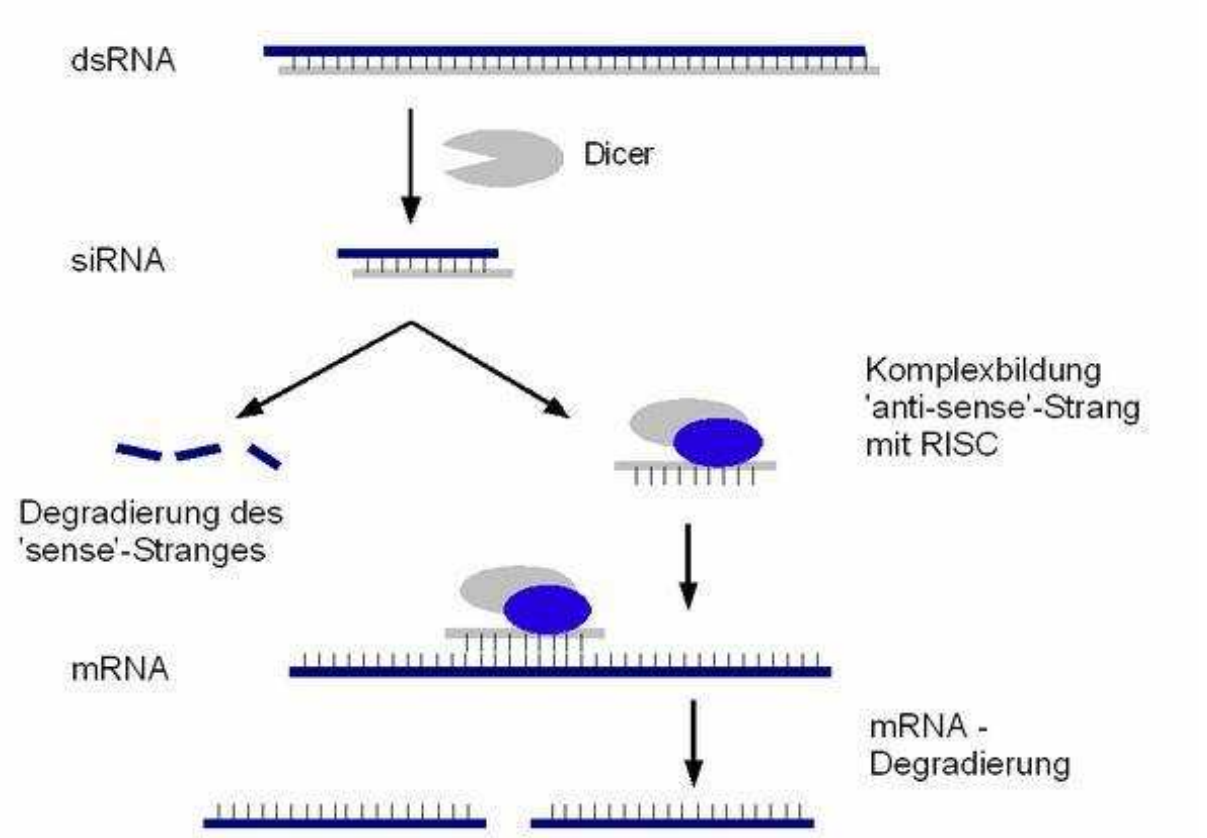

Abb.2: Die dsRNA wird durch den Proteinkomplex Dicer in siRNA geschnitten. Es kommt zur Degradierung des Sense-Stragnes und zur Bilung des RISC-Komplexes mit einem Antisense-Strand, so dass der RISC-Komplex an die mRNA komplementär binden kann, was zur Degradierung der mRNA führt.

2.1.9. Zielsetzung

Ziel dieser Arbeit ist die Etablierung und Charakterisierung der Marmoset Stammzelllinie cjes001. Außerdem sollen die Kultivierungsbedingungen im Hinblick auf den Erhalt der Pluripotenz und die Undifferenziertheit optimiert werden. Dazu sollten die unter den ESC als Kokultur kultivierten MEF auf ihren Einfluss bezüglich auf das Differenzierungsverhalten der ESC und im Vergleich zwischen unterschiedlichen Inaktivierungsmethoden untersucht werden.
Ein weiterer Teil ist die Manipulation der embryonalen Stammzellen mittels shRNA im Hinblick auf die Immunogenität.

2.2. Ergebnisse/Diskussion

Die in den beigefügten Artikeln gezeigten Ergebnisse sind im Folgenden zusammengefasst und erörtert.
Die Charakterisierung der embryonalen Stammzelllinie cjes001 war die Grundvoraussetzung für alle weiteren Arbeiten. Es wurde gezeigt, dass die Zelllinie pluripotent ist und alle Eigenschaften einer Stammzelllinie aufweist. Des Weiteren wurde der Einfluss der kokultivierten MEF auf die ESC bezüglich der optimalen Kulturbedingungen untersucht. Ferner wurde mittels RNAi die Immunogenität der ESC manipuliert. Der MHC konnte herunterreguliert werden und das Silencing durchflusszytometrisch sowie mittels Real Time PCR nachgewiesen werden.

Charakterisierung der embryonalen Stammzelllinie cjes001

Die Zelllinie cjes001 konnte positiv als embryonale Stammzelllinie charakterisiert werden. Die Expression der Pluripotenzmarker wurden positiv durch immunhistochemische Färbungen sowie durch PCR nachgewiesen. Die Zellen konnten differenziert werden und verschiedene Zelltypen konnten detektiert werden. Die für undifferenzierte Stammzellen typisch hohe Expression an reverser Transkriptase konnte ebenfalls ermittelt werden. Die Gesamtheit dieser Ergebnisse spricht für den Stammzellcharakter und die Pluripotenz der Zelllinie cjes001.

Die über 89 Passagen kultivierten embryonalen Stammzellen wiesen die typische Morpholgie auf. Sie zeigten klare Abgrenzungen der aus vielen hundert Zellen bestehenden Kolonien, die in sich homogen erscheinen (Sasaki et al. 2005). Die nach 64 Passagen durchgeführte Karyotypisierung zeigte einen normalen Karyotyp mit 64 Chomosomen, XX.
Die spezifischen Stammzellmarker für Pluripotenz Oct 3/4, Nanog und Sox-2 konnten alle in der Zelllinie cjes001 nachgewiesen werden. Diese Marker spielen eine große Rolle in der Embryonalentwicklung und sind hoch exprimiert (Boyer et al. 2005). Für diese Marker wurde zusätzlich die mRNA mittel RT PCR nachgewiesen. Außerdem konnten die Oberflächenmarker SSEA-3, SSEA-4, Tra-1-60 und Tra-1-81 immunhistochemisch nachgewiesen werden.

Durch Real Time PCR wurde eine fünffach stärkere Expression an mRNA der reversen Transkriptase nachgewiesen als es in Maus-Fibroblasten der Fall ist. Dieses Ergebnis spricht ebenfalls für den undifferenzierten Status der Zellen.
Embryonale Stammzellen lassen sich in andere Zellen differenzieren. Ihre Fähigkeit sich in alle drei Keimblätter (Mesoderm, Entoderm, Ektoderm) auszubilden, dient als Pluripotenznachweis. Die verwendeten Zellen cjes001 differenzierten spontan unter gegebenen Kulturbedingungen ungerichtet in Embryoid bodies. Es konnten verschiedene Zelltypen in den EB nachgewiesen werden. Außerdem konnten Zelltypen, hervorgegangen aus allen drei Keimblättern in den induzierten Teratomen nachgewiesen werden.

Einfluss der MEF auf ESC

Um die Kulturbedingungen für die embryonalen Stammzellen zu optimieren, wurde ein Vergleich zwischen verschiedenen Arten der Inaktivierung von MEF sowie zwischen verschiedenen verwendeten Mausstämmen durchgeführt. Zur Inaktivierung der MEF wird üblicherweise eine der folgenden zwei Methoden angewendet: Zum einen eine chemische Inaktivierung mittels Mitomycin C oder zum anderen Bestrahlung mit einer γ-Strahlenquelle. Es konnte gezeigt werden, dass die Methode der Inaktivierung eine Auswirkung auf die ESC hat. Es gibt signifikante Unterschiede zum einen zwischen den metabolischen Aktivitäten der verschieden behandelten Zellen als auch zwischen den sezernierten Faktoren der unterschiedlich inaktivierten MEF. Außerdem scheinen die ESC je nach Inaktivierungsmethode schneller zu differenzieren, was ein Einfluss der von den MEF sezernierten Faktoren zu sein scheint.

Grundsätzlich hat es den Anschein, dass es von der Zelllinie abhängig ist, welche Art der Inaktivierung für ein optimales Wachstum der Zellen zu wählen ist. Es wurde publiziert, dass bei der Kultivierung von B-LCLs die Form der Inaktivierung eine große Rolle spielt. So ist die Proliferation der B-LCL 100-fach höher, wenn γ-bestrahlte MEF im Gegensatz zu Mitomycin C behandelten MEF verwendet werden (Roy et al. 2001). Gegenteilig wurde aber auch gezeigt, dass die Methode der

Inaktivierung in anderen Fällen keinen Einfluss auf die Kultivierung der Zellen hat (Ponchio et al. 2000). Bei der Kultivierung der ESC scheint die Methode der Wahl die Inaktivierung mittels Bestrahlung zu sein.

Der MTT-Test zeigte signifikante Unterschiede zwischen den verschiedenen Inaktivierungsmethoden bei denen γ-bestrahlte MEF eine wesentlich höhere Aktivität aufwiesen als durch Mitomycin C chemisch inaktivierte. Alle behandelten Zellen wiesen eine geringere metabolische Aktivität auf als unbehandelte Zellen. Dies spiegelt den Stress wieder, den die Behandlung auf die Zellen ausübt. Die geringere Aktivität der chemisch inaktivierten Zellen spricht für eine größere Belastung als es die Bestrahlung zu sein scheint.
Für die Kultivierung von ESC auf MEF scheint eine hohe metabolische Aktivität der MEF von Vorteil. Die sezernierten Faktoren haben Einfluss auf den undifferenzierten Status der Zellen.
Mit dem *bead-based-multiplex-array* wurden die Unterschiede in der Sezernierung verschiedener Faktoren dargestellt. Mit Mitomycin C behandelte MEF und γ-bestrahlte MEF sonderten unterschiedliche Mengen an Stoffwechselprodukten ab. Diese sezernierten Faktoren scheinen einen großen Einfluss auf das Wachstum, die Proliferationsrate sowie die Pluripotenz der ESC zu haben.
Der *bead-based-multiplex-array* zeigte, dass einige Faktoren von Mitomycin C behandelten Zellen in größeren Konzentrationen, andere von bestrahlten MEF in höheren Konzentrationen abgegeben werden. Insgesamt zeigte sich, dass das Ergebnis des MTT-Test bestätigt wird und die Mitomycin C behandelten MEF eine geringere metabolische Aktivität aufweisen; die Konzentrationen an abgegebenen Faktoren im Medium waren deutlich geringer als bei den bestrahlten Zellen.
Standardmäßig werden in verschiedenen Laboren unterschiedliche Mausstämme für die Gewinnung von MEF genutzt. Die meistgenutzten Stämme sind CF1 und NMRI. Beide Stämme wurden ebenfalls mit den gleichen Untersuchungsmethoden, die schon bei dem Vergleich der Inaktivierungsmethoden genutzt wurden, untersucht. Es konnten von fast allen gemessenen Faktoren höhere Konzentrationen in den aus CF1 präparierten MEF gemessen werden. Da aber sowohl die Faktoren höher konzentriert sind, welche die Differenzierung beeinflussen als auch jene, die die Pluripotenz erhalten, lässt sich keine eindeutige Aussage über Vorteil des einen oder

anderen Mausstamms treffen. Es scheint, als wären beide Mausstämme gleich gut geeignet, um MEF für die Kultivierung von ESC zu gewinnen.
Embryonale Stammzellen werden gewöhnlich zwischen 5 und 7 Tagen auf den gleichen MEF kultiviert. Um eine Aussage über den Einfluss der MEF auf die ESC im Bezug auf die Zeit zu treffen, wurden über 24 Stunden verteilt Proben genommen und bezüglich ihrer metabolische Aktivität anhand der sezernierten Faktoren beurteilt. Es konnte dabei kein Unterschied zwischen bestrahlten und Mitomycin C behandelten MEF festgestellt werden. Bei beiden Inaktivierungsmethoden wurden die Faktoren gleichmäßig stark in das Medium abgegeben. Somit haben beide Inaktivierungsmethoden im Zeitverlauf den gleichen Einfluss auf die ESC. Da die Anfangskonzentrationen aber unterschiedlich waren, müssen die Ergebnisse von den anderen Tests mit in die Überlegung welche Art der Inaktivierung man wählt mit einbezogen werden.
Morphologisch betrachtet scheint die Methode der Inaktivierung einen Einfluss auf die ESC zu haben. Bestimmt man die Proliferationsrate, so ist eine geringere Anzahl Stammzellkolonien auf den mit Mitomycin C inaktivierten MEF zu finden. Es wurden zu Beginn des Versuchs gleiche Zellzahlen ausgesät. Außerdem sind auf den bestrahlten MEF weniger differenzierte oder andifferenzierte Kolonien zu finden. Die höheren Konzentrationen sezernierter Faktoren von den bestrahlten MEF scheinen also die Pluripotenz der Stammzellen besser erhalten zu können.

Immunogenität

Die Manipulation der Immunogenität der embryonalen Stammzelllinie cjes001 konnte erfolgreich durchgeführt werden. Dieser Ansatz bietet gute Möglichkeiten die therapeutische Behandlung mit ESC zu vereinfachen. Werden körperfremde Zellen transplantiert, werden über den MHC diese Zellen als solche erkannt und eliminiert. Ist der MHC herunterreguliert, können Zellen mit einem geringen Risiko der Abstoßung transplantiert werden. Denkbar wäre eine Herunterregulierung des MHC in embryonalen Stammzellen, die dann gezielt in die benötigte Richtung differenziert werden können.

Die Regulation des MHC wurde mittels RNA-Interferenz durchgeführt. Es wurden fünf verschiedene sequenzspezifische 19-21 Basenpaar lange Fragmente entworfen, die komplementär zur beta-2-mikroglobulin Region des Marmoset MHC sind. Diese sogenannten shRNAs wurden mit lentiviralen Vektoren in die Zielzellen eingebracht. Dort binden sie spezifisch an die Ziel-RNA und hemmen so die Expression des Gens. Die erfolgreiche Transduktion der Ziel-Zellen konnte durchflusszytometrisch gezeigt werden, da der lentivirale Vektor GFP markiert wurde. Es konnten Transduktionsraten von bis zu 48% erreicht werden. Die Herunterregulierung des MHC wurde ebenfalls durchflusszytometrisch als auch auf mRNA Ebene mittels Real Time PCR bestimmt.

Die Methode wurde bereits erfolgreich an humanen Zelllinien durchgeführt (Figueiredo et al. 2006). Es wurden lentivirale Vektoren, die eine shRNA gerichtet gegen das humane ß2m auf dem MHC exprimieren, eingesetzt.

Um die Methode zu etablieren und die Effektivität der fünf verschiedenen shRNAs beurteilen zu können wurde der Versuch zunächst an Fibroblasten durchgeführt, da diese im Allgemeinen leicht zu transduzieren sind. Es konnte gezeigt werden, dass es mit allen fünf verschiedenen Konstrukten möglich ist Marmoset Zellen zu transduzieren und den MHC herunterzuregulieren. Im durchflusszytomertrischen Nachweis auf Proteinebene gelang eine Verminderung der Expression um bis zu 85%. Auf mRNA Ebene ermittelt durch Real Time PCR, konnte eine Herunterregulierung um bis zu 50% nachgewiesen werden. Die Herunterregulierung des MHC in Marmoset ESC konnte mit einer supprimierten MHC Expression in einer Real Time PCR von bis zu 35% gezeigt werden. Die Ergebnisse zeigen, dass eine Herunterregulierung des MHC in embryonalen Stammzellen nicht humaner Primaten möglich ist.

Schlussfolgerung/Perspektiven

In dieser Arbeit wurde die embryonale Stammzelllinie cjes001 charakterisiert. Die Pluripotenz konnte mit verschiedenen Markern nachgewiesen werden, ebenso die Möglichkeit der Differenzierung in verschiedene Zelltypen, z.B. Keimzellen.
Des Weiteren wurde der Einfluss der Inaktivierungsmethode der MEF auf die ESC mit dem Ergebnis untersucht, dass für die embryonale Stammzelllinie cjes001 die Inaktivierung der MEF durch Bestrahlung effektiver bezüglich des Erhaltes der Pluripotenz ist. Um den undifferenzierten Status der Stammzellen erhalten zu können, scheinen hohe Konzentrationen an LIF und bFGF von Vorteil, wie sie im Überstand von bestrahlten MEF in höheren Konzentrationen als bei chemisch inaktivierten MEF zu finden sind. Bestrahlte MEF sezernieren aber auch einige Faktoren, die die Differenzierung beeinflussen, wie z.B. VEGF, in größeren Mengen als chemisch inaktiverte MEF. Sieht man alle Faktoren und durchgeführten Versuche zusammen, scheint die Bestrahlung im Gegensatz zu der chemischen Inaktivierung durch Mitomycin C die bessere Inaktivierungsmethode für die Stammzelllinie cjes001 zu sein.
Durch shRNA vermittelte Herunterregulierung des MHC konnte die Expression von MHC Klasse I in Fibroblasten und ESC des Marmoset verringert werden. Der Silencing-Effekt konnte durch die gezielte Manipulation der ß2m-Region des MHC erzielt werden und betrug je nach verwendeter Sequenz bis zu 85%.
Zusammenfassend lässt sich sagen, dass die embryonale Stammzelllinie cjes001 detailliert charakterisiert worden ist. Die Kulturbedingungen im Bezug auf den Einfluss der MEF auf die Stammzelleigenschaften der ESC wurden eingehend untersucht. Die Zellen konnten erfolgreich in Bezug auf ihre MHC-Expression manipuliert werden.
Die im Rahmen der Promotion gesetzten Ziele konnten vollständig erreicht werden. Die Zelllinie wurde charakterisiert und es konnten Erkenntnisse über die Optimierung der Kulturbedingungen und den Einfluss der kokultivierten MEF gewonnen werden. Zudem konnte gezeigt werden, dass eine Herunterregulierung des MHC in Marmoset Zellen möglich ist.
Weiterführend soll nun eine stabile Herunterregulierung des MHC in Marmoset ESC erreicht werden. Außerdem ist es für therapeutische Anwendungen unabdingbar Einzelzellklone herunterregulierter ESC zu kultivieren, da nur so gewährleistet

werden kann, dass alle Zellen der Population keine MHC-Expression mehr aufweisen. Diese Einzelzellklone sollen gezielt in hämatopoetische Zellen differenziert werden. Ein Transplantationsversuch in Marmosets wäre anschließend vorstellbar.

3. Veröffentlichungen

3.1. A novel stem cell line derived from the common marmoset monkey (*Callithrix jacchus*) exhibiting germ cell-like characteristic

Title

A novel stem cell line derived from the common marmoset monkey (*Callithrix jacchus*) exhibiting germ cell-like characteristics

Author

Müller T, Fleischmann G, Eildermann K, Mätz-Rensing K, Horn PA, Sasaki E, Behr R

In Review at

Human Reprouction, 2008

Kommentar: Zum Zeitpunkt der Veröffentlichung der Doktorarbeit akzeptiert in Human Reproduction, 27.02.2009, http://humrep.oxfordjournals.org/cgi/content/full/dep012v1

A novel stem cell line derived from the common marmoset monkey (Callithrix jacchus) exhibiting germ cell-like characteristics

Journal:	*Human Reproduction*
Manuscript ID:	draft
Manuscript Type:	Original Articles
Date Submitted by the Author:	n/a
Complete List of Authors:	MÃ¼ller, Thomas; German Primate Center, Stem Cell Research Group Fleischmann, Gesine; Hannover Medical School, Institute for Transfusion Medicine Eildermann, Katja; German Primate Center, Stem Cell Research Group MÃ¤tz-Rensing, Kerstin; German Primate Center, Department of Infectious Pathology Horn, Peter; Hannover Medical School, Institute for Transfusion Medicine Sasaki, Erika; Central Institute for Experimental Animals, Laboratory of Applied Developmental Biology, Marmoset Research Department Behr, Rüdiger; German Primate Center, Stem Cell Research Group
Keywords:	ANIMAL MODEL, EMBRYOLOGY, GERM CELLS, STEM CELLS
Specialty:	Embryology

A novel stem cell line derived from the common marmoset monkey (*Callithrix jacchus*) exhibiting germ cell-like characteristics

Thomas Müller[1], Gesine Fleischmann[2], Katja Eildermann[1], Kerstin Mätz-Rensing [3], Peter A. Horn[2], Erika Sasaki[4], Rüdiger Behr[1]

[1] Stem Cell Research Group, German Primate Center, Kellnerweg 4, 37077 Göttingen, Germany

[2] Institute for Transfusion Medicine, Hannover Medical School, 30625 Hannover, Germany

[3] Department of Infectious Pathology, German Primate Center, Kellnerweg 4, 37077 Göttingen,

[4] Laboratory of Applied Developmental Biology, Marmoset Research Department, Central Institute for Experimental Animals, Kawasaki, Japan

Correspondence should be addressed to:

Dr. Rüdiger Behr
German Primate Center
Stem Cell Research Group
Kellnerweg 4
37077Goettingen
Germany

Phone: ++49 - 551 3851 132
Fax: ++49 - 551 3851 288
Email: rbehr@dpz.eu

Request for cells should be addressed to Dr. Erika Sasaki.

Key words: Embryonic stem cell, common marmoset, germ cell, non-human primate, pluripotency

Abstract

Background

Embryonic stem cells (ESC) hold great promise for the treatment of degenerative diseases. However, before clinical application of ESC in cell replacement therapy can be achieved, the safety, performance and feasibility has to be tested in animal models with numerous independent ES cell lines. The common marmoset monkey (*Callithrix jacchus*) is readily used as a preclinical non-human primate model due to its physiological similarities to man. Yet, only a very limited number of marmoset ESC lines exist and potential differences in their developmental potential remain dubious.

Methods

Blastocyst collection and immunosurgery was performed as described (Sasaki et al., 2005). Cjes001 cells were tested for euploidy by karyotyping. Presence of markers for pluripotency and differentiation was tested by immunofluorescence staining, histology of teratoma, embryoid body formation, RT-PCR and real time PCR.

Results

The cjes001 cells displayed a normal 46, XX karyotype. Strong telomerase and alkaline phosphatase activity was confirmed. The cells express the ESC transcription factors OCT4, NANOG and SOX-2. Glycan surface markers exhibited the typical primate ESC signature with presence of SSEA-3, -4, TRA-1-60, and TRA-1-81. Teratoma formation assay displayed derivatives of all three embryonic germ layers. Upon non-directed differentiation the cells exhibited strong germ cell-specific gene expression of *VASA, BOULE, GCNF*, and the meiosis marker *SCP3*.

Conclusions

The cjes001 cells represent a new ESC line which accomplishes all criteria of a pluripotent ESC line with evidence for enhanced spontaneous differentiation potential into germ cells. This cjes001 line will be very valuable for comparative studies on primate ESC biology.

Introduction

Cell replacement therapy using pluripotent or multipotent stem cells holds great promise for regenerative treatment of a vast number of degenerative diseases. However, it is still disputed if embryonic stem cells (ESC), somatic stem cells (SSC) or recently described induced pluripotent stem (iPS) cells are best suited for cell replacement studies in preclinical and possible future clinical applications (Meissner et al., 2007; Okita et al., 2007; Wernig et al., 2007; Yu and Silva, 2008). This open issue needs extensive further investigation with all cell types being considered as sources for cell replacement therapies. Therefore, the safety and potential of all respective cell types have to be tested in preclinically relevant animal models.

Mouse embryonic stem cells differ from primate and human ES cells with respect to cell culture requirements, morphology, physiology and gene expression, reflecting significant differences between mouse and primate embryogenesis and general organismic physiology (Fougerousse et al., 2000; Ginis et al., 2004; Turnpenny et al., 2006). Therefore, it is of fundamental importance to study primate ESC. But, beside ethical concerns as well as legal limitations in several countries using human ES cells, further doubts are related to the clinical safety of the potential therapies using pluripotent stem cells (Stojkovic et al., 2004). It has to be ensured that transplanted cells are not tumorigenic and that cell replacement therapy does not cause other harmful long-term side effects. Hence, although previous results from mouse ESC have provided invaluable insight in stem cell biology, the potential of clinical stem cell applications in humans must be pioneered in nonhuman primate species (Wolf et al., 2004) like the macaques and marmoset monkey.

The common marmoset monkey (*Callithrix jacchus*) is readily available as a non-human primate animal model that exhibits many physiological similarities to humans (Eslamboli, 2005; Mansfield, 2003; Michel and Mahouy, 1990; Zuhlke and Weinbauer, 2003). However,

only a few embryonic stem cell lines of this species exist to date. More than ten years ago Thomson et al. (1996) already created eight ES cell lines from the common marmoset (Thomson et al., 1996). But, these lines are no longer available (J. Thomson, personal communication). We recently continued research with marmoset ESC by creating and characterizing the lines CMES20, CMES40 and CMES52 (Sasaki et al., 2005). Nevertheless, the more ES cell lines from one species are available the better and the more profound the knowledge about a certain stem cell type from a certain species will be. For instance, human and non-human primate ESC lines diverge in their karyotype, gene expression and differentiation potential (Chen et al., 2008; Heins et al., 2004). To have a broad collection of ESC lines isolated from embryos at different developmental stages and sex on-hand will help to investigate the spectrum of intra- and inter-cell line-specific varieties and epigenetic stability.

In this work, we established and characterized a fourth marmoset ESC line (cjes001) that could be cultivated successfully for over 24 months (P84). Besides its potential to develop into somatic cell types it also revealed strong potential to develop into germ cells upon spontaneous differentiation.

Material and Methods

Recovery of blastocysts and initial culture

All procedures were carried out according to German and Japanese Animal Experimentation Law and all animal experiments in Japan were approved by the institutional animal care and use committee, and were performed in accordance with institutional guidelines..Animals were housed according to standard German and Japanese Primate Centre practice for the common marmoset. The method of blastocyst recovery was described before in detail (Sasaki et al., 2005). Briefly, marmoset preimplantation embryos were recovered from female adult marmosets kept in the marmoset colony at the CIEA (Kawasaki, Japan) 8 days after putative ovulation (10.7 ± 1.3 days after Prostaglandin F2α administration) by uterus-flush. Fifteen immunosurgically isolated inner cell masses (ICM) were plated on an irradiated MEF feeder layer, 3 ICMs were cultured for more than 10 passages and, finally, the cjes001 line resulted from one ICM. All ICM-derived cells showed flat, packed, and tight colony morphology and a high nucleus : cytoplasm ratio corresponding to the morphology reported for other primate ESCs, including humans, rhesus and cynomolgus monkeys. Cjes001 were cultured as described (Sasaki et al., 2005).

Immunosurgery and maintenance of ESC

The immunosurgery, isolation and culture of ESC lines was performed as described in detail before (Sasaki et al., 2005). The zona pellucida of the marmoset blastocyst was removed by digestion in 0.1% pronase in PBS. To remove the trophoblast, the blastocysts were first incubated for 45 minutes at 37°C in 5% CO_2 with a 10-fold dilution of anti-marmoset fibroblast rabbit serum in DMEM. After three washes with DMEM, the blastocysts were incubated with a fivefold dilution of guinea pig complement (Invitrogen) in DMEM for 30 minutes at 37°C in 5% CO_2. Then, the trophoblast was mechanically removed from the ICM

by pipetting the incubated blastocyst up and down. Then the ICM was plated on 3,500-rad γ-irradiated mouse embryonic fibroblast (MEF) feeder layer. First passaging of the ICMs was performed after 10–14 days by physical removal of the ICM colony and dissociation by vigorously pipetting. The medium used in the ESC culture comprised 80% Knockout DMEM (Invitrogen) supplemented with 20% Knockout Serum Replacement (KSR; Invitrogen), 1 mM L-glutamine, 0.1 mM MEM nonessential amino acids, 0.1 mM ß-mercaptoethanol (2-ME; Sigma), 100 IU/ml penicillin, 100 µg/ml streptomycin sulfate, 250 ng/ml amphotericin B, and 10 ng/ml leukemia inhibitory factor. For passaging, ESC colonies were treated with trypsin-EDTA (0.25% trypsin,1 mM $CaCl_2$, 20% KSR in DMEM) to remove ESC from feeder layer, mechanically dissociated into clumps of 10 to 50 cells and replated on a new irradiated MEF feeder layer. The cjes001 line was maintained up to the present under these culture conditions for 24 months (P84).

Immunofluorescence staining

The cjes001 colonies were grown on gamma-irradiated mouse embryonic feeder cells (MEFs) in foil-coated 24-well plates (Greiner-Biopro, Stuttgart, Germany) for 2-4 days, fixed with acetone (30 sec) and washed 2 x with 1 x PBS. The staining with primary antibodies was done according to the manufacturer's recommendations. A complete list of all primary and secondary antibodies used in this study is provided in Table 1. After 60 min the cells were washed twice with PBS, incubated for another 60 min with the respective secondary antibody covalently linked to Alexa dye A488 or A568. Images were taken on a Zeiss Axio Observer Z1 and a Zeiss LSM 510 confocal microscope, respectively. Counterstaining reagents were propidium iodine (1:10000, 5 min) or Hoechst 33258 (Sigma-Aldrich).

Alkaline phosphatase staining

For alkaline phosphatase staining, the Alkaline Phosphatase staining kit (Dako Universal LSAB Kit, K0679 HRP) was used according to the manufacturer's instructions. For histochemistry, cells were fixed with 4% paraformaldehyde for 30 sec, washed 2x with PBS and incubated with 0.3 % H_2O_2 for 30 min at room temperature.

Histology of teratoma

Teratoma were fixed in Bouin's solution (0.9 % picrinic acid, 9.6% formaldehyde, 4.8 % acetic acid) for 5h, then transferred into 70% - to 96% EtOH until the tissue was dehydrated. After incubation in Xylene and embedding in paraffin the tissue was sectioned at 5 µm and hematoxylin / eosin stained.

Karyotypic analysis

Confluent ESC colonies were incubated for 4h in ESC medium containing 0.02µg/ml Colcemid (Invitrogen GmbH, Karlsruhe, Germany), then washed once with 1xPBS and trypsinized (15 min, 37C). After detaching the colonies were centrifuged (200xg, 10 min) and the pellet was resuspended in 3 ml of prewarmed (37°C) 8 mM KCl / 15 mM sodium citrate solution for 25 min at room temperature. After centrifugation (200xg, 10 min) 2 ml of the KCl/sodium citrate solution were removed and 4 ml icecold MetOH/acetic acid (3:1) slowly added to the vial. After 5 min incubation at room temperature the cells were pelleted again (200xg, 10 min), 4.5 ml of supernatant removed, the remaining liquid carefully dropped on glass cover slips and dried over night. For Giemsa staining (Sigma, 0.4% (w/v) in buffered methanol solution, pH 6.8) the dye was added for 2 min on the coverslip, then washed 10 times with aq. bidest and dried again. For chromosome analysis, the cells were incubated after fixation on the coverslips for 5 min in McIlvaine-buffer (pH4.6) including 0.01 µg /ml fluorescence dye (Hoechst 33258, Sigma), then washed 10 times with aq. bidest. McIlvaine-buffer containing 5µg/ml Quinacrine mustard (Sigma Q2876) was added for 20 min. After the

second staining, the cells were washed again 10 times with aq. bidest, incubated for 5 min in McIlvaine-buffer again and embedded in mounting media (Citifluor Ltd, London, UK). The chromosome analysis was performed with a Leica CW 4000 system with a modified chromosome template based on data from Sherlock and colleagues (Sherlock et al., 1996).

Telomerase detection

Cjes001 telomerase activity was determined by Biomax Telomerase detection kit (Biomax Inc., Ijamsville, MD, USA) according to the manufacturer's references. Briefly, $1x10^6$ CJES01 cells were lysed and the cell extract was added to a quantitative telomerase determination pre-mix in a real time PCR reaction utilizing SYBR green for 37 cycles. As controls served MEFs, immortal green monkey kidney cells (COS7) and SYBR green exclusive of cell extract.

Reverse Transcription (RT-) PCR

RNA from cjes001 or teratoma was isolated by RNeasy kit (Qiagen, Hilden, Germany) according to manufacturer's recommendation. First strand cDNA was synthesized with Omniscipt RT Polymerase (Quiagen) and cDNA was amplified in 35 cycles (denaturation 95 C 1 min / annealing 60° C 30 sec / elongation 72° C 60 sec, respectively) with 2.5 U BiothermStar TAQ Polymerase (Genecraft, Luedinghausen, Germany) in PCR reaction buffer (160 mM $(NH_4)2SO_4$, 670 mM Tris-HCl, pH 8.8, 15 mM $MgCl_2$, 0.1% Tween 20), 0.2 mM dNTP, 0.5 mM of each primer. cDNA from mouse embryonic feeder cells and mock reverse transcription without RT provided negative controls. A complete list of oligonucleotides used in this study provides Table 2. If marmoset DNA sequences were unavailable, the expected sizes of the PCR products were deduced from alignments of the homologous human and mouse sequences. Selected RT-PCR products were verified by DNA sequencing (data not

shown). Normal monkey tissues exhibiting considerable expression of the respective genes served as positive controls.

Embryoid body formation

To study EB formation, undifferentiated ESC were removed from the MEF layer, dissociated using 0.25% trypsin in PBS with 20% KSR and 1 mM $CaCl_2$, and cultured in hanging drop cultures for 14 – 20 days in DMEM (10% FBS) with a medium change every 3 days. The forming EBs were frozen in OCT Compound (Tissue-Tek, Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands) for cryosections or used in parallel for RT-PCR.

In vitro differentiation

To spontaneously differentiate the cjes001 cells, the colonies were removed from the MEF layer, dissociated using 0.25% trypsin in PBS with 20% KSR and 1 mM $CaCl_2$, and cultured on gelatine-coated petri dishes (Nunc) in MEF-medium. After 7 to 10 days the cells had lost their characteristic undifferentiated morphology and were collected for RT-PCR.

In vivo differentiation analysis

Eight weeks after subcutaneous injection of 1-3x10^6 cjes001 cells tumor formation could be observed in NOD/SCID mice. The tumors were resected from the mice, fixed in bouin's fixative or snap frozen in liquid nitrogen for RNA analysis. Histological and RT-PCR analysis were performed as described above. As positive control for teratoma formation murine ESC were utilized, for negative control PBS was injected.

Western blot analysis

Western blot analysis was performed as described before (Quintana et al., 1993). Briefly, ~ 50 mg of testis tissue or stem cell culture material was mechanically homogenized (3x 30 sec) in

2.5ml IMP buffer (0.15M NaCl, 20mM HEPES, 1mM EDTA and a protease inhibitor cocktail 1:10 (Sigma #P8340), at pH 7.4) using a tissue homogenator, centrifuged (10 min, 3200rpm, 4C) and resuspended in 2.5 ml lysis buffer (IMP buffer + 0.5% Nonidet P40). The protein content was determined by BCA protein assay kit (Novagen #71285-3) and equal amounts of protein per lane were loaded onto a SDS gel. A protein marker (Novex sharp prestained protein standard, Invitrogen) served as size standard. After the run the gel content was transferred to a PVDF membrane (Amersham Hybond-P) in a electrophoresis chamber (Roth, 100V, 1.2h, 300mA). The membrane has been washed 3x5min in PBS, incubated 1h in blocking solution and stained with the primary antibody (1:500) over night (4C). After 3x5 min wash in PBS the secondary, HRP conjugated antibody (1:10000) was added for 1h and again washed 2x15min. The detection was carried out with ECL Kit (Amersham #RPN2209) in an Ecomaxx x-ray Film developer.

Results

Cjes001 show typical ESC Morphology and a normal Karyotype

Out of 15 inner cell masses initially cultured, the cjes001 line could be established. This line conserved its typical ESC morphology (Figure 1A, (Sasaki et al., 2005)) and marker expression for 24 months (84 passages) and remained positive for Alkaline Phosphatase (Figure 1B). The doubling time of cjes001 monitored by BrdU was roughly 19 hours (data not shown). Karyotyping analysis after 64 passages showed a regular 46, XX chromosome set (Figure 2).

Cjes001 express ES cell marker molecules

Immunofluorescence staining revealed the expression of the transcription factors OCT4, NANOG and SOX2 (Figure 3), which serve as markers for pluripotency of ES cells (Figure

3A-I) (Boyer et al., 2005). Upon differentiation of the colonies the expression of these markers vanishes (data not shown). For *OCT4*, *NANOG*, and *SOX2* we have demonstrated this also at the mRNA level (Figure 4). The pluripotent cell surface antigens (Lanctot et al., 2007) stage-specific embryonic antigen SSEA-3 and SSEA-4 (Figure 5A-I) and keratan sulfate antigens TRA-1-60 and TRA-1-81 (Figure 6A-F) were also strongly expressed by undifferentiated ES cells. Quantitative real time PCR analysis showed high levels of telomerase reverse transcriptase mRNA. The detected levels were 5-fold higher than in highly proliferative embryonic mouse fibroblasts and approximately twice as high as in the SV40 virus-mediated immortalized monkey control cell lines COS7 (Figure 7).

Cjes001 cells can form different types of embryonic bodies and teratoma

As shown in Figure 8, cjes001 cells can form cystic as well as compact types of embryonic bodies. The cystic bodies developed to a size of ~1.000 µm in diameter. Semi-thin sections (data not shown) showed that the wall of the cyst consisted of a flattened epithelium, whose apical surface was oriented to the lumen of the cyst. The outer cells of the wall of the cyst had mainly mesenchymal appearance. The compact types of embryoid bodies consisted of mesenchymal cells and primitive epithelia (data not shown). Embryoid body formation showed a striking cellular differentiation potential typical of pluripotent cells even after one week of differentiation. Beyond in vitro differentiation in EBs we also tested the pluripotency of the ES cells in vivo by teratoma formation in NOD /SCID mice (Figure 9 A-D). This assay allowed prolonged differentiation of the cjes001 cells. Histological sections of the subcutaneously developed encapsulated tumor exhibited fully differentiated tissues of different embryonic origins. Adenomatous and columnar epithelia, mesenchyme, neuroglia, chondrocytes and bone including bone marrow besides other cell types developed within the teratoma (Figure 9 A-E). Differentiation into derivatives of all three embryonic germ layers

was also confirmed by the detection of α-Fetoprotein (endoderm), Brachyury (mesoderm) and β-III Tubulin (ectoderm) by RT-PCR (Fig. 9 F).

Evidence for germ cell specification in differentiating cjes001 cells

Interestingly, concurrently with the downregulation of *OCT4, SOX2*, and *NANOG* during spontaneous ESC differentiation (Figure 4) specific marker mRNAs for germ line / germ cell development (*VASA, SCP3, GCNF*) were up-regulated (Figure 10A). Furthermore, the germ cell marker *BOULE* was expressed at high levels already in those cells defined as undifferentiated and its expression was maintained in differentiated cells, which also express the above mentioned germ cell markers (Figure 10A). To further corroborate the development of germ line cells we attempted to localise VASA protein in embryoid bodies. Immunofluorescence staining demonstrated a distinct cytoplasmic signal for the germ cell marker VASA (Castrillon et al., 2000) in clusters of cells of the embryoid bodies (Figure 10B). The absence of OCT4 from these embryoid bodies was accompanied by sporadic expression of caudal type homeobox transcription factor 2 (CDX2) (data not shown), which is a marker for trophoblast cells (Strumpf et al., 2005). To ensure that the VASA antibody detects a protein of the correct size (72 kDa) also in the marmoset, we performed western blot analysis with protein from a marmoset testis (Figure 10 C, left lane). In addition to the testis, we also obtained a specific and robust western blot signal for VASA with differentiated cjes001 cells, further confirming germ line differentiation in cultures of these marmoset ESCs. In control mouse feeder cells (which support the growth of cjes001 cells) we detected only a faint VASA signal (~25 % of the signal intensity of the middle lane, normalised to β-ACTIN) most likely originating from mouse primordial germ cells present in the feeder cell preparation.

Discussion

Non-human primate embryonic stem cells are an attractive tool to study aspects early embryonic development (Behr et al., 2005; Rodda et al., 2002) and carry great hope for regenerative medicine (Murry and Keller, 2008). Creating new monkey ESC lines for characterization purposes in vitro and in vivo is an important step to guarantee the safety, performance and reproducibility of anticipated medical procedures prior to clinical trials. A wide range of different lines at hand will help to mimic epigenetic scatter, because human and non-human primate ESC lines diverge in karyotype (Thomson et al., 1996), gene expression and differentiation potential (Chen et al., 2008; Dighe et al., 2008; Heins et al., 2004). In this study we established and characterized a novel ESC line from the common marmoset monkey, named cjes001. As standards for successful establishment we judged morphology and utilized a panel of molecular signatures including transcription factors, surface antigens, lineage-specific gene expression and enzyme activity. Long term cultivation up to passage 84 with normal karyotype demonstrates the reliability of culture conditions, media composition and MEF density.

The morphology of cjes001 matched with those of other undifferentiated primate ESC colonies in other reports (Sasaki et al., 2005; Thomson et al., 1996) namely the distinct colony boundaries and the high nucleus : cytoplasm ratio with prominent nucleoli. However, we never observed any ES cell colony resembling morphologically an early embryo consisting of regularly structured tissues in terms of embryonic germ layer formation as it was described previously by Thomson (Thomson et al., 1996). The strong histochemical staining for alkaline phosphatase is also characteristic of undifferentiated ESC. The surface antigen composition with strong expression of SSEA-3, and -4 and keratan sulfate antigens TRA1-60 and TRA1-81 matched with other reports of undifferentiated non-human primate and human ESCs and

human iPS cells (Sasaki et al., 2005; Takahashi et al., 2007; Thomson et al., 1998; Thomson et al., 1995; Thomson et al., 1996). SSEA-1 was not detected which is primarily present in rodent ES cells (Lanctot et al., 2007). As for transcription factors, the strong presence of OCT4, NANOG and SOX2 indicates the pluripotency of ESC, which is also reflected by the enhanced levels of telomerase mRNA detected by real time PCR at P64. Interestingly, NANOG staining was located in this ES cell line mainly at the cell surface associated with the cell membrane and not, as expected from previous studies in other species, in the nucleus. This result was repeatedly obtained with 3 different antibodies. Recent studies reported a complex regulation of NANOG expression and function (Chambers et al., 2007; Fujita et al., 2008). Therefore, we speculate that the regulation and possibly also the function of NANOG might be regulated in marmoset ESC by subcellular localisation. However, interestingly, a recent report on the stem cell identity of testicular germ cell tumor cells also showed clear cytoplasmic localisation of human NANOG in a substantial portion of germ cell tumor cells (Clark, 2007). Nevertheless, future studies on marmoset NANOG will reveal potential differences between the marmoset and other mammalian NANOG proteins.

All pluripotency transcription factors tested were significantly down-regulated upon ES cell differentiation; other differentiation-specific genes were switched on like *CD34* for hematopoietic progenitors, *NESTIN* for neuronal progenitors, as well as *FOXD3* (data not shown). This forkhead transcription factor is required in the mouse for the establishment of the epiblast from the inner cell mass and is hence also a factor representing differentiation (Hanna et al., 2002). Subcutaneous injection of cjes001 cells into immuno-deficient mice resulted in tumors expressing marker mRNAs representing all three embryonic germ layers (β3 tubulin for differentiated neural cells (ectoderm), Brachyury for mesoderm and α-fetoprotein for endoderm). Also, histological evaluation of the teratoma revealed tissues indicative of a tumor derived from pluripotent cells such as chondrocytes, bone tissue, bone

359 marrow, mesenchyme, muscle, nerves and epithelia. Altogether, the data show that this novel
360 marmoset ESC line can form teratoma and, thus, is pluripotent.
361
362 Interestingly, RT-PCR analysis of differentiated ES cells revealed the presence of genes
363 considered specific for germ cells. VASA can be detected in vivo in migrating and
364 postmigratory primordial germ cells as well as in gonocytes in the fetal testis and in
365 premeiotic, meiotic and postmeiotic testicular germ cells (Castrillon et al., 2000). In contrast,
366 in undifferentiated human ESC *VASA* mRNA and protein could not be detected (Clark et al.,
367 2004), thus, being presently the best and most reliable marker for germ cell development in
368 cultures of pluripotent cells. Since we also have not detected *VASA* mRNA in undifferentiated
369 ESC but strongly in differentiated ones, we have shown that germ cells spontaneously
370 develop in cultures of cjes001 cells. To substantiate this finding we have also demonstrated
371 the presence of VASA protein in differentiated ESC by immunofluorescence and Western
372 blot analysis. Immunofluorescence of embryoid bodies showed that VASA-positive cells are
373 organized in clusters. This is in agreement with the previous report on VASA expression in
374 embryoid bodies derived from human ESC (Clark et al., 2004). To provide further evidence
375 for spontaneous germ cell development from cjes001 we also proved the expression of the
376 germ cell marker mRNAs synaptonemal complex protein 3 (*SCP3*), *BOULE*, and germ cell
377 nuclear factor (*GCNF*). SCP3 is a specific structural component of the meiotic synaptonemal
378 complex and is essential for male fertility and for proper oogenesis in mice and serves as an
379 excellent marker for meiotic germ cells (Di Carlo et al., 2000; Yuan et al., 2000). Expression
380 of *SCP3* strongly indicates the presence of germ cells in early meiotic stages in spontaneously
381 differentiating marmoset ES cells. DAZ, DAZL, and BOULE are germ cell-specific RNA-
382 binding proteins essential for gametogenesis in several species (Xu et al., 2001). While DAZ
383 is lacking in the marmoset (Gromoll et al., 1999), DAZL (Deleted in Azoospermia-like) and
384 its ancestral pendant BOULE are expressed in the common marmoset testis in late

spermatocytes / early spermatids and in early meiotic germ cells, respectively (Gromoll et al., 1999; Wistuba et al., 2006). In the present study, *BOULE* mRNA is expressed in undifferentiated as well as in differentiated marmoset ES cells. Since it is unknown whether *BOULE* is also expressed in earlier developmental germ cell stages in the marmoset we can only speculate that *BOULE* expression in rather undifferentiated ESC reflects early stages of germ cell develoment in this peculiar species. Currently we have no solid explanation for the absence of *DAZL* from the ESC. Since DAZL is, at least in human fetal germ cells, expressed in both sexes, even the female karyotype of our line can not serve as an explanation for this. Possibly this finding simply reflects that the germ cells differentiating in ESC cultures in the absence of their natural environment are not totally in concordance with their natural counterparts. However, in addition to *VASA*, *SCP3*, and *BOULE*, we also detected germ cell nuclear factor (*GCNF*) at significant levels in differentiating cells, while this mRNA was almost absent from undifferentiated cells. Although *GCNF* is not absolutely germ cell-specific (Chung and Cooney, 2001), these data altogether show that the cjes001 ES cells can not only differentiate into cell types representing the three embryonic germ layers but also into germ line cells further strengthening the view that these cells are indeed pluripotent. Moreover, this culture system will allow interesting studies on the developmental control points distinguishing somatic differentiation from germ line maintenance. Future studies will also reveal the potential to develop postmeiotic gametes from these marmoset ES cells applying directed differentiation protocols as already established for mouse ES cells (Geijsen et al., 2004; Hubner et al., 2003).

In conclusion, we have established and characterized a novel primate ES cell line from the common marmoset which exhibits beside its potential do develop into many different somatic lineages also the capacity to spontaneously develop into germ cells.

Acknowledgements

We thank Reiko Hirakawa, Ayako Sugawara, Marion Niebeling and Kirsten Elger for excellent technical assistance. These studies were supported by an inhouse grant of the German Primate Center and the DAAD German-Japanese Exchange program (D/06/19948) as well as the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) for the Cluster of Excellence REBIRTH (From Regenerative Biology to Reconstructive Therapy).. A part of this study was supported by funds from Technology Agency tand from Special Coordination Funds for Promoting Science and Technology of Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government to E.S..

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Legends to Figures

Fig.1:

A) Light microscopy shows tightly packed cell colonies with distinct boundaries. B) Positive staining for alkaline phosphatase.

Fig.2:

Cjes001 cells exhibit a normal 46, XX karyotype after 64 passages.

Fig.3:

Positive staining for transcription factors OCT4 (A-C), NANOG (D-E) and SOX2 (G-I). B, E and H show red PI counterstaining; C, F and I show green Alexa 488 staining. A, D, and G, show the merged pictures. Note the cytoplasmic / membraneous staining for NANOG.

Fig.4:

Comparison of the mRNA expression the pluripotency transcription factors *OCT4*, *NANOG*, and *SOX2* in undifferentiated cjes001 (ESC) cells versus differentiated ES cells (ESCD). This figure clearly indicates significant down-regulation of the respective mRNAs after one week of spontaneous differentiation.

Fig.5:

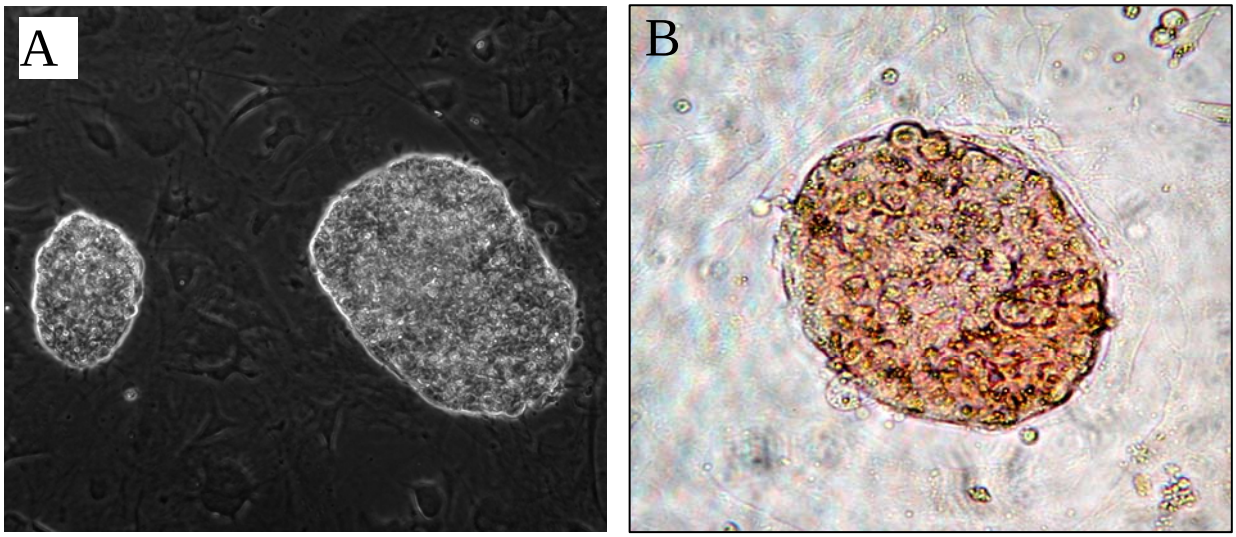
Stage specific embryonic antigens. Negative staining for stage specific antigens SSEA-1 (A-C), but positive signals for SSEA-3 (D-E) and SSEA-4 (G-I). B, E and H show counterstaining by PI; C, F and I show Alexa 488 staining and A, D, and G the merged pictures.

Fig.6:

570 Positive staining for tumor rejection antigens TRA-160 (A-C) and TRA-181 (D-E). B and E
571 show counterstaining by PI; C, F and I shows Alexa 488 staining, A, and D the merged
572 pictures.
573
574 **Fig.7:**
575 Telomerase reverse transcriptase mRNA levels reflecting telomerase activity quantified by
576 real time PCR. MEF: mouse feeder cells, COS7: immortalized green monkey kidney cell line,
577 cjes001: marmoset ESC. Note the almost 2-fold increased number of *TERT* molecules even
578 compared to the immortalised COS7 cell line.
579
580 **Fig.8:**
581 Cjes001 cells can form embryoid bodies with a (A) cystic or (B) compact (solid) phenotype.
582 The different types of embryoid bodies also exhibit different histological features (see text).
583
584 **Fig.9:**
585 Cj001 cells form tumors showing derivatives of all three embryonic germ layers. A) Nerval
586 structures within the tumor represent ectoderm. B) Osteogenic and hematopoietic
587 differentiation show mesoderm formation. C) Chondrocytes and osteogenesis with adnate
588 muscles tissue also represent mesoderm. D) Adenomatous epithelium. E) Columnar
589 epithelium possibly representing endodermal differentiation. F) RT-PCR analysis of two
590 teratoma tissue samples (S1, S2) showed expression of the marker mRNAs βIII-Tubulin,
591 Brachyury, and α-Fetoprotein, which represent ektoderm, mesoderm, and endoderm
592 formation, respectively.
593
594 **Fig.10:**

595 A) Analysis of germ cell-specific gene expression by RT-PCR. The germ cell markers *VASA*
596 and *SCP3* are not detectable in undifferentiated ES cells. In contrast, in differentiated ES cells
597 these germ cell markers are expressed at significant levels almost reaching testicular
598 expression levels (C, positive control). Also, *GCNF* was cleary up-regulated in differentiated
599 ESC. In contrast to these regulated germ cell markers *BOULE* was not found to be regulated
600 during ES cell differentiation and could be detected in both conditions. *DAZL* could be
601 detected in neither condition. B) Immunofluorescence staining of cystic embryoid bodies
602 showed a distinct cytoplasmic signal for VASA in some clusters of cells within the embryoid
603 body. C) Western blot analysis detecting specificly VASA protein in marmoset testis protein
604 extracts (left lane) and in differentiated cjes001 cells. A significantly weaker signal was also
605 obtained with a protein extract from mouse embryonic feeder cells alone suggesting that the
606 feeder cell population also contains mouse germ cells.
607

Figure 1:



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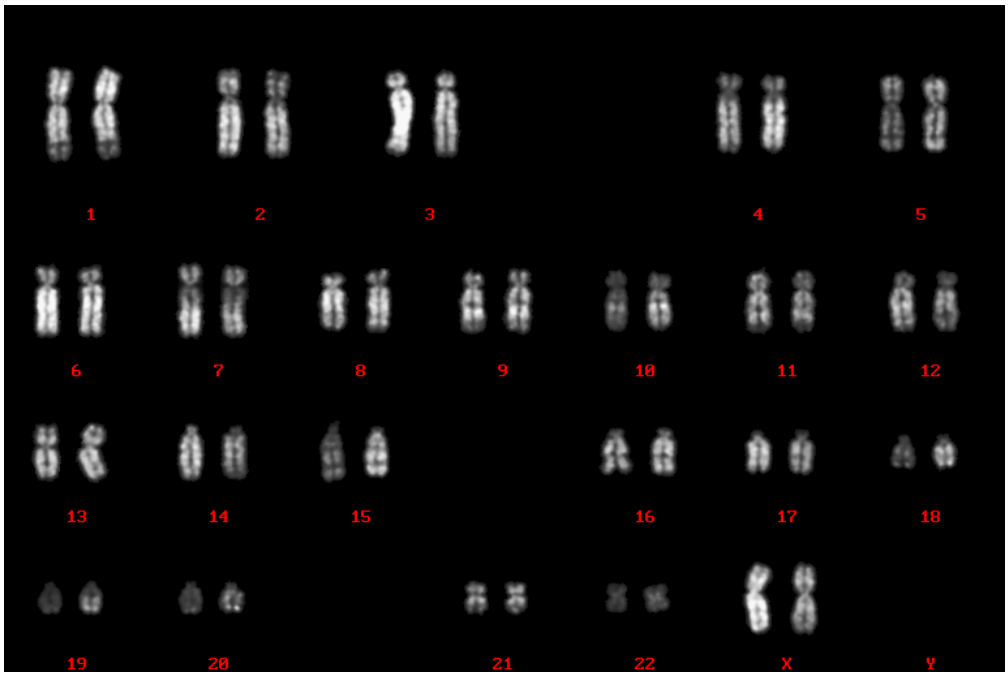

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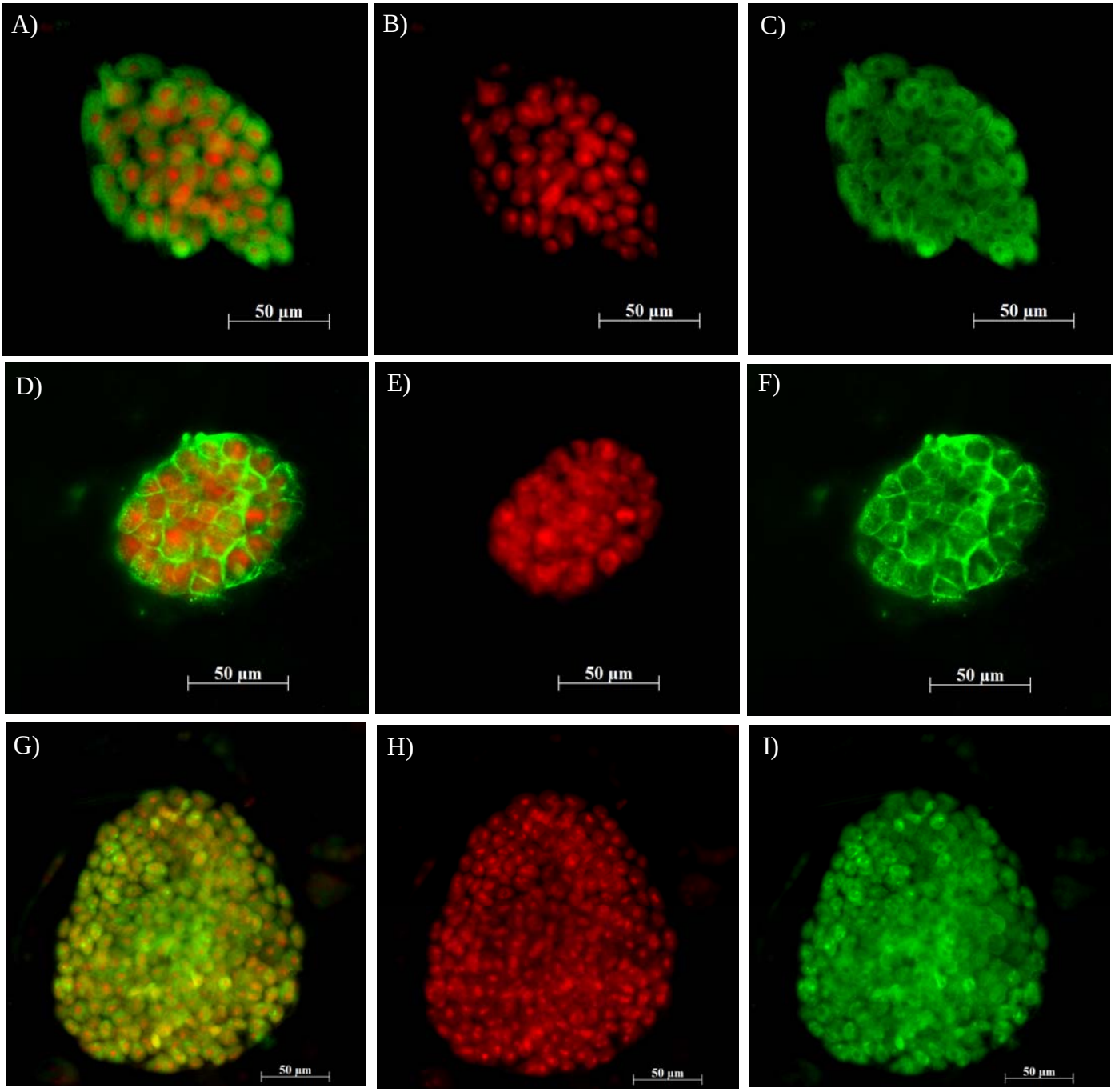

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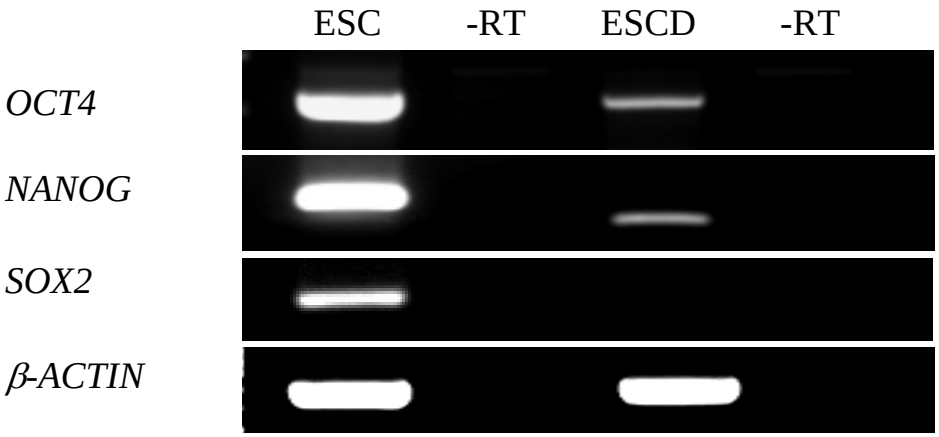

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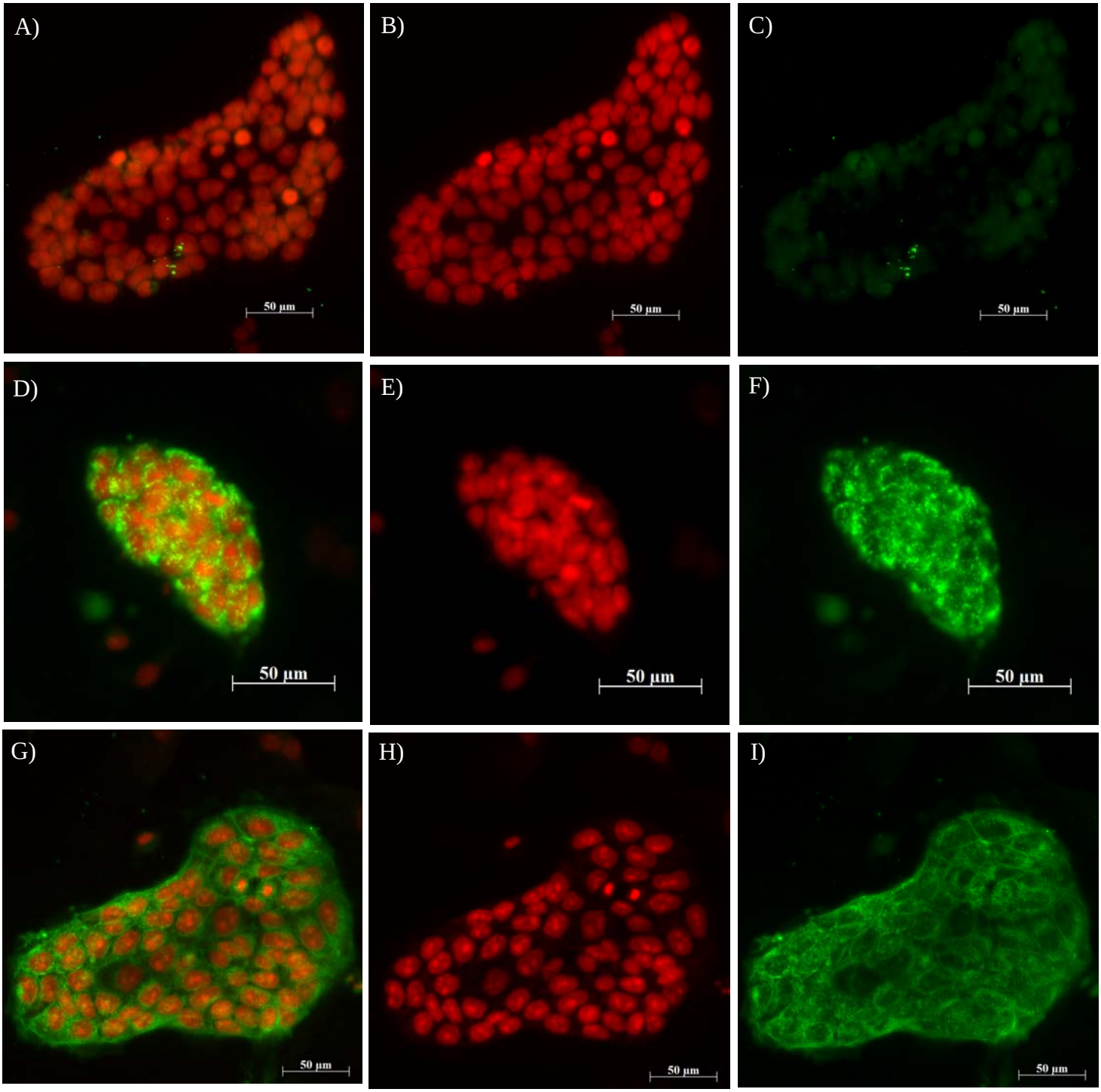

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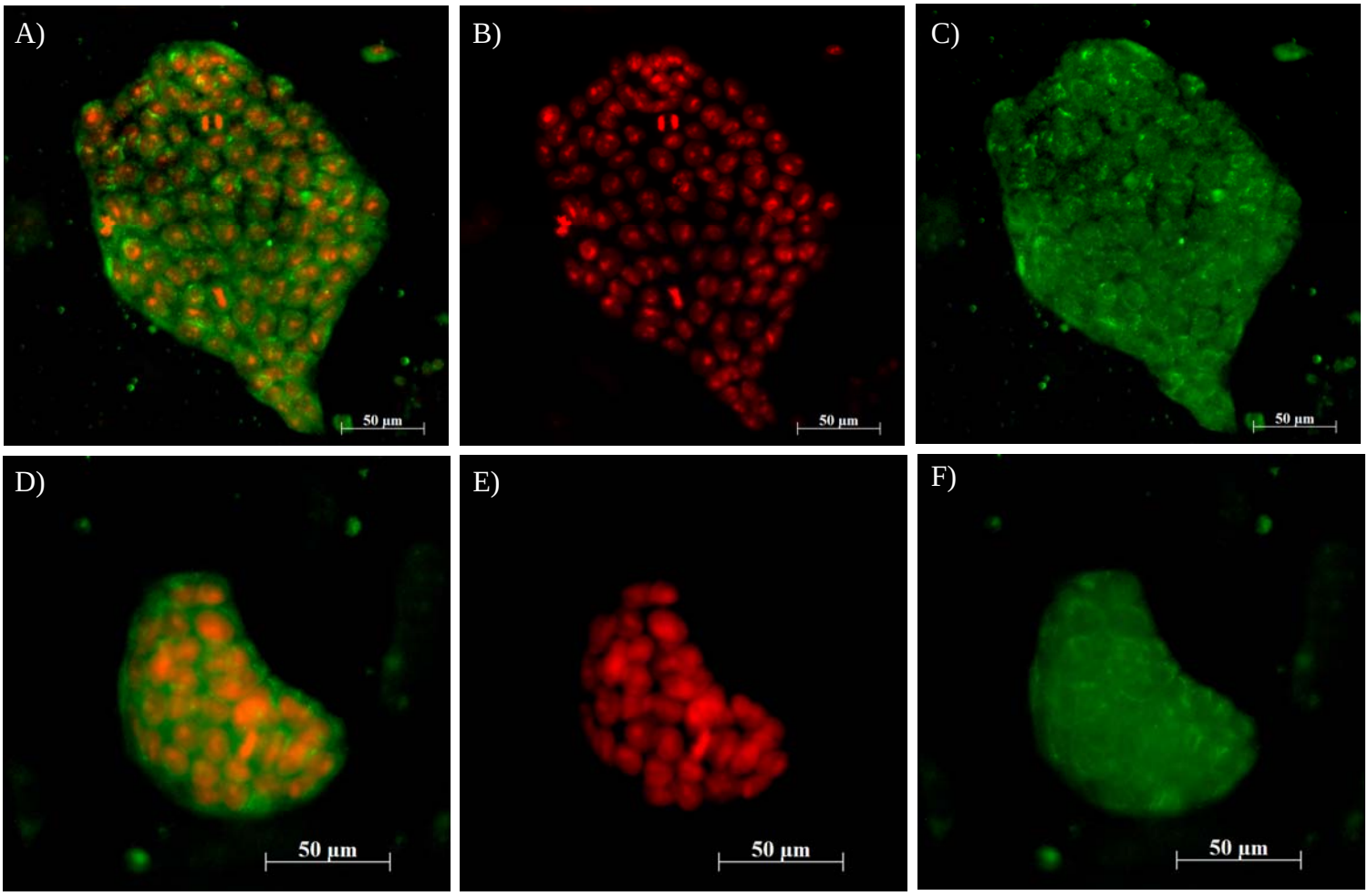

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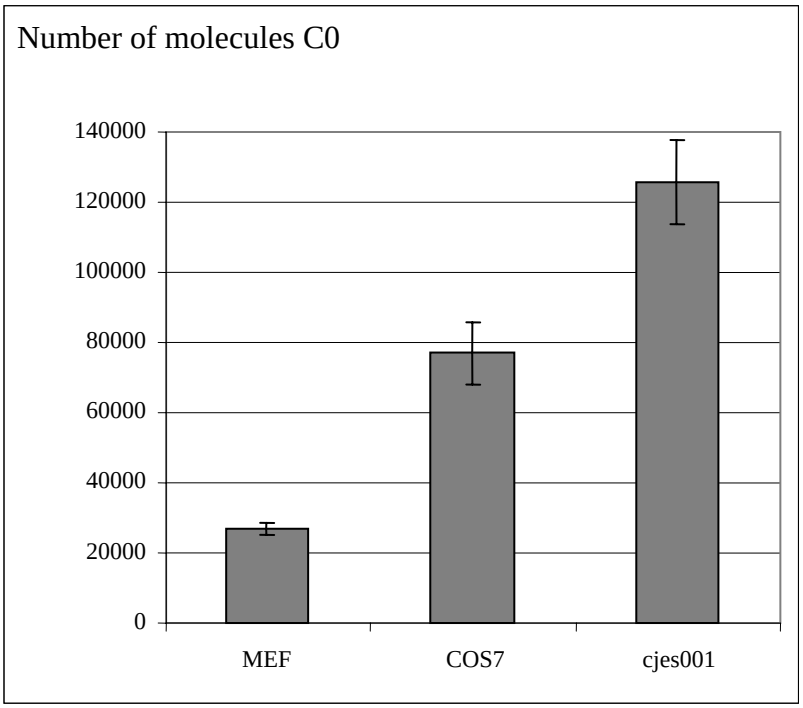
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120000
100000
80000
60000
40000
20000
0
MEF
COS7
cjes001

Figure 8:

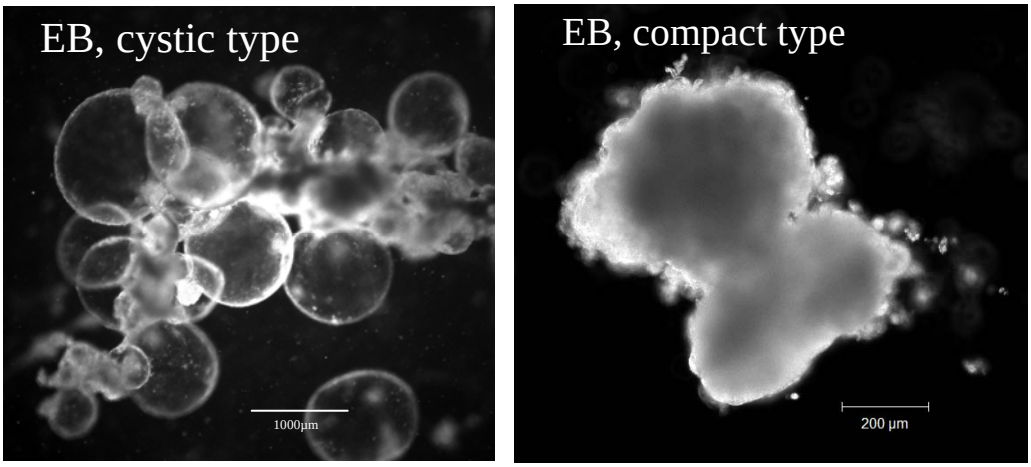
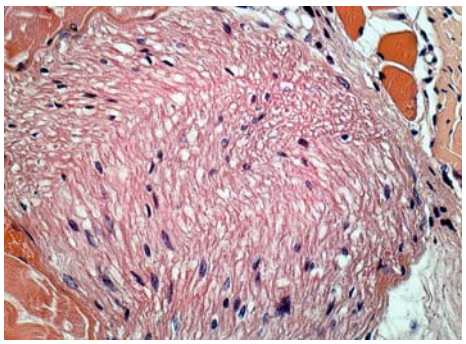
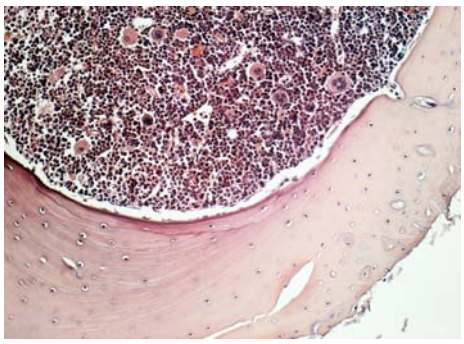
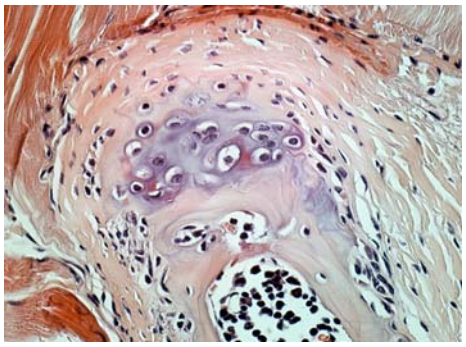
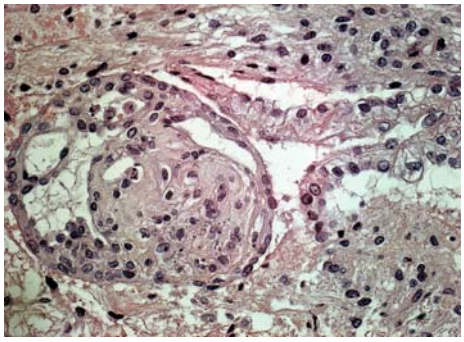


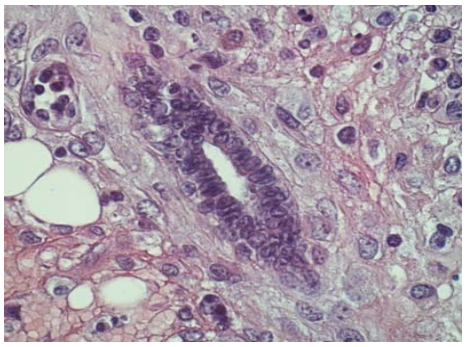
Figure 9:

A) 

B) 

C) 

D) 

E) 

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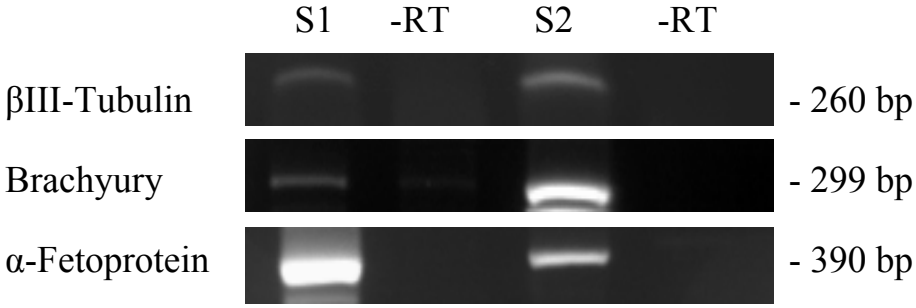
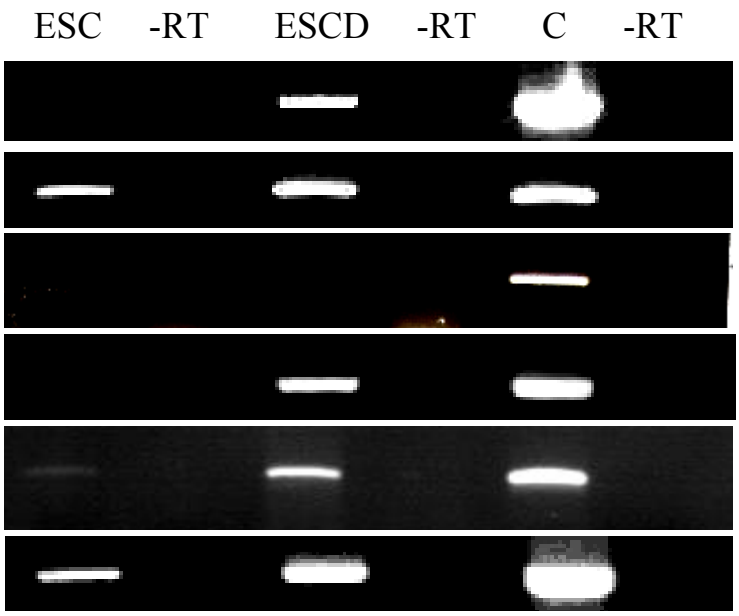


Figure 10:

A)



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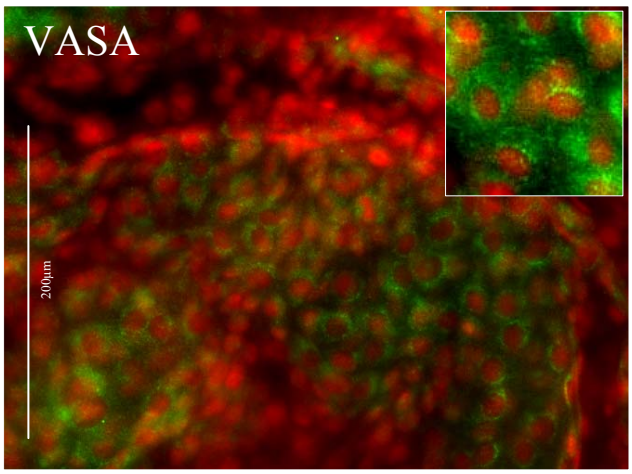

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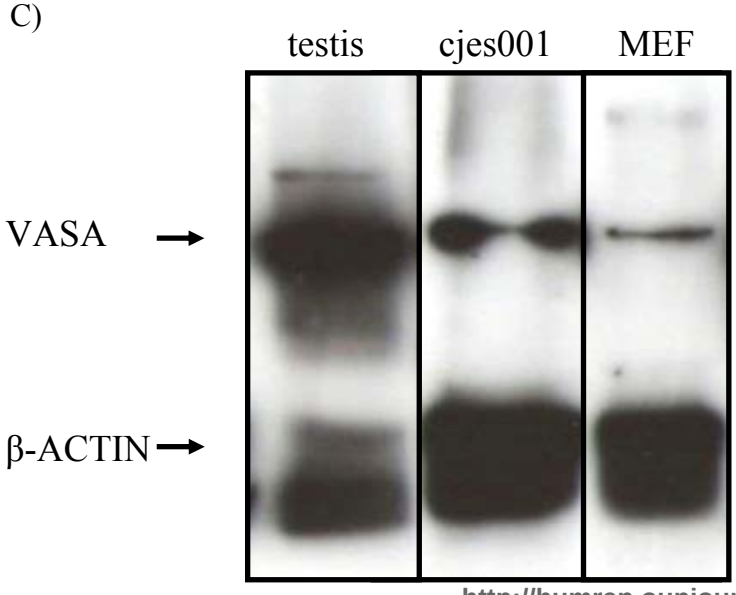

Table 1: Antibodies used in this study

Antibodies	Host	Company	Cat-No
anti-DDX4/MVH/Vasa	rabbit	Biozol	Ab 13840-100
anti-Nanog	rabbit	Abcam	Ab 21603-100
anti-Nanog	goat	Santa Cruz	SC-30329
anti-human Nanog	goat	R&D Systems	AF1997
anti-Oct3/4	rabbit	Santa Cruz	SC-9081
anti-Sox2	rabbit	Chemicon	AB5603
anti-ß-Actin	mouse	Sigma	A1978
anti-SSEA1	mouse	Chemicon	MAB 4301
anti-SSEA3	rat	Chemicon	MAB 4303
anti-SSEA4	mouse	Chemicon	MAB 4304
anti-TRA-1-60	mouse	Chemicon	MAB 4360
anti-TRA-1-81	mouse	Chemicon	MAB 4381
Alexa Flour 488	goat	Invitrogen	#A31620
Alexa Flour 488	donkey	Molecular probes	#A311034
Anti rabbit HRP	goat	Santa Cruz	#sc-2301
Anti mouse HRP	donkey	Jackson ImmunoResearch	#715-035-150

Table 2 : List of primers used and their respective PCR fragment sizes

Name	primer	annealing	exp. size (bp)
Nanog	5'-A A ACAGAAGACCAGAACTGTG -3' 5'-AGTTGTTTTTCTGCCACCTCT-3'	60	190
Oct3/4	5'- CCTGGGGGTTCTATTTGGGA-3' 5'-T T T-GAATGCATGGGAGAGCC-3	60	530
FoxD3	5'-CGACGAC-GGGCTGGAGGAGAA-3' 5'-ATGAGCGCGATGTAC-GAGTA-3'	60	356
Sox2	5'-AGAACCCCAAGATGCACAAC-3' 5'-GGGCAGCGTGTACTTATCCT-3'	60	200
CD34	5'-AGCCT-GTCACCTGGAAATGC-3' 5'-CGTGTTGTCTTGCT-GAATGGC-3'	60	627
Nestin	5'-GCCCTGACCACTCCAGTTTA-3' 5'-GGAGTCCTGGATTTCCTTCC-3'	60	200
Boule	5’-GCGACGCAAACATCAAACCAG-3’ 5’-GAACACATCCACCATCCTGTG-3’	58	187
Dazl	5’-CCAGTCCTCATCAGCTGCAAC-3’ 5’-CAACATAGCTCCTTTGCTCCC-3’	58	306
ß-Actin	5’-CATG GAGAAGATCTG GCACCAC-3’ 5’ –GATCTCCTTCTGCATCCTGTC-3’	58	689
ßIII-Tubulin	5’-CATGTCCATGAAGGAGGTGGA-3’ 5’ -GTGAACTCCATCTCATCCATG-3’	58	260
AFP	5’-CAGAAAYACATCSAGGAG AG -3’ 5’-GAGCTTGGCACAGATCCTTG-3’	58	390
Brachyury	5’ -CTGCYTAYCAGAAYGAGGAGA -3’ 5’-GGTTGGAGARTTGTTCCGATG -3’	58	299

Table 2 : Primers used and their respective PCR fragment sizes

Name	primer	annealing	exp. size (bp)
Nanog	5'-A A ACAGAAGACCAGAACTGTG -3' 5'-AGTTGTTTTTCTGCCACCTCT-3'	60	190
Oct3/4	5'- CCTGGGGGTTCTATTTGGGA-3' 5'-TTTGAATGCATGGGAGAGCC-3	60	530
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Boule	5’-GCGACGCAAACATCAAACCAG-3’ 5’-GAACACATCCACCATCCTGTG-3’	58	187
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3.2. Growth characteristics of the non-human primate embryonic stem cell line cjes001 depending on feeder cell treatment

Title

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Authors

Fleischmann G, Müller T, Behr R, Blasczyk R, Sasak E, Horn PA

Submitted in

Cloning and Stem Cells, 2008

Kommentar: Zum Zeitpunkt der Veröffentlichung der Doktorarbeit akzeptiert in Cloning and Stem Cells, Volume 11, Number 2, 2009

Cloning and Stem Cells

Cloning and Stem Cells Manuscript Central: http://mc.manuscriptcentral.com/cloning

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Journal:	*Cloning and Stem Cells*
Manuscript ID:	CLO-2008-0064
Manuscript Type:	Original Paper
Date Submitted by the Author:	29-Sep-2008
Complete List of Authors:	Fleischmann, Gesine; Hannover Medical School, Institute for Transfusion Medicine Müller, Thomas; German Primate Center, Stem Cell Research Group Sasaki, Erika; Central Institute for Experimental Animals, Laboratory of Applied Developmental Biology, Department of Marmoset Research; Center for Human Metabolomic System Biology, Keio University, School of Medicine Blasczyk, Rainer; Hannover Medical School, Institute for Transfusion Medicine Horn, Peter; Hannover Medical School, Institute for Transfusion Medicine
Keyword:	Primate, Embryo Stem Cells, Stem Cells, In Vitro

Growth characteristics of the non-human primate embryonic stem cell line cjes001 depending on feeder cell treatment

Gesine Fleischmann[1], Thomas Müller[2], Rainer Blasczyk[1], Erika Sasaki[3, 4], Peter A. Horn[1]

1 Institute for Transfusion Medicine, Hannover Medical School, Hannover, GERMANY

2 Stem Cell Research Group, German Primate Center, Göttingen, GERMANY

3 Central Institute for Experimental Animals, Laboratory of Applied Developmental Biology, Department of Marmoset Research, Kawasaki, JAPAN

4 Center for Human Metabolomic System Biology, School of Medicine, Keio University, Tokyo, JAPAN

Address all correspondence to:

Peter A. Horn, M.D.
Institute for Transfusion Medicine
Hannover Medical School
Carl-Neuberg-Straße 1
30625 Hannover
Tel.: +49 511 532 8704
Fax: +49 511 532 2079
Horn.Peter@mh-hannover.de

Abstract

Embryonic stem cells (ESC) hold tremendous potential for therapeutic applications, including regenerative medicine, as well as for understanding basic mechanisms in stem cell biology (Wobus 2001; Mountford 2008). Since numerous experiments cannot be conducted in human ESC because of ethical or practical limitations, nonhuman primate ESC serve as invaluable clinically relevant models (Fischbach and Fischbach 2004; Nikol'skii et al., 2007, Gruen, 2006).

The novel marmoset (*Callithrix jacchus*) ESC line cjes001 was characterized using different stem cell markers. The cells were stained positively with Oct3/4, SSEA-3, SSEA-4, Tra-1-60, Tra-1-81 and Sox-2 underscoring their status as undifferentiated ESC. ESC are typically grown on mouse embryonic fibroblasts (MEF) as feeder cells whose proliferation is arrested either by treatment with Mitomycin C or by γ-irradiation (Ponchio et al., 2000; Conner 2001).

To assess the impact of these treatments on the ability of MEF to support the growth of undifferentiated ESC, we used an MTT assay to evaluate the cellular metabolic activity of growth arrested feeder cells (Mosmann 1983). There was a significant ($p<0.02$) difference in γ-irradiated cells displaying a higher metabolic activity compared to Mitomycin C inactivation. Also we quantified 69 soluble factors in the supernatant of both Mitomycin-treated and γ-irradiated MEF by bead-based multiplex analysis and thus established a profile of MEF-secreted factors. The time course of secretion was analyzed by monitoring the supernatant at 0, 6, 12 and 24 hours after changing the medium. Comparing γ-irradiated and Mitomycin-treated MEF suggested higher amounts of some cytokines including FGF or SCF by the former. We also assessed whether the method of inactivation had an effect on growth kinetics and differentiation of primate ESC. There appeared to be a trend to a lower number of

differentiated ESC colonies on the γ-irradiated feeder cells, suggesting that this may be the preferable method of growth arrest.

Keywords: Non-human primate embryonic stem cells, *Callithrix jacchus*, Mouse embryonic fibroblasts, Mitomycin C, γ-irradiation

Introduction

Embryonic stem cells (ESC) hold tremendous potential for therapeutic applications because of their ability to differentiate into multiple, clinically applicable cell types (Wobus 2001; Mountford 2008; Fleischmann, 2007). Mouse embryonic stem cells are very commonly used and methods for culture and differentiation of these stem cell lines are well established. However, mouse embryonic stem cells significantly differ from human embryonic stem cells in their characteristics concerning culture, morphology and gene expressions. It is at best unclear to what extend results established in murine models can be transferred to the human setting. Also, many experiments cannot be conducted in human ESC because of ethical problems thus elevating nonhuman primate ESC as invaluable clinically relevant models (Nakatsuji and Suemori 2002; Fischbach and Fischbach 2004; Horn et al., 2006; Suemori H. 2006; Nikol'skii et al., 2007; Mountford 2008). Especially the common marmoset *Callithrix jacchus* serves as a very useful nonhuman primate model because of its small size, the unproblematic breeding and long lifespan.

For clinical applications and to prevent uncontrolled differentiation ESC need to be cultured under defined standardized growth conditions abolishing all undefined compositions of media and feeder cells.

The approaches to cultivate ESC under feeder free conditions are indeed encouraging but are currently not fully developed to be transferred to all different ESC lines. The specific method of cultivation also depends on the species the ESC are derived from. For human and rhesus ESC it has been described that feeder-free cultivation is possible with special media and cultivation terms (Hong-mei and Gui-an

2006; Zhang et al., 2006). Here, both the additives and the dish-surfaces used play an important role.

There are more published approaches where human ESC are cultivated under completely feeder-free conditions (Xu, 2001; Beattie et al., 2005; Amit and Itskovitz-Eldor 2006a; Amit and Itskovitz-Eldor 2006b; Bigdeli et al., 2008). In these cases additives such as leukemia inhibitory factor (LIF) were substituted to keep the ESC in an undifferentiated and pluripotent status (Rose-John 2002, Humphrey, 2004).

In addition, the O_2 tension is thought to play an important role to keep the cells in an undifferentiated status which may be due to the fact that early stage embryos develop in low O_2 concentration (hypoxia). However human ESC are typically cultured in 21% O_2 (normoxia) conditions under which they tend to differentiate spontaneously (Ezashi et al., 2005; Kurosawa et al., 2006).

At present the majority of laboratories typically grow ESC on MEF whose proliferation is arrested either by treatment with Mitomycin C or by γ-irradiation. Mitomycin C is an antibiotic covalently intercalating the cells' DNA, preventing dissociation essential for replication and transcription finally causing apoptosis. The damage for the DNA by Ionizing radiation is not fully understood, though it is commonly accepted that the inhibition of transcription and apoptosis is mediated here by upregulation of tumorsuppressorprotein p53. It appears that ESC receive signals from the feeder layer via cytokines and extracellular matrix–cell surface molecule interaction, which may result in intracellular signal transduction. There is no unique reliable protocol established for feeder free cultivation of most human and nonhuman primate ESC and unfortunately the interaction of MEF and ESC is not fully understood. Identifying the best MEF inactivation method in terms of ESC growths and understanding the interaction between MEF from different mouse strains and ESC in more detail could

be an important step to develop such a feeder-free protocol. In this study, MEF from two different mouse strains (CF1 and NMRI) were inactivated by Mitomycin C or by γ-irradiation and used to assess the influence of all four conditions on ESC.

We evaluated the impact of these different treatments on the capacity of the MEF to support undifferentiated growth of primate ESC. Also the secreted factors by MEF and the metabolic activities were measured to point out the differences between the inactivation methods.

Material and Methods

Cultivation of the embryonic stem cell line cjes001

Methods for cultivation and characterization of the undifferentiated status of ESC were performed as previously published (Müller et al, 2008, in review).

In brief, ESC colonies were cultured in medium consisting of Knockout-DMEM (Dulbecco's Modified Eagle Medium) (Gibco Invitrogen GmbH, Karlsruhe, Germany) with 20% Knockout-Serum-Replacement (Gibco Invitrogen GmbH), 1% Pen-Strep (c·c·pro, Oberdorla), 1% MEM non-essential amino acids (Gibco, Invitrogen GmbH), 1 mM L-Glutamine (c·c·pro), 0,2 µM β-Mercaptoethanol (Gibco Invitrogen GmbH), and 10 ng/ml bFGF (basic fibroblast growth factor) (peprotech, Hamburg, Germany).

Cultivation was performed on MEF, which were inactivated either by Mitomycin C (10µg/ml) (Sigma, Steinheim, Germany) treatment or γ-irradiation. MEF were seeded on plates coated with 0,1% gelatine (Stem Cell Technologies Inc, Palo Alto, USA) for 30 minutes at 37°C. The ESC were split once a week 1 : 2 to 1 : 4, depending on density, and plated on MEF which had been seeded out 24 hours earlier.

Characterization of cjes001

The characterization of cjes001 was performed by immunofluorescent staining with stem cell markers Oct3/4 (Octamer 3/4), SSEA-3 (Stage-Specific Embryonic Antigen-3), SSEA-4 (Stage-Specific Embryonic Antigen-4), Tra-1-60, Tra-1-81and Sox-2. Detection of alkaline phosphatase (AP) was performed by immunohistochemical staining (Chemicon International, USA).

Immunofluorescent staining was performed after fixation with 4% PFA for 2-3 min at room temperature. Cells were washed twice with PBS and incubated with primary antibody for 30 min at 4℃. Primary antibodies were diluted 1:50 in PBS. After another washing step with PBS cells were incubated with the secondary antibodies Alexa488 (Invitrogen GmbH, Germany) which were diluted 1:50 in PBS. Analysis was performed using the fluorescent microscope BZ-8000 from Keyence (Osaka, Japan).

Preparation of MEF

MEF were prepared by extraction from 13,5 day old embryos of CF1 and NMRI mice, respectively. Limbs, head and tail were removed and the tissue was minced by grinding between two frosted glass slides with 10ml PBS. After centrifugation at 4℃ for 5 min at 200xg, the pellet was resuspended in 20 ml Trypsin-EDTA (0,25%) (c·c·pro) and incubated for 15 min at 37℃. 5 ml FCS were added, the mixture was given through a 70µm cell-strainer (bd biosciences, Heidelberg) and flushed with MEF-media (DMEM, 10% FCS, 1% sodium pyruvate, 1% MEM-non-essential-amino-acids, 1% Antibiotic, Antimycotic). After final centrifugation (400xg, 5 min) the cells were resuspended from the pellet and seeded in a density of $20x10^6$ cells on a

500cm² cell culture plate (Corning, New York, USA). The cells could be expended for 2-3 passages and were then inactivated as described below.

Inactivation of MEF

The inactivation of MEF by Mitomycin C (10 µg/ml) was performed for 4 hours at 37°C, 5% CO_2 followed by 2x washing the cells with 5 ml PBS.

Alternatively, cells were inactivated by γ-irradiation with 30 Gy at 2 Gy/minute using a Cs-137 source (IBL437C, CIS GmbH, Dreieich, Germany). In both cases MEF were thereafter plated on gelatine coated cell culture dishes at a density of $0,5*10^6$/6-well.

MTT Assay

We utilized a colorimetric microtiter (MTT) assay to quantify the metabolic activity of cells. In general the cells were incubated with the eponymous substance 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (ICN Biomedicals Inc., Ohio, USA) and the metabolic activity can be quantified by the reduction of MTT into blue-violet hydrophob formazan crystals. In our experiments the feeder cells were seeded ($0,17 \times 10^5$ cells/well) in a 96-well plate and cultivated overnight at 37°C, 5% CO_2. After 12h the medium was changed to 100 µl medium and 25 µl MTT-reagent (5 mg MTT in 1 ml PBS) followed by an incubation for 2 hours at 37°C. Eventually, 50 µl lysis-buffer (20% SDS in 1:1 DMF : H_2O, pH 4,7) was added and incubated for further 12 hours. 100µl medium + 25µl MTT-reagent + 50 µl lysis-buffer were used as control and reference in an Elisa plate reader at 562 nm (anthos bt3, Anthos Labtec Instruments, Wals, Austria). Further analysis was performed with WinRead software, version 2.36. The test was repeated for three times. Each part (negative control,

Mitomycin C treated cells, irradiated cells, frozen/thawed cells) was performed in an 8x approach.

Cytokine quantitation using a multiplexed immunoassay

Different cytokines and other soluble factors were quantified using a bead-based multiplex quantitative analysis (Rules Based Medicine, Austin, Texas, USA). 69 factors were measured to compare different concentrations of these factors in media of Mitomycin C-treated and γ-irradiated MEF and also in MEF of different mouse strains. To compare the different mouse strains and inactivation types a time course of secreted factors was analyzed by monitoring the supernatant at 0, 6, 12 and 24 hours.

Counting colonies and Morphology of cjes001

ESC were cultured in 6-well-plates (greiner bio-one, Cellstar, Frickenhausen, Germany). The number of colonies per well was quantified by counting under white field microscopy (Olympus, IMT-2, Japan). The undifferentiated cells grow as compact, multicellular colonies with a well-defined border and appear to “gleam” compared to the feeder layer. We anticipate that this is a helpful light microscopy effect originating in the tight packing of the cells in an intact colony. In contrast, differentiated colonies’ borders are blur and lack of the above mentioned light effect.

Growth rate and differentiation status

Considering the above mentioned differences in secretion of soluble factors in each feeder cell setup, we assumed a direct effect of feeder cell treatment on ESC growth characteristics, namely in growth rate and also rate of spontaneous differentiation. To calculate the rate of cell division of ESC on different MEF types we visually counted

the number of colonies every 6th day after splitting for 8 weeks. As a second criterion, the “lack shininess/blurred colony borders” vs “shiny and clear borders” of the counted ESC colonies was recorded.

Statistical analysis

The statistical package for social sciences (SPSS version 15 for windows, Chicago, IL) software was used for the statistical analysis. Initially, descriptive statistics were employed and important parameters such as mean, standard deviation and standard error were determined. To compare the means of different groups, an analysis of variance was performed with subsequent pairwise post hoc-tests. In addition, a Kruskal-Wallis-H-test was used to define differences between groups. P-values < 0.05 were considered significant for testing the hypothesis.

Results

Characterization of cjes001

The expression of different pluripotency markers in cjes001 was determined by immunofluorescence. Positive immunofluorescent staining of the embryonic stem cell markers was detected for Oct 3/4, SSEA-3, Tra-1-60, SSEA-4, Sox-2, Tra-1-81 (Fig. 1A-F). High alkaline phosphatase expression could be detected by immunohistochemistry (Fig. 1G). In comparison figure 1 shows an unstained embryonic stem cell colony (Fig. 2).

MTT-assay

MTT assay was used to assess the metabolic activity of MEF after Mitomycin C treatment or γ-irradiation, respectively. The assay displayed a significant ($p<0.02$) difference between the two methods of inactivation with γ-irradiated cells (30 Gy) namely a higher metabolic activity than Mitomycin C treated cells. One cycle of freezing and thawing of cells, as typically would be done in most laboratories, further decreased metabolic activity of the feeder cells by roughly 16% (Fig. 3).

Soluble factors secreted by MEF

A bead-based multiplex quantitative analysis (Rules-Based Medicine, Inc., Austin, TX 78759, USA) was performed to assess differences in concentrations of cytokines and other soluble factors secreted by MEF. Both, the method of inactivation (either by Mitomycin C treatment or γ-irradiation), and MEF of two different mouse strains (NMRI vs. CF1) were compared. In addition, the kinetics of the release of soluble factors by MEF over time by quantitation after 0, 6, 12 and 24 hours was measured.

The analysis displayed significant differences in concentrations of both cytokines and soluble factors for Mitomycin C / γ-irradiation as well as between NMRI and CF1. As result, we show here the five outstanding factors as example for the differences in inactivation and in mouse strains.

Mitomycin C treatment vs. γ-irradiation

Comparing between Mitomycin C treatment and γ-irradiation, Stem Cell Factor (SCF), Fibroblast Growth Factor (FGF) and Vascular Endothelial Cell Growth Factor (VEGF) have a markedly higher concentration in γ-irradiated cells than in Mitomycin C-treated cells. In contrast, the concentration of Leukemia Inhibitory Factor (LIF) is higher in Mitomycin C-treated cells than in γ-irradiated (Fig. 4)

NMRI vs. CF1

The comparison between the different mouse strains NMRI and CF1 showed that in all cases for LIF, SCF, FGF, VEGF, and Tumor Necrosis Factor-alpha (TNF-alpha) the CF1-derived MEF secreted higher amounts than MEF prepared from NMRI mice (Fig. 4).

Time response

As expected, the levels of most soluble factors secreted by MEF increases over time. Some, such as Tissue Inhibitor of Metalloproteinase Type-1 (TIMP-1) and Fibrinogen increase rather continuously over time while others, such as TNF-alpha, Oncostatin M (OSM), LIF, Interleukin-7 (IL-7) and SCF increase very rapidly and then remain on a plateau (Fig. 5). No significant difference in the kinetics of secretion were seen when comparing either the different inactivation methods or the different mouse strains.

Effect on ESC

Although the differences in secretion of soluble factors in each feeder cell treatment group suggest quick effects on ESC growth rate, we just detected no significant difference between Mitomycin and irradiated MEF over 5 weeks (Fig. 6). In addition, there also appeared to be slightly, but not significantly more differentiated ESC colonies on Mitomycin C-treated feeder cells than on γ-irradiated feeder cells (Fig. 7, statistics not shown).

Discussion

Embryonic stem cells are commonly maintained and expanded on inactivated feeder cell layers. The method of inactivation and the utilized mouse strain is thought to have an effect on growth rate and differentiation status of the ESC (Ponchio et al., 2000). Moreover the secreted cytokines, soluble factors and the metabolic activity of MEF should have an effect on growth and differentiation of ESC (Prowse et al., 2007). In this study differences between the inactivation methods and the two mouse strains CF1 and NMRI were examined.

As expected, untreated MEF in general display a higher metabolic activity in a MTT assay, than either irradiated or chemically treated cells. Apparently, the inactivation by γ-irradiation leaves the cells with a 10% higher basic metabolic activity compared

to Mitomycin C, thus seems to the more “gentle” method for MEF inactivation. Whether a higher metabolic activity is essential for enhanced growth of the ESC or the opposite, remains unclear. On the one hand, enhanced cytokine levels could stimulate ESC growth, on the other enhance spontaneous differentiation. For other cells it has previously been published that the treatment makes no difference for cultivation (Ponchio et al., 2000). However, there is evidence in the literature that for other cell types, such as B lymphocytes, γ-irradiation of the feeder layer is much more effective in terms of cell expansion than Mitomycin C treatment (Roy et al., 2001). As a side note, MEF with a higher metabolic rate persist longer as a coherent feeder layer on the plastic well and improve hence the overall handling and splitting of ESC.

The information obtained from the cytokine array, namely the low metabolic activity of Mitomycin C treated MEF, is consistent with the MTT results, since it can be expected that cells with high metabolic activity also secrete higher levels of cytokines and other soluble factors. Especially the levels of SCF and FGF are higher after irradiation. SCF is suspected to influence the undifferentiated status of the ESC likewise and FGF as a growth factor and regulatory protein. Both factors also play an important role in embryonic development (Marie 2003; Dvorak and Hampl 2005; Lavine et al., 2005; Xu et al., 2005; Yu and Ornitz 2008). It can be speculated, that due to that increased concentrations of SCF and FGF expressed by irradiated MEF, the ESC displaying a slight tendency to less spontaneous differentiation.

Another key player being described to play an important role in differentiation of embryonic stem cells is VEGF (Sone et al., 2007). In our case, VEGF has also a much higher metabolic activity in irradiated MEF than in Mitomycin C treated MEF. It has been described to have an effect on differentiation process from ES cells to

vascular cell components. For keeping ESC in undifferentiated status the concentration of secreted VEGF likely should be minimized.

Interestingly, LIF is lower in irradiated MEF than in Mitomycin C treated. LIF is described as cytokine for maintaining both proliferation and the developmental potential of non-primate stem cells (Metcalf 1991; Fry 1992; Li et al., 2007). A high level of LIF secreted by MEF may be beneficial for cultivation of non-primate ESC in an undifferentiated status, we see no effect of LIF in marmoset ESC culture.

The comparison of the different mouse strains displayed higher concentrations of almost all factors in CF-1 mice MEF. All keyplayers known to affect ESC differentiation like VEGF or TNF-alpha but also the factors which are important to maintain the undifferentiated status are higher. For ESC lines in need of high levels of cytokines, we clearly recommend CF-1 feeder cells, for ESC with marginal cytokine requirements NMRI MEF would be appropriate.

In cytokine arrays, the concentration of most important factors promoting differentiation like VEGF (Hehlgans and Pfeffer 2005; Sone et al., 2007) increased linearly over time whereas SCF reaches a plateau after 18 hours indicating its halflife. From this background, the empirically discovered strict media change each 24 hours in stem cell cultures becomes comprehendible. On the other hand, the levels of LIF with its ability to prevent differentiation abilities reaches likewise a plateau after 12 hours maybe thus annihilating the differentiation by other factors. The imbalance of pluripotency-supporting factors vs. differentiation-inducing factors accumulating in ESC media older than 24 hours may explain the rapid spontaneous differentiation of ESC on “older” feeder layers. It would therefore be interesting to monitor these

factors over a longer period of time or artificially increase concentrations of single factors immediately after splitting of ESC.

Interestingly, the morphology of ESC seems to be different depending on the feeder cells though the quantification of this effect remains difficult. From visual assessment there was a higher number of blur bordered, “non-shiny”, that is differentiated ESC colonies on the Mitomycin C-treated feeder cells than on the γ-irradiated. Maybe this observation is caused by the lower level of cytokines or maybe by other soluble factors not being measured here being secreted by the Mitomycin C-treated MEF.

In conclusion, a clear-cut general solution for cultivation of stem cells can certainly not be deduced from this study. However, we find significant differences in radiation and Mitomycin treatment of MEF, namely significantly higher cytokine and metabolite levels in irradiated cells. For optimizing the cell culture conditions for each ESC line, we encourage researchers to test if the specific line used in culture is a “high level cytokine” or “low level cytokine” line and thereby improving ESC performance from the beginning.

Acknowledgements

These studies were supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) for the Cluster of Excellence ReBirth (From Regenerative Biology to Reconstructive Therapy). We furthermore would like to thank Kirsten Elger and Marion Niebeling for excellent technical assistance and the working group of LEBAO, especially Prof. Dr. Ulrich Martin and Dr. rer. nat. Kristin Schwanke for excellent cooperation.

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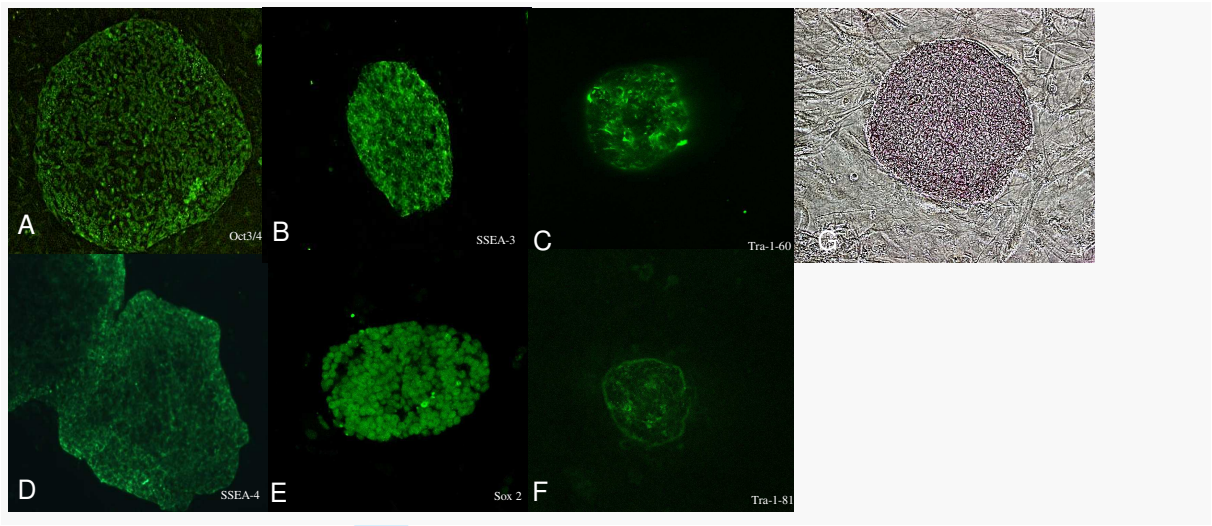

Fig 1: Immunofluorescent staining with stem cell markers Oct3/4, SSEA-3, SSEA-4, Tra-1-60, Tra-1-81 and Sox-2 (A-F). Immunohistochemical staining of alkaline phosphatase (AP) (G). The positively staining underscores their undifferentiated status.

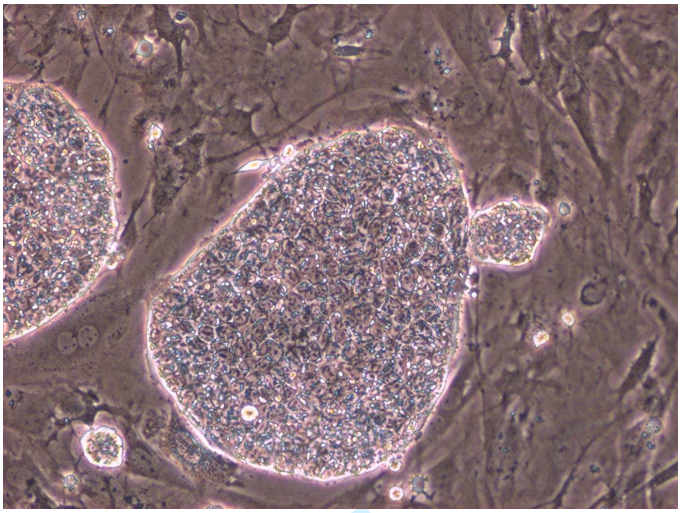

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Fig 2: Lightmicroscopy picture of a cjes001 colony on inactivated MEF, 4 days after splitting.

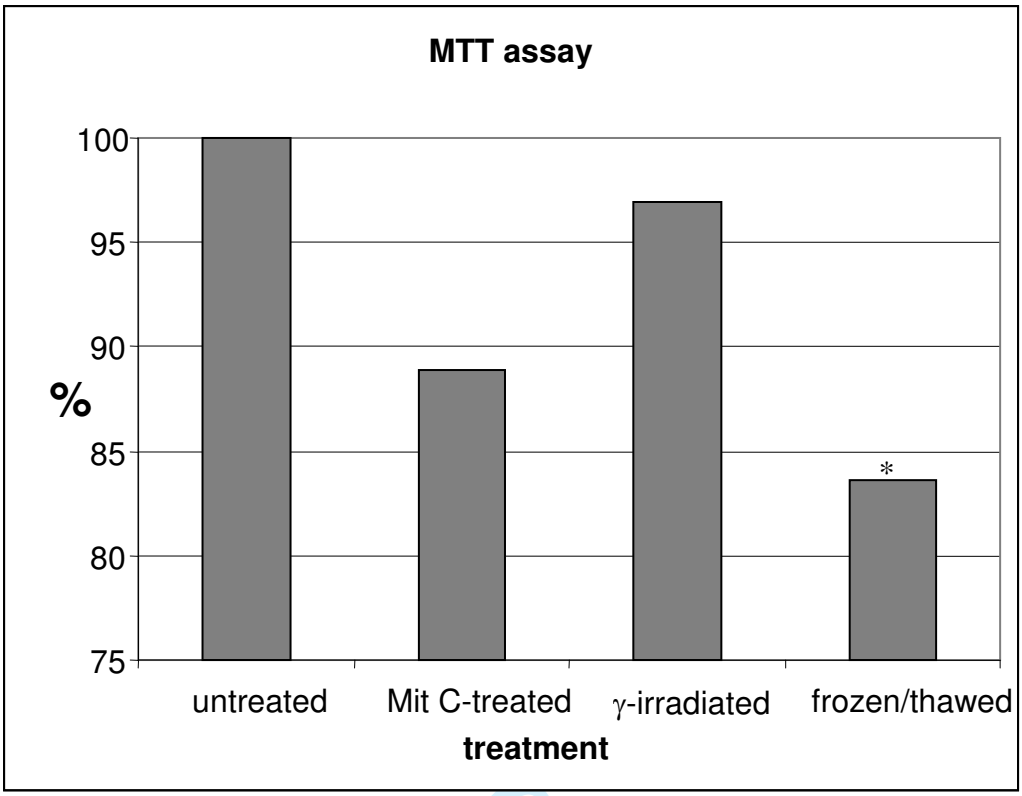


Fig 3: Metabolic activity (%) of inactivated MEF, analyzed by MTT assay. Student's T-test unreveals a significant ($p<0.02$) difference of metabolic activity between γ-irradiated vs. chemically treated cells by Mitomycin C. Furthermore, freezing as well decreases the metabolic activity significantly (indicated by asterisk).

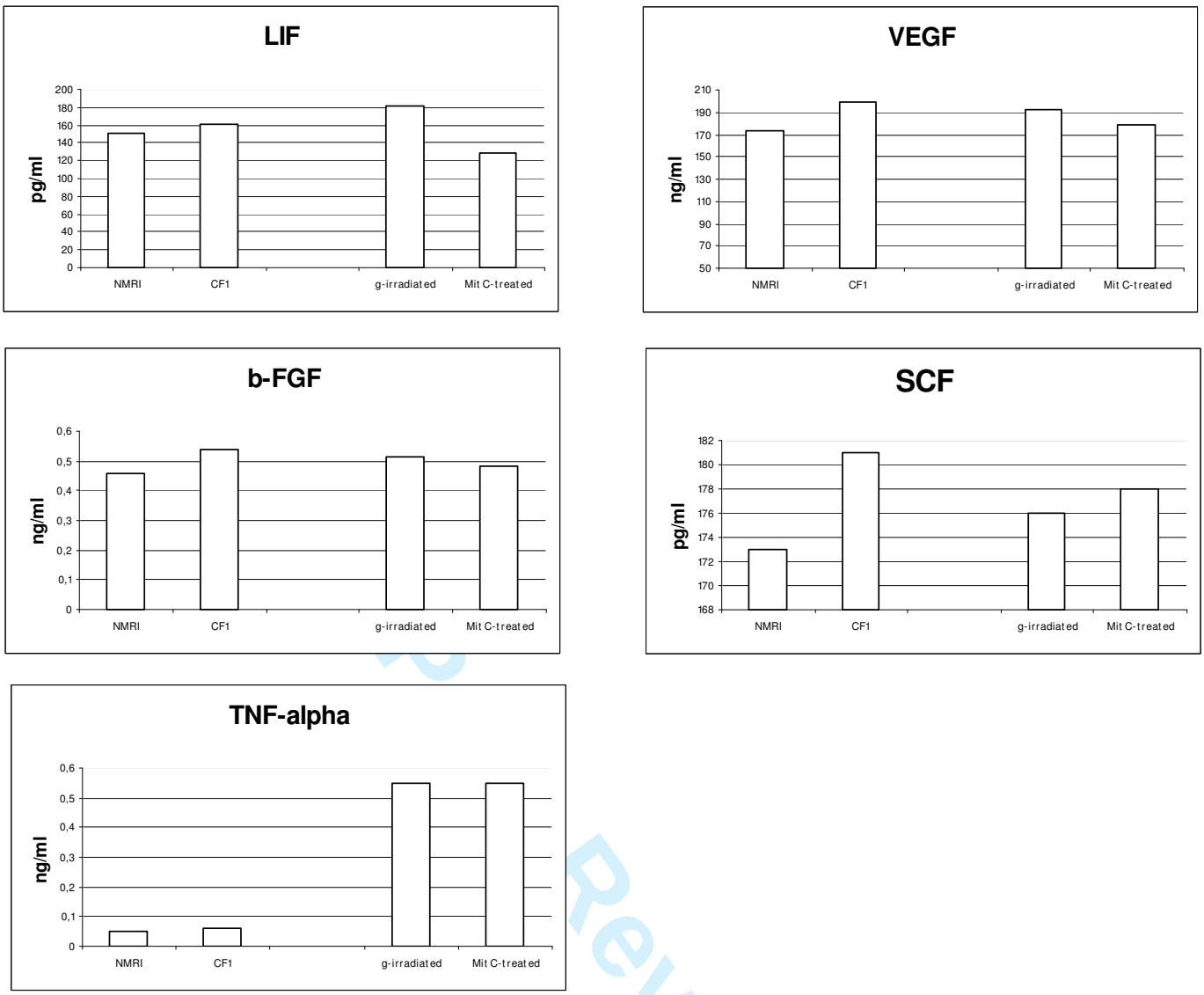


Fig 4: Comparison of 5 soluble factors in NMRI mice vs CF1 mice and γ-irradiated vs Mitomycin C-treated, respectively. With exception of LIF, a generally higher concentration of cytokines in supernatants of CF1-MEF than in NMRI could be observed. Furthermore the concentrations of LIF, b-FGF and VEGF are significantly higher in irradiated MEF. Interestingly, SCF values indicate higher concentration in chemically treated cells, whereas TNF-alpha values are equal independently of treatment.

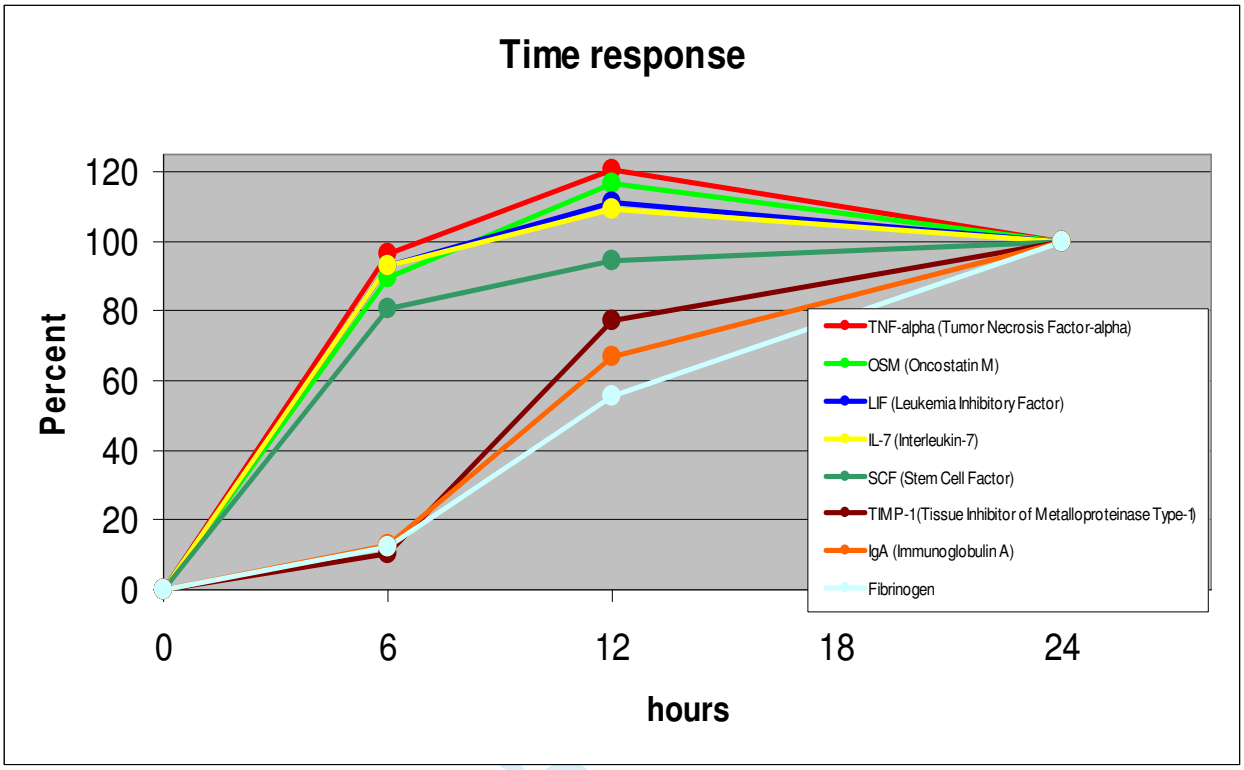


Fig 5: The level of soluble factors secreted by MEF (irradiated, NMRI) monitored over time. Some factors such as TIMP-1 and Fibrinogen increase continuously while others, such as TNF-alpha, OSM, LIF, IL-7 and SCF increase very rapidly and then remain on a plateau after 12-18 hours.

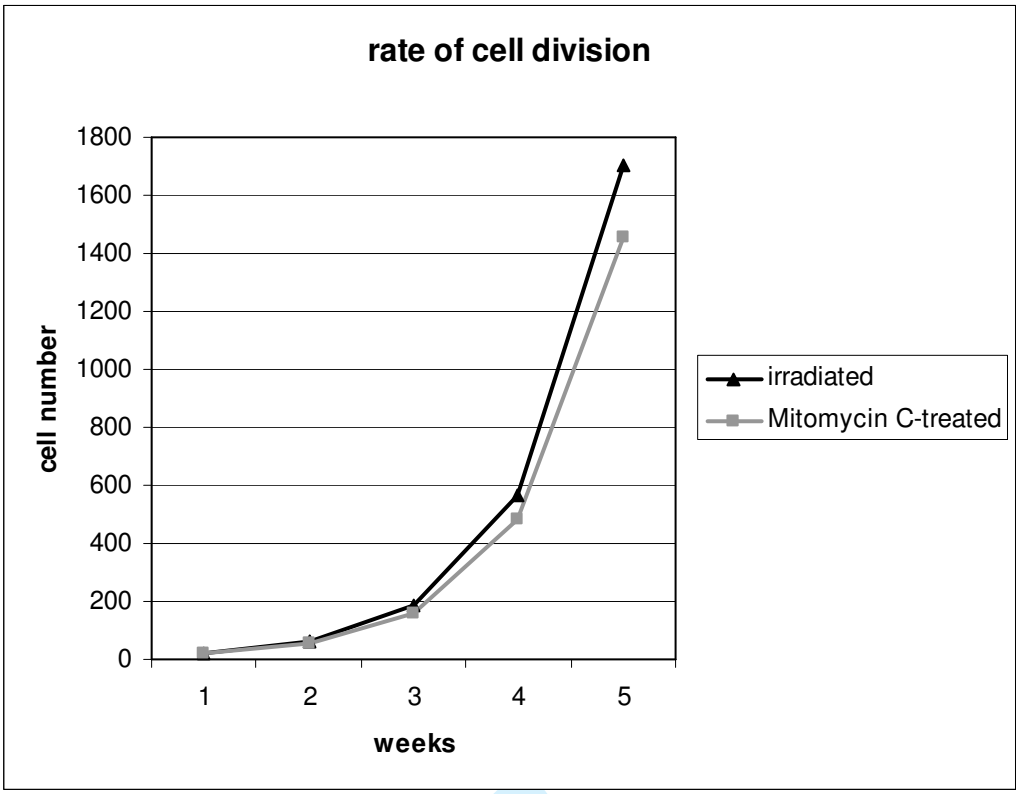


Fig 6: The graph shows the proliferation rate of ESC colonies in comparison to colonies grown on Mitomycin C-treated MEF and on γ -irradiated MEF.

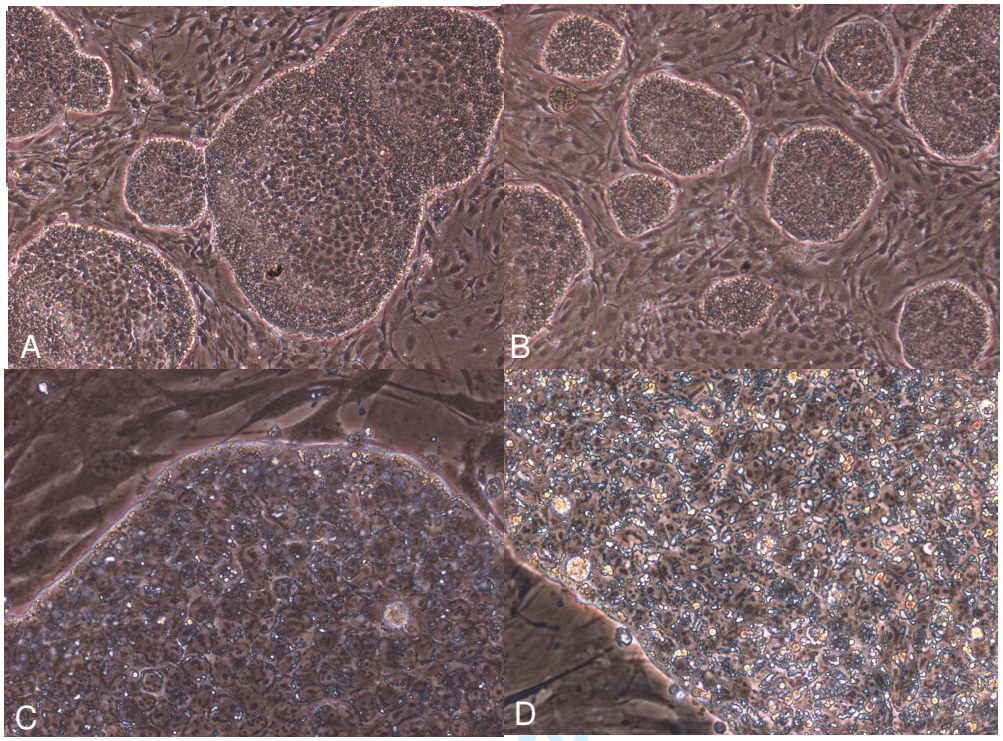


Fig 7 (A-D): Larger ESC colonies with blur borders on Mitomycin C treated MEF (A, C), “shiny” ESC colonies with distinct borders grown on γ-irradiated MEF (B, D).

3.3. Embryonic Stem Cells: MHC Expression and Immunogenicity of Stem Cell-Derived Cellular Therapeutics

Title

Embryonic Stem Cells: MHC Expression and Immunogenicity of Stem Cell-Derived Cellular Therapeutics

Authors

Fleischmann G, Figueiredo C, Seltsam A, Blasczyk R, Horn PA

Preliminary accepted in

Stem Cell Applications in Diseases, Nova Science Publishers, Inc. (Editor F. Columbus), 2008

Embryonic Stem Cells: MHC Expression and Immunogenicity of Stem Cell-Derived Cellular Therapeutics

In: Stem Cell Applications in Diseases, Nova Science Publishers, Inc.
Editor: Frank Columbus

Gesine Fleischmann[1,2], Constança Figueiredo[2], Axel Seltsam[2], Rainer Blasczyk[2], Peter A. Horn[1,2]

[1]Institute for Transfusion Medicine, University Hospital Essen, Germany

[2]Institute for Transfusion Medicine, Hannover Medical School, Hannover, Germany

Grant Support: Part of this work is supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) for the Cluster of Excellence REBIRTH (From Regenerative Biology to Reconstructive Therapy). CF was supported by the Portuguese Foundation for science and technology (SFRH/9687/2002)

SUMMARY

Embryonic stem cells (ESC) represent a theoretically inexhaustible source of precursor cells that can be differentiated into any cell type. These pluripotent, endlessly dividing cells have been hailed as a possible means for treating numerous diseases including degenerative, genetic or malignant diseases, or injury caused by trauma, infection, or inflammation. In addition, ESC are an invaluable research tool to study stem cell development and can serve as a platform to develop and test novel therapies.

Beside the ethical limitations associated with the use of human embryonic stem cells, the application of cellular therapeutics derived from ESC is mainly hindered by two technical hurdles: The risk of tumor formation from undifferentiated ESC on the one hand and rejection of transplanted cells by the host's immune system on the other hand.

The emergence of stem cell-based regenerative medicine as a potential therapy for substitution of organs and tissues is therefore intimately correlated with the necessity to inhibit the host immune response to the modified autologous or allogeneic stem cells. One of the most sought after goals in cell as well as tissue and organ transplantation is therefore the ability to induce specific immunologic tolerance to transplantation antigens since this would allow the replacement of host organs or tissues without the need for immunosuppression. In principle, this may be achieved by either altering properties of the host's immune system, achieving a state of specific tolerance (or ignorance), or alternatively by altering the properties of the transplanted cells and tissues, making these less immunogenic. Most desirable would be a stable, normal graft function in the complete absence of a requirement for maintenance of immunosuppression. Alternatively, the concept of employing tolerogenic strategies to permit graft acceptance with dramatically reduced immunosuppression requirements would already be a significant improvement.

In addition to discussing the therapeutic potential of human ESC, this chapter will cover limitations to using ESC for replacement cell therapy and strategies to overcome these limitations, focusing in particular on difficulties associated with the immunogenicity of these cells. We will review the current state of the art in cell replacement strategies using embryonic stem cells and present novel research results on the immunogenicity of embryonic stem cells fom non-human primates.

INTRODUCTION

Regenerative medicine

The scarcity of organ donors and the fact that untreatable diseases arise from the loss or malfunction of specific cell types in the body led to an emerging field of treatment called cell therapy or regenerative medicine. This science is based on the concept of producing new cells to replace malfunction or to treat diseases such as Alzheimer's disease, Parkinson's disease, type II diabetes, heart failure, osteoarthritis and aging of the immune system as well as medical conditions resulting from injury follow-on trauma, infarction and burns (Down and White-Scharf 2003, Shi et al. 2004, Shufaro and Reubinoff 2004, Surbek and Holzgreve 2002). The replacement of damaged cells with fully functional ones might be a useful therapeutic strategy in treating many of these diseases.

The field of regenerative medicine is also seen as an emerging market. Although the current sales are less than $100 million, in contrast with the approaching $200 billion of medical implant industry, it is expected that cell-based therapies will be a $100 billion business. Here, four key issues are considered of critical relevance for the commercial and medical success of new developed products. These consider the cell source, off-the-shelf availability, matrix and immune acceptance (Ahsan and Nerem 2005, Strom et al. 2002).

Cell-based therapies are classified in two main categories with respect to the use of autologous or allogeneic approaches and the majority of the companies have chosen to adopt just one. Patient's own cells are used in autologous therapies. The cells are collected, modified or expanded *ex vivo* for posterior delivery to the same patient. The major advantage of this approach is that the patient's immune system recognize the therapeutic cells as "self" and does not reject them. However, this strategy raises economic, logistic and regulatory problems as all therapeutic applications must be custom made for each single, individualize patient. In addition, every laboratory would treat the cells with a different protocol, which might lead to significant phenotypic and functional discrepancies (Down and White-Scharf 2003, Strom et al. 2002).

Nuclear transfer technologies have been successfully developed for a broad range of mammalian cells and have brought closer the possibility of human

therapeutic cloning, which aims the production of pluripotent stem cells carrying the nuclear genome of the patient. These cells were then be induced to differentiate into replacement cells and used for treatment. Although this approach tries to solve the critical problem of immune incompatibility, these cells may present immunogenic peptides resulting from the mitochondrial compartment. Moreover, reconstituting the cells into tissues or organs may represent a hard obstacle (Gimble 2003, Odorico et al. 2001, Shufaro and Reubinoff 2004, Strom et al. 2002, Tirziu and Simons 2005).

An alternative to the use of autologous or cloned cells is the exploitation of allogeneic cell sources, in which a particular cell type is used for a given therapeutic indication and applied to transplant all patients. This may contribute to overcome cell availability and economical factors, which represent important obstacles of custom made cell therapy. Also, the use of off-the-shelf products allows standardization of protocols between different laboratories. The use of immunosuppressants or cell encapsulation has been applied to prevent graft rejection, but confers infection susceptibility to the patient or further challenges such as the development of an adequate material to construct capsules. Nevertheless, the allografting approach must escape the immune system, which has specialized to identify and eliminate “non-self” cells (Ahsan and Nerem 2005, Down and White-Scharf 2003).

Stem Cells

Stem cells are characterized by the ability to renew themselves and differentiate into a diverse range of specialized cell types. They can be classified in embryonic stem cells that are found in blastocysts and in adult stem cells that are found in adult tissues. They are divided in four different groups: totipotent, pluripotent, multipotent and unipotent (Gage 2000).

Totipotent stem cells are cells which have the ability to from a complete organism. These cells are only found in the early days of the embryo. After the morula stage the cells lose their totipotency. These cells are no longer able to form a complete organism but they still have the capability to form all other cell types of the embryo, a property coined pluripotency (Donovan and Gearhart 2001). The pluripotent cells differentiate in their development to the three germ

layers mesoderm, ectoderm and entoderm. They are able to differentiate into more than 200 different cell types. These cells can be isolated from the blastocyst at day 5 to 14. Other more differentiated cell types are called multipotent. They have the capability to form two or three other cell types like for example hematopoetic stem cells. All fully differentiated cell types like for example fibroblasts are called unipotent.

Stem cell type	Description	Examples
Totipotent	Each cell can develop into a new individual	Cells from early (1-3 days) embryos
Pluripotent	Cells can form any (over 200) cell types	Some cells of blastocyst (5 to 14 days)
Multipotent	Cells differentiated, but can form a number of other tissues	Fetal tissue, cord blood, and adult stem cells

Figure 1. Overview about different stem cell types.

Stem cells are classified in totipotent, pluripotent and multipotent cells. For each cell type the description shows the abilities of the cells to differentiate. Third line shows examples where these cells can be found.

Embryonic stem cells are pluripotent cells derived from the inner cell mass (ICM) of blastocysts at day 5 to 6 (Martin 1981). ESC were first isolated from the ICM of mice in 1981(Evans and Kaufman 1981). Today ES cell lines of different species like mouse, human and non-human primates are established (Martin 1981) (Graves and Moreadith 1993) (Thomson et al. 1995) (Thomson et al. 1998). The culture conditions of ESC differ widely depending on the species they are derived from (Ginis et al. 2004).

Stem cell characteristics and the pluripotent status of ESC can be shown by different methods. The expression of stem cell markers is one characteristic. Detection of this markers could be done by immunofluorescent/ immunohistochemical staining or by flow cytometry with different stem cell markers including Oct3/4, SSEA-3, SSEA-4, Sox-2, Tra-1-60, Tra-1-81 and Alkaline Phosphatase. Undifferentiated pluripotent stem cells are positive for all

these markers. Upon differentiation embryonic stem cells gradually lose the expression of these markers. Undifferentiated colonies look relatively homogeneous by light microscopy with a bright “shiny” border, growing as compact multicellular colonies (Fig. 2 h). The common marmoset ES cell line cjes001 was stained positive for all named markers in immunofluorescent staining (Fig. 2 a-c, e-g). Positive detection of AP is demonstrated in figure 2 d. Expression of all these stem cell markers could be confirmed by flow cytometry. Detection of Oct3/4, SSEA-3, Sox-2, c-kit and Tra-1-81 could be verified in ~90% of cells. SSEA-4 and Tra-1-60 could be detected in ~80% of measured cells (Fig. 3)

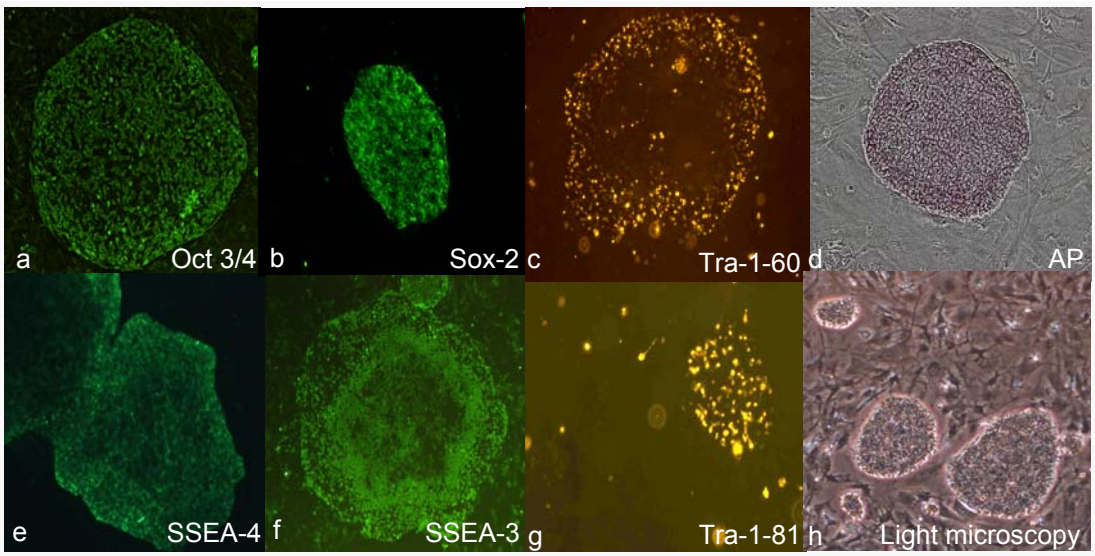


Figure 2. Staining for stem cell markers.

Immunofluorescent staining for stem cell markers Oct3/4, Sox-2, Tra-1-60 (a-c) and SSEA-4, SSEA-3, Tra-1-81 (e-g). Detection of alkaline phosphatase (AP) with immunohistochemical staining (d). The positive staining underscores their undifferentiated status. Unstained ESC colonies as negative control are shown in the lower right picture (h).

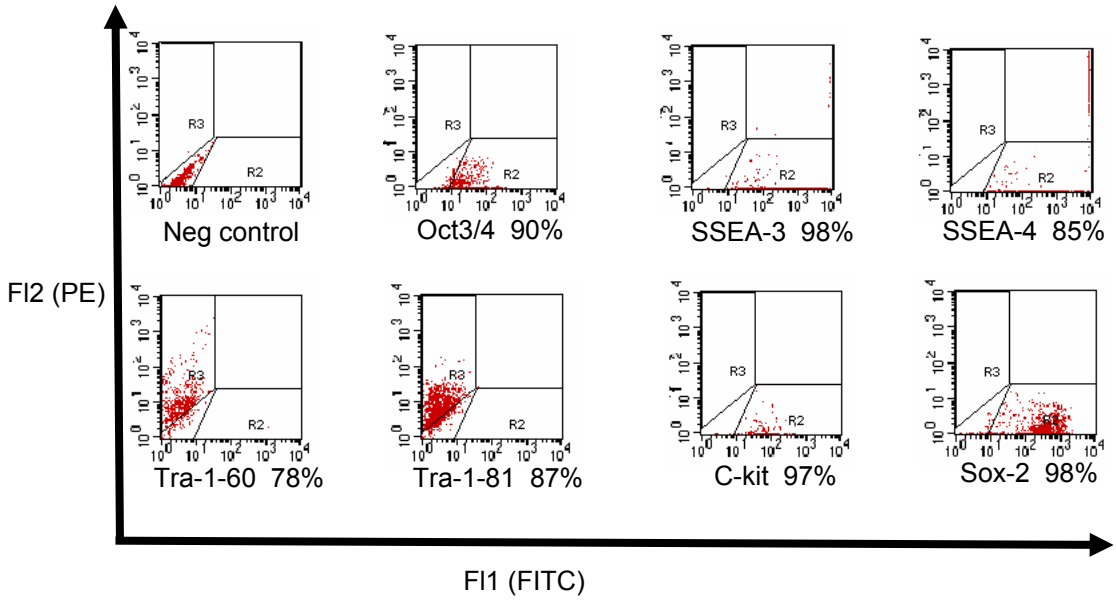


Figure 3. FACS analysis of stem cell markers.

The different dot plots show ESC stained with stem cell markers Oct3/4, SSEA-3, SSEA-4, Tra-1-60, Tra-1-81, c-kit and Sox-2 analyzed by flow cytometry. The positive staining underscore the undifferentiated status of the ESC.

The ability to form all three different germ layers leads to tumor growth if ESC are injected in immunodeficient mice. This is a frequently used method that is used as a functional proof of pluripotency of ESC. The injection of marmoset ES cells of the cell line cjes001 led to tumor growth in NOD/SCID-mice. Cell types of all three germ layers could be found in the teratoma. Figure 4 shows sections of a teratoma formed of cjes001.

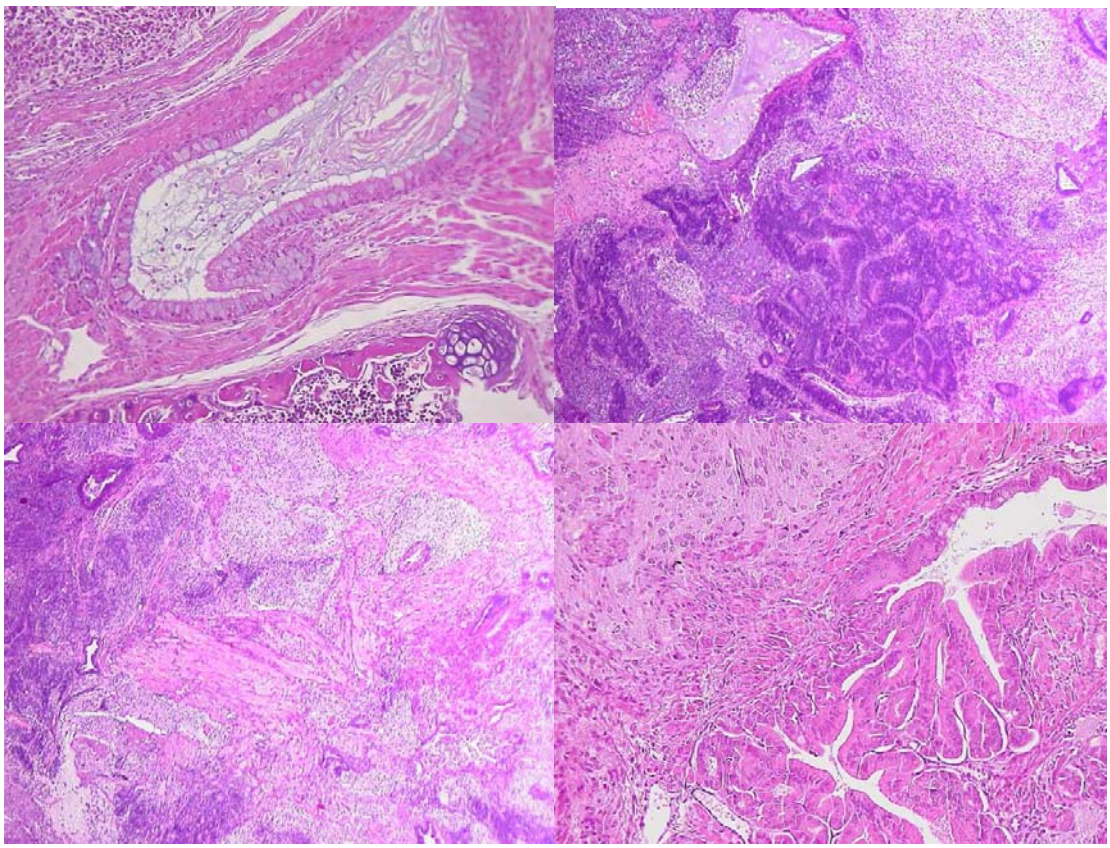


Figure 4. Teratoma formation of marmoset ESC in inmunodeficient mice.
The picture shows different sections of a teratoma formed by injection of ESC in immunodeficient NOD/SCID-mice. Note the highly sophisticated tissue organization found therein containing derivatives from all three germ layers.

Embryonic stem cell lines can be cultured for an unlimited period in vitro. ESC are typically grown on mouse embryonic fibroblasts (MEF) as feeder cells and form two dimensional monolayer colonies on them. The feeder cells have to be inactivated either by γ-irradiation or by Mitomycin C-treatment to avoid an overgrowing of feeder cells to the ESCs. It appears that ESC receive signals from the feeder layer via cytokines and extracellular matrix-cell surface molecule interaction, which may result in intracellular signal transduction. It is not well understood which inactivation method is the most effective for maintenance of ESC, or in other words, which method leads to least differentiation of the ES cells. There are significant differences in radiation and mitomycin C treatment of MEF, namely significantly higher cytokine and metabolite levels in irradiated cells (Fleischmann 2008). However, a clear-cut general solution for cultivation of stem cells can certainly not be deduced.

Research/Therapeutical aspects

Embryonic stem cells are of increasing interest because of their unique properties in comparison to all other cell types. There are some important advantages of stem cells compared to other cells like their ability to differentiate and the lower chance of rejection after transplantation. That is the reason why stem cells are thought to change the treatment of many human disease types by being used to repair specific tissues or to (re)grow organs.

To use embryonic stem cells in clinical research, one of the main focuses is in targeted differentiation. It is possible to differentiate the cells into several cell types like liver, heart muscle or kidney cells. Most differentiation protocols are still dependent on the formation of embryoid bodies (EB). EBs are cell clumps of ESC which are formed spontaneously if stem cells have no feeder layer to attach. They are three dimensional clumps which flotage free in the medium. Cells in embryoid bodies differentiate into several cell types and pass through different differentiation steps. In the first step there is the formation of an endodermal layer which is round of the EBs. In the middle of the embryoid body develop mesodermal structures and in between an ectodermal like cell layer. In differentiation protocols it is possible to separate required cell types and begin a targeted differentiation. An example is the differentiation of ESC over EB to HSCs and Monocytes (Karlsson, 2008). The advances to PBMCs are that the donor variability and the culture variability could eliminate.

Other promising differentiation models are the differentiation of ESC into Insulin producing cell clusters (IPCC) (Boyd, 2008) and the work on tissue repair cells (Hock, 2007).

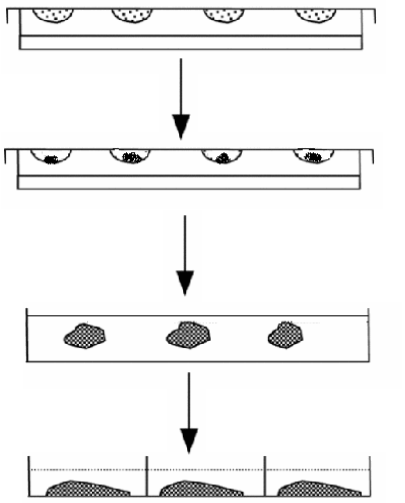


Figure 5. Schematic diagram of EB formation.

The figure shows a schematic diagram of the formation of embryoid bodies. At day 0 a single cell suspension was sowed out in hanging drops. At day 1-3 embryoid bodies were formed in bottom of hanging drops. Hanging drops are given in suspension culture on day 3-5. After another few days single aggregates can be plated out.

Limitations of cellular therapeutics

MHC incompatibility is the most relevant hurdle in transplantation and to the development of cell-based therapies. The host immune system recognizes the transplanted cells or organs as non-self, which leads to the destruction of the graft. This process is mainly mediated by the huge polymorphism of MHC molecules, but also by SNPs occurring in polymorphic proteins that become susceptible to be presented as minor histocompatibility antigens in a transplantation context. In the past decades strong efforts have been made to improve histocompatibility including the development of more efficient immunosuppression protocols, optimization of HLA match between donor/recipient pairs and tolerance induction. Despite the progresses made in this field, HLA incompatibility is still responsible for large waiting lists, graft failure and more recently constitute an obstacle to the development and standardization of cell-based therapies.

We here describe a strategy to create a situation of immunological blindness that may induce acceptance of allogeneic grafts. Several groups have previously demonstrated the feasibility of using siRNA or shRNAs as a tool to knockdown the target expression in a sequence-specific manner. Here, we used RNAi as a technique to silence the expression of MHC class I antigens. For this purpose, the dimeric structure of HLA class I molecules was used to define three main targets. To induce a MHC class I-specific silencing, we defined β2m as a target.

One crucial step in RNAi-based approaches is the identification of siRNA effectors able to efficiently knockdown the target expression. We used a constitutive and lentiviral-based system for the delivery of shRNAs targeting β2m in order to achieve a stable suppression of MHC class I.

Controlling HLA expression in genetically modified cell-based therapeutics may help to overcome the limitation of immunological rejection, and facilitate the improvement and standardization of protocols in the regenerative field of medicine.

Immunology

The major histocompatibility complex (MHC) and antigen presenting cells

T-cells as the main effectors of adaptive immunity require the degradation of the antigenic protein and the presentation of single antigen-derived peptides in the context of cellular receptors, generally denoted as major histocompatibility complex (MHC) or human leukocyte antigen (HLA), when referring to humans (Crawford et al. 2006, Haque and Blum 2005). Two major classes of HLA molecules are involved in immune responses to invasive pathogens, those are HLA class I and HLA class II molecules. These two classes of HLA molecules differ in structure and pattern of expression.

The heterodimeric structure of HLA class I molecules consist of two subunits: an approximately 40 KD heavy α-chain containing a transmembrane domain and a light 11 KD ß2-microglobulin (ß2m) chain. The HLA class I heavy chain structure is divided in three domains named α1, α2 and α3 domains. The variable α1 and α2 domains form a cleft where typically peptides of 8 to 10 amino acids can bind (Fig. 6a). Peptides bound to HLA class I molecules are recognized by a subset of T cells called cytotoxic T cells (CTLs), characterized by the expression of CD8 molecules. These molecules bind to the conserved α3 domain and act as a co-receptor of the T cell receptor (TCR).

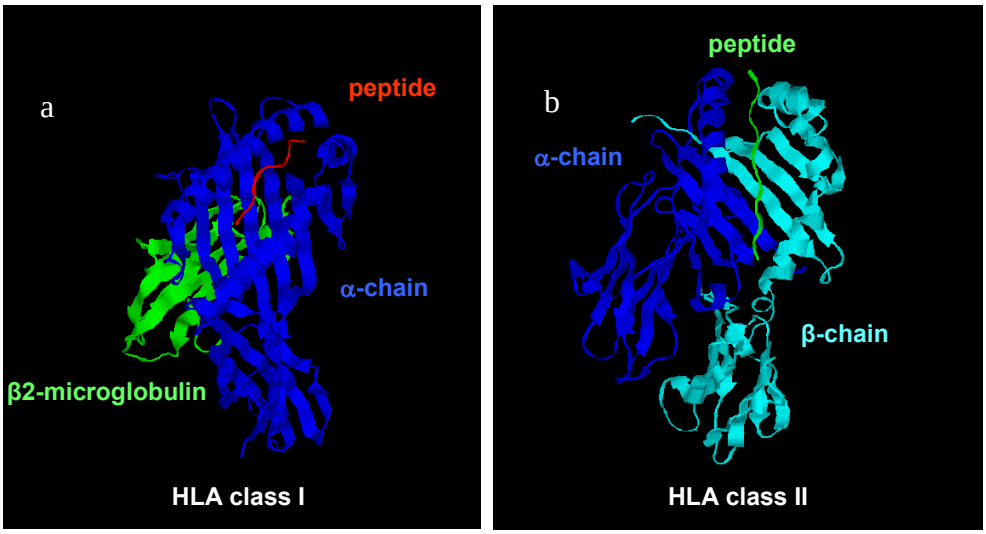


Figure 6. A ribbon-representation of the three-dimensional structure of HLA class I (a) and HLA class II (b) molecules loaded with a peptide.

HLA class II molecules are composed of two non-covalently linked subunits, the α- and the β-chain that comprise a total of four domains named α1, α2, β1 and β2. The HLA class II groove is formed by the α1 and β1 domains and allows the binding of peptides longer than 10 amino acids (Fig. 6b). The α2 and β2 domains contain transmembrane segments that permit the anchorage of HLA class II molecules to the cell surface. CD4 T helper (T_h) cells are involved in the recognition of peptides bound to HLA class II molecules. CD4 serves as co-receptor for the TCR signalling and binds the β2 domain of HLA class II antigens (Schafer et al. 1995, Springer et al. 1977).

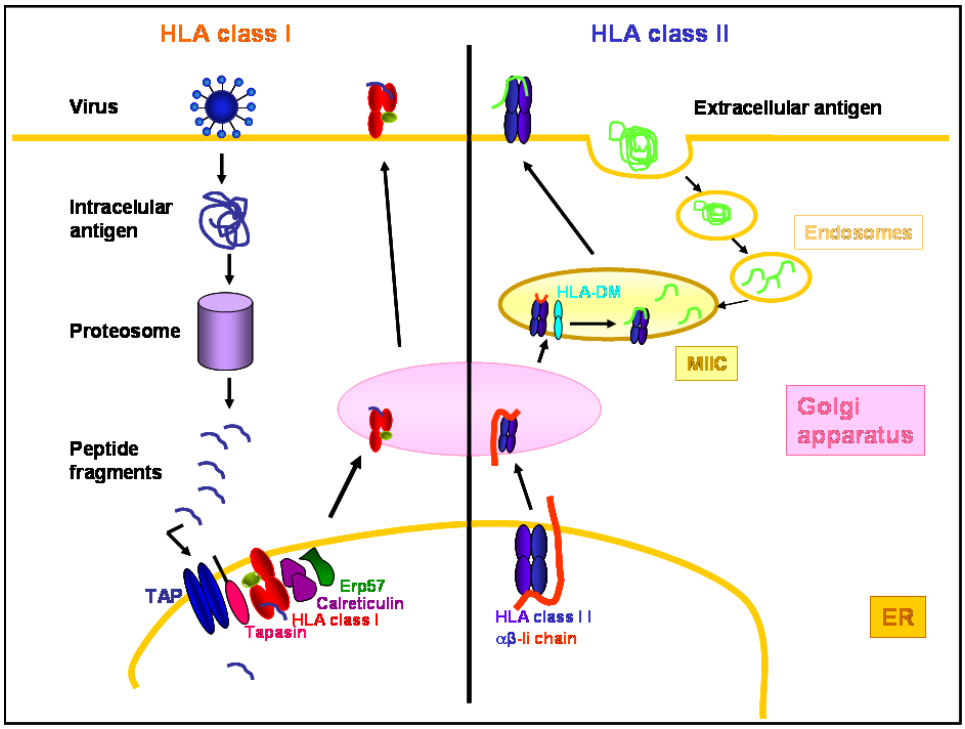


Figure 7. Schematic representation of the assembly and intracellular traffic of HLA molecular complexes.

HLA class I molecules are loaded with peptides derived from intracellular antigens that were degraded in the cytosol by the proteasome and internalized into the ER, where HLA class I molecules are assembled. In contrast, HLA class II molecules present peptides derived from extracellular antigens that were internalized into the APC by endocytosis. HLA class II associates with Ii in the ER and is transported to MIIC compartments, the site of peptide loading. HLA-DM molecules are required for the generation of stable HLA class II-peptide complexes.

HLA class I molecules are expressed in virtually all cell types and evolved to confer resistance to viral infections. Those molecules present peptides derived from proteasomal cleavage of intracellularly synthetized antigens. On the other hand, the constitutive expression of HLA class II antigens is restricted to professional antigen presenting cells (APCs), which include dendritic cells, B cells and macrophages. Professional APCs are also characterized by the constitutive expression of costimulatory molecules. In contrast to HLA class I, HLA class II molecules bind antigens derived mainly from extracellular pathogens that were internalized into the APCs by endocytosis, phagocytosis or macropinocytosis (Chaperot et al. 2000, Heath and Carbone 2001).

T Lymphocytes

T cells are lymphocytes that have their origin in the bone marrow but migrate to the thymus for maturation and during this phase are known as thymocytes. In the thymus, the proteins related with T cell function such as CD4, CD8, the CD3 complex and TCR will start to be expressed. Two lineages of T cells undergo differentiation in the thymus, the majority in number are α:β T cells and a minority maturate as γ:δ T cells. The dynamic relocation of lymphoid progenitors into, within and out of the thymus offers an exposition to multiple environments that determine the generation of functional mature T cells. Thymocyte development involves a stringent repertoire selection in which only 1-3% of thymocytes succeeds in survival and export from the thymus (Benz et al. 2004, Joachims et al. 2006, Lauritsen et al. 2006).

In the thymus, the motility of thymocytes is mediated by the expression of chemokine receptors such as CCR4, CCR7, CCR9 and CXCR4. The differential expression of those receptors allows the co-localization of thymocytes with specific thymic microenvironments (Beismann-Driemeyer and Tampe 2004).

Lymphoid progenitor T cells begin their development into T cells through the development pathway that is commonly identified by the expression profiles of CD25 and CD44. The intial thymocyte development until the CD4-CD8-CD25+CD44- stage (CD4 and CD8 double negative stage) is promoted by signals provided by the cortical thymic epithelial cells (cTECs) which comprises Notch-mediated signals delivered by the binding of Delta ligands and signals

delivered by IL-7 (Laky et al. 2006, Lehar et al. 2005, Zamisch et al. 2005). Double negative thymocytes begin to rearrange their Tcrb locus in the thymic cortex. The successful thymocytes in generating the in-frame Tcrb rearrangement begin assembling TCRβ and pre-TCRα chain. The cell surface expression of the pre-TCR complex along with Delta-Notch interaction provides the signals necessary for the further development into $CD4^+CD8^+$ double positive (DP) thymocytes (Laky et al. 2006).

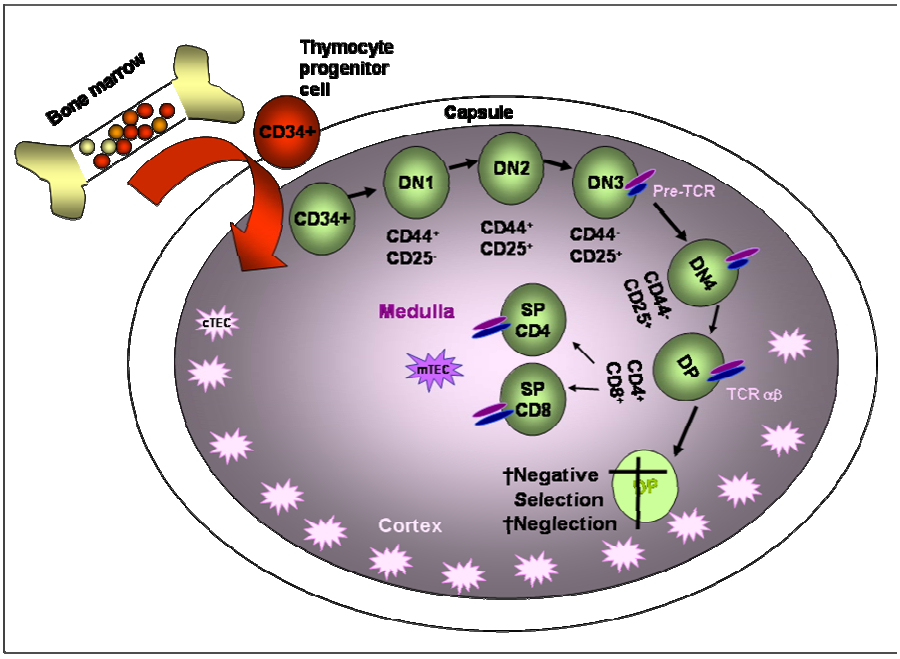


Figure 8. T cell maturation in the thymus.

A committed hematopoietic stem cell migrates from the bone marrow to the thymus, where the maturation process begins. During differentiation, the thymocytes move from the subcapsular region to the thymic cortex where they are positively selected. The double-positive thymocytes that survive to the positive selection migrate to the medullar region where a negative selection process will eliminate the thymocytes that have escaped this process in the cortex. Then the thymocytes differentiate to a single-positive stage and leave the thymus as fully functional, although naïve T cells.

Natural Killer cells

Human Natural Killer (NK) cells constitute 10% of the lymphocytes circulating in the blood. They are phenotypically characterized by the surface expression of CD16, CD56 and lack of CD3 antigens. Conversely to T and B cells, NK cells do not express clonally distribution of antigen-specific receptors (Costello et al. 2004, Draghi et al. 2005, Farag et al. 2002).

Natural killer cells were first identified by their ability to kill cancer cells and virus infected cells known to have lost partially or completely MHC class I antigens. In the last decade, it was shown that NK cells express two distinct families of receptors involved in the fine regulation of NK cell-mediated cytotoxicity. The first family comprise the immunoglobulin-like NK receptors (KIR, LIRL, NCRs, p75/AIRM1, IRp60, 2B4/CD244, NTB-A, DNAM1/CD266 and LAIRL). C-type lectin-like receptors constitute the second family of NK cell receptors, which are in humans represented by CD94/NKG2, NKG2D, NKp80 and NKRP1 (Carbone et al. 2005, Demanet et al. 2004, Verheyden et al. 2004). These receptors are characterized by triggering either activating or inhibitory signals necessary for the NK cell function.

Several NK cell receptors recognize HLA class I antigens (KIR, CD94/NKG2 and LIRL). Those receptors are distributed through the NK cell population and their interaction with HLA class I alleles expressed by the target cells prevent NK cell cytotoxicity. In contrast to the TCR, KIRs have a limited view of HLA class I polymorphisms. Only the HLA class I specificities HLA-A3, HLA-A11, HLA-Bw4, HLA-Cw1 and HLA-Cw2 epitopes, are recognized by NK cells and, contribute to the inhibition of NK cell cytotoxicity. Nevertheless, it was shown that at least one inhibitory receptor for a self HLA class I molecule is expressed in all NK cell clones. The decision of kill is based on a balance provide by activating and inhibitory NK cell receptors (Brumbaugh et al. 1998, Demanet et al. 2004, Farag et al. 2002, Ljunggren and Karre 1990) (Fig. 9).

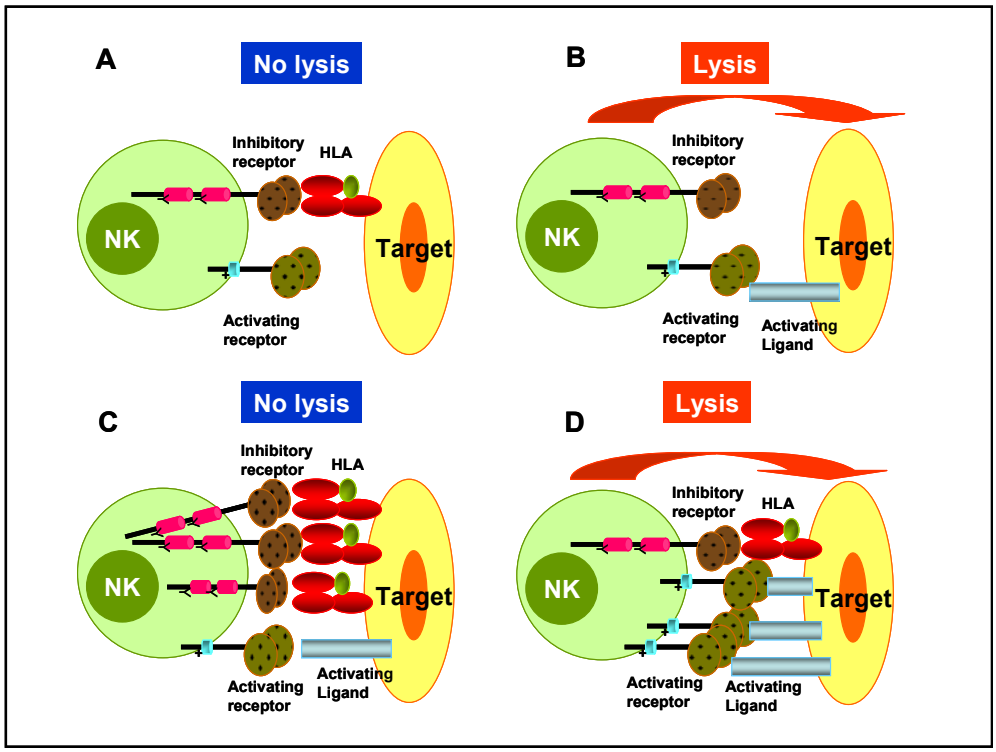


Figure 9. Balance of inhibitory and activating signals in NK cell cytotoxic response.

Inhibitory receptors (e.g. CD94/NKG2A or inhibitory KIRs) bind HLA class I molecules in the surface of their targets and deliver an inhibitory signal. Activating receptors (CD94/ NKG2C, CD94/NKG2D or activating KIRs) bind HLA or non-HLA ligands and trigger an activating signal which results in the target cell lysis. (A) When the target cell presents a ligand to the inhibitory receptors on the NK cell and lacks activating ligands, the cytotoxic NK cell response will be inhibited. (B) In contrary, when the target cells only display ligands for the NK cell activating receptors, they will be lysed due to lack of inhibitory signals. (C) When inhibitory receptor/ligand interactions predominate over the activating signals, the targets are protected from lysis. (D) Conversely, if the activating signal is stronger than the inhibitory signal, the cell targets are lysed (adapted from Farag, 2002).

Immunology of transplantation

Cellular rejection

Alloreactive T lymphocytes are obligatory mediators of allograft rejection and are present at extremely high frequencies in the T cell repertoire of an individual. Between 0,1% and 10% of the T cell repertoire are able to react against alloantigens compared to less than 1/100.000 for nominal peptide antigens (Heeger 2003).

The ability of a T cell to acutely or chronically damage the graft is conferred by previous priming, whereby a naive T cell undergoes activation and clonal expansion after stimulation by the alloantigen on a surface of an APC. Nevertheless, self-restricted T cell clones can also recognize allogeneic HLA/ peptide complexes expressed on the allograft, independently of which peptide is contained in the binding groove. Two main pathways of allorecognition are considered to be involved in T cell priming which further leads to transplant injury. Studies performed over the last two decades have identified two distinct allorecognition pathways: the direct allorecognition and the indirect pathway (Benichou et al. 1999, Heeger 2003).

Direct pathway of allorecognition

Donor DCs can directly prime $CD4^+$ or $CD8^+$ T cells through the recognition of HLA/ peptide complexes present on the donor cells and not endogenous to the recipient. The overall three-dimensional structure of all HLA molecules is similar, regardless of the high number of polymorphic residues occur in the peptide binding groove. The low affinity of any given TCR for its ligand permits recognition of more than one HLA/ peptide complex by each T cell. Thus, the high frequency of alloreactive T cells in the host is expected to be due to molecular mimicry (cross-reaction of a specific TCR with an allo-HLA/ peptide complex). Even in HLA matched donor-recipient pairs, direct recognition might occur, triggered by donor derived minor histocompatibility antigens, resulting from proteosomal processing of polymorphic molecules shared by the donor and recipient (Wallgren et al. 2005) (Fig. 10).

Alloreactive T cells directly primed by donor DCs produce a type-1 proinflammatory cytokine response. Those T cells mediate direct cytotoxicity of

target cells and delayed type hypersensitivity (DTH) reactions, both of which can contribute to a damage of the graft (Ridge et al. 1998, Schoenberger et al. 1998).

Alternatively, immunomodulatory cytokines (i.e. IL-4, TGF-β) and the presence or absence of specific costimulatory signals may lead that naïve alloreactive T-cell precursors become noninflammatory or regulatory T cells, promoting graft tolerance.

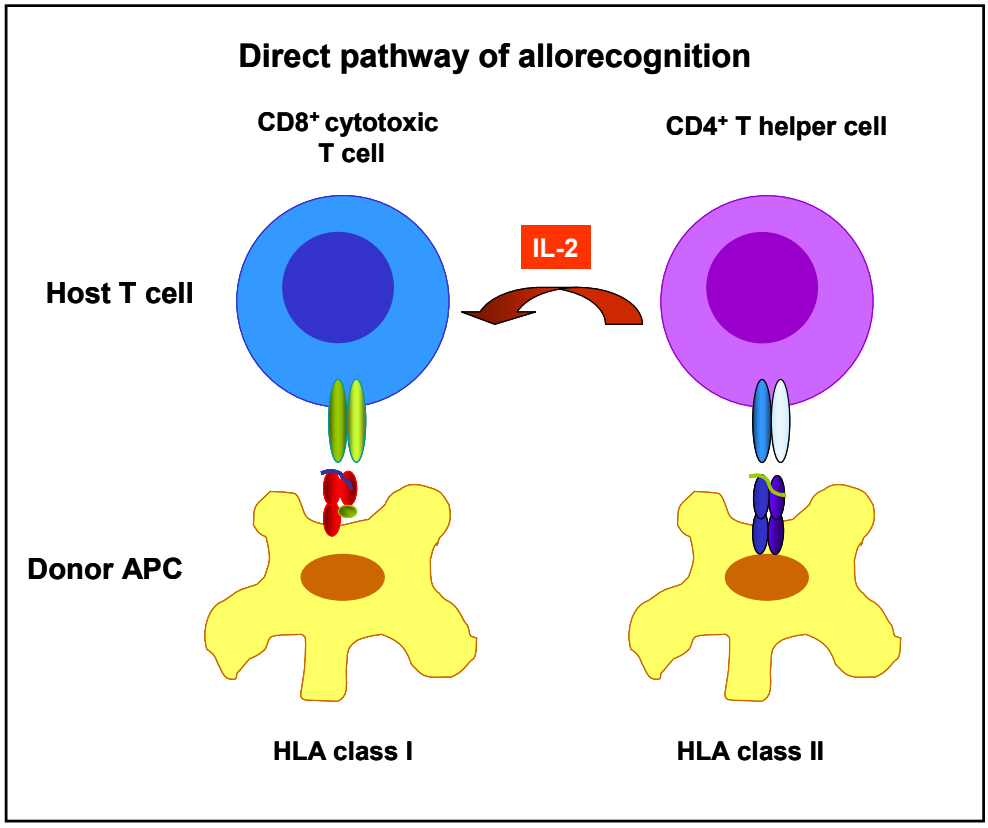


Figure 10. Direct pathway of allorecognition.

Primed effector T cells can directly recognize donor-derived peptides on HLA class I molecules presented by donor antigen presenting cells (APCs) or endothelial cells. The direct allorecognition pathway might receive help from cytokine-derived signals provided by the indirect pathway (right).

The direct allorecognition pathway is likely to be time-limited, because donor APCs are destroyed during the priming process. However, it will lead to the formation of memory T cells, which can virtually reactivate in absence of new direct priming at any time-point of post-transplantation. Non-hematopoietic cells may also activate $CD8^+$ T cells by the direct pathway of allorecognition. Indeed, it has been shown the capability of graft endothelial cells, fibroblast or muscle

cells to trigger CD8+ T cell recognition, representing an ongoing source of direct allopresentation (Benichou et al. 1999, Matesic et al. 1998).

Indirect pathway of allorecognition

As proposed by Lechler and Batchelor, an indirect pathway of allorecognition is involved in T cell priming in which donor-derived antigens are processed and presented by recipient APCs to recipient T cells (Batchelor and Lechler 1982). Several mechanisms might promote indirect allopresentation (Fig. 11). Donor-derived peptides bound to HLA molecules on the surface of recipient APCs found in the secondary lymphoid organs might be a consequence of endocytose of donor DCs that migrate to the lymphoid tissue by the recipient APCs. Alternatively, recipient macrophages may penetrate the donor tissue and endocytose donor antigens and present them on recipient HLA molecules to recipient T cells. A third mechanism could have origin in processing and presentation of soluble HLA molecules, released from the transplant, by recipient APCs in the secondary lymphoid organs. Several of the peptides formed during these processes are derived from polymorphic proteins including variable regions of the donor HLA molecules. As those result of endocytose of exogenous material, they are presented by HLA class II molecules on the surface of recipient APCs, which acquire the capability to prime recipient CD4+ T cells (Benichou et al. 1992, Benichou et al. 1997, Lechler et al. 1991). However, an alternative antigen processing pathway allows the presentation of peptides derived from extracellular antigens by HLA class I molecules and therefore might indirectly prime recipient CD8+ T cells. This cross-priming mechanism was originally demonstrated in a skin transplant model by Matzinger and Bevan (Arnold et al. 1995, Matzinger and Bevan 1977, Owens et al. 1984).
Similarly to the direct pathway, indirectly primed CD4+ T cells differentiate into a pro-inflammatory type-1 cytokine secreting phenotype and provide helper signals to activate cytotoxic CD8+ T cells and to induce alloantibody formation, both capable to cause injury to the graft (Heeger et al. 2000).
Recent evidences suggest that the frequency of indirectly primed CD4+ T cells may be correlated with the outcome of the transplant survival, whereby higher

frequencies of primed CD4+ T cells are associated with acute rejection while lower frequencies may mediate graft fibrosis and vasculopathy (Ardehali et al. 2002, Rose 1997, Szeto et al. 2002, Tran et al. 2003).

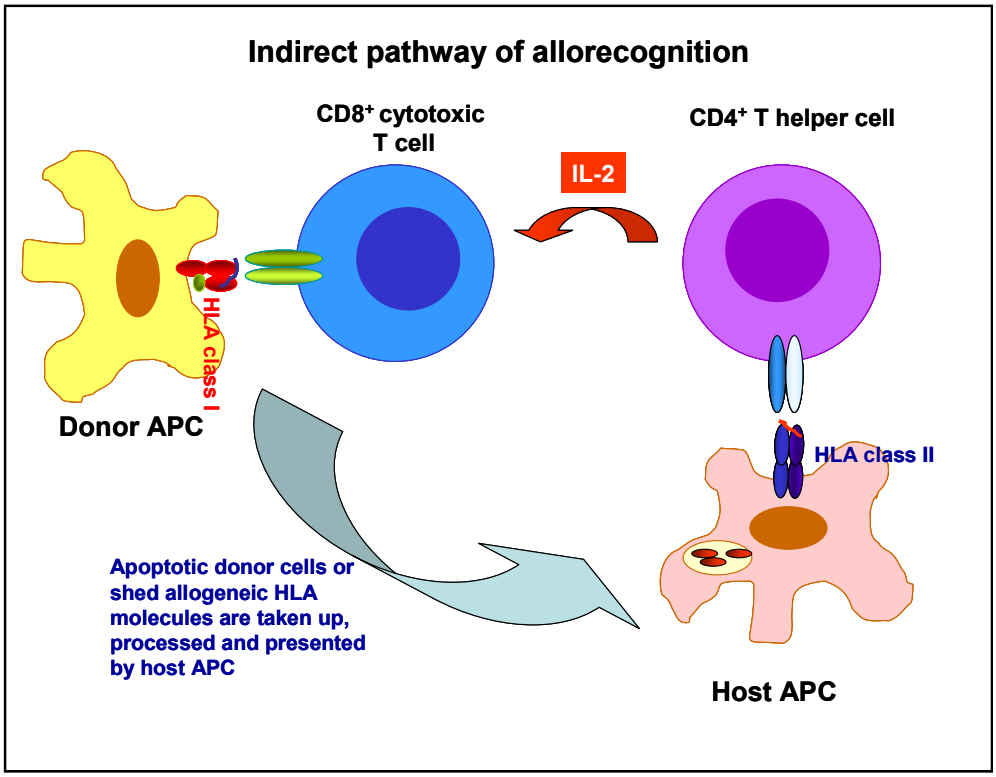


Figure 11. Indirect pathway of allorecognition.

A recipient monocyte/ macrophage can migrate to the donor graft, take up the donor antigen and return to the recipient lymphoid organ to prime T cells. Also, a donor antigen presenting cells (APC) can migrate to the recipient, be endocytosed by a recipient APC (while undergoing apoptosis), resulting in the processing and presentation of donor antigens on the HLA class II molecules. A third possibility involve HLA molecules shed from the graft that can be processed by recipient APCs resulting in donor-derived peptides presented to recipient T cells on recipient HLA class II molecules.

RNA interference

RNA interference (RNAi) is a mechanism similar to the posttranscriptional gene-silencing (PTGS) cosuppression present in lower animals, plants and fungi. The RNAi term arose from the discovery that double-stranded RNA (dsRNA) in the nematode *Caenorhabditis elegans* leads to specific knockdown of transcripts with high homology to the dsRNA (Elbashir et al. 2001).

RNA interference is associated with virus resistence, development control and heterochromatin formation in eukaryotes and has evolved to protect the genome against mobile genetic elements (Elbashir et al. 2001).

Gene silencing mediated by dsRNA is a regulatory process in which the steady-state level of a specific messenger RNA (mRNA) is reduced by sequence-specific degradation of the transcript, without affecting the transcription rate of the target gene. Nevertheless, RNAi may also be elicited by aberrant single-stranded RNA (ssRNA) which may serve as a template for RNA-dependent RNA polymerases, responsible for initiation and amplification of RNAi process (Huppi et al. 2005).

RNAi can be triggered by the delivery of dsRNA in the form of small interfering RNAs (siRNAs), which are duplexes of 19-23-base pairs with two nucleotides 3'-overhangs at both ends. The siRNA duplex contains 5'-phosphates and 3'-hydroxyls. The target mRNA is sequence-specificly degraded in a siRNA-programmed effector complex denoted as RNA induced silencing complex (RISC) (Elbashir et al. 2001, Elbashir et al. 2002, Zamore et al. 2000). One of the RISC proteins is a member of the Argonaute family (in humans, Ago2/ EIF2C) containing a Piwi/ Argonaute/ Zwille (PAZ) domain, which binds RNA. The helicase activity of RISC promotes the undwind of the siRNA duplex which allows the load of the resultant ssRNA into the RISC. This step induce the activation of RISC, which will be guided toward the target mRNA by the loaded antisense strand. After hybridization with the target, the ribonuclease activity of RISC will cleave the mRNA strand at a position approximately 10 nucleotides from the 5'-end of the antisense siRNA sequence. Without the protection of 5'-cap and 3'-polyA tail, the target mRNA is quickly degraded. *In vivo*, siRNAs are the product of Dicer activity, but RNAi can be also mediated by the transfection of synthetic siRNAs (Elbashir et al. 2001, Harborth et al. 2003, Huppi et al.

2005, Williams and Rubin 2002, Zamore et al. 2000).

The selection of siRNA sequences capable of efficiently and specifically knockdown the target expression is a central step required for the use of RNAi in the development of therapeutic approaches. In the following some important points to consider for the design and selection of siRNAs are described:

- Asymetric loading of antissense strand – Previous studies showed that the strand most easily unwound in the 5'-3' direction will be preferentially assembled with RISC. Some nucleotide combinations at specific positions which increase stability for nucleotides 5-10 of the 5'-3' antisense strand have demonstrated higher loading probabilities into RISC. Nevertheless, the significance of these observations is still under discussion.
- Targeting polymorphic regions – It might be important to consider the frequency and position of single nucleotide polymorphisms (SNPs) as well as alternatively the presence of splicing sites to ensure the same efficacy of the siRNA sequence within the same species.
- Off-target effects – The efficiency of reducing the target expression mediated by a mismatched siRNA effector sequence is significantly reduced compared with a fully matched sequence. However, it was shown that non-target transcripts with 11 consecutive nucleotide matches with the siRNA sequence may also be downregulated causing off-targets effects. The selection of siRNA sequences should avoid consecutive matches in particular between nucleotides 2-12 of the siRNA antisense strand. The likelihood of off-target effects mediated by the interaction of the siRNA sense strand with non-target mRNA should be considered.
- Non-specific dsRNA responses – Mammalian cells are able to trigger several non-sequence specific dsRNA dependent responses. Some proteins such as protein kinase dsRNA-dependent (PKR) and 2'-5' oligoadenylate synthetase play critical roles in those responses. These unspecific responses are generally overcome by shortening the antisense strands (e.g. by using siRNAs) and by reducing the concentration of the RNAi effector used. The most common dsRNA response is mediated by the interferon-γ pathway which results in an unspecific reduction of all transcripts.

The first applications of RNAi in mammalian cells have used chemically synthesized siRNAs (Elbashir et al. 2001). However, the low transfectability of some cell types and the transient silencing effect led to the construction of plasmid or viral-based vectors containing RNAi expression cassettes, for a stable delivery of short hairpin RNAs (shRNAs). The RNAi cassette used in vector constructs consist of a PolIII promoter, two complementary siRNA sequences (sense and antisense strand) separated by a linker sequence (which is intracellular removed by intracellular processing) and an appropriate terminator sequence (Ardehali et al. 2002).

Stable HLA silencing

To overcome the transient effect of synthetic siRNAs, we constructed VSV-G pseudotyped lentiviruses to deliver HLA-A gene-specific or ß2m-specific shRNAs into HeLa cells (typed as homozygous for HLA-A28). Lentiviral constructs were transduced with a multiplicity of infection (MOI) of 3. HeLa cells stably expressing HLA-A-specific shRNA reduced HLA-A surface expression by up to 70% after 3 months in culture compared to the expression rate in non-transduced cells (Fig. 12a). HeLa cells transduced with lentiviruses encoding ß2m-specific shRNAs suppressed ß2m protein expression by 90% (Fig. 12b). B-LCL cells expressing gene-specific shRNAs showed a reduction of HLA-A expression by up to 80% (Fig. 12c). B-LCLs expressing HLA class I-specific shRNAs showed a reduction of ß2m expression by up to 70% (Fig. 12d).

HLA-A expression remained unaffected in HeLa and B-LCL cells stably expressing nonsense shRNAs or shRNAs directed against lamin A/C (Fig. 12a-d). Silencing of lamin A/C in HeLa and B-LCL cells transduced with the lamin A/C-specific vector was determined by western blot after 3 months (data not shown). Lack of inhibition of ß-actin expression in HeLa and B-LCL cells transduced with HLA-A heavy chain- or ß2m-specific shRNAs (Fig. 12 e and f) as well as lack of inhibition of HLA-B expression in HeLa cells transduced with HLA-A heavy chain specific shRNAs (data not shown) documented the specificity of the shRNA sequences used.

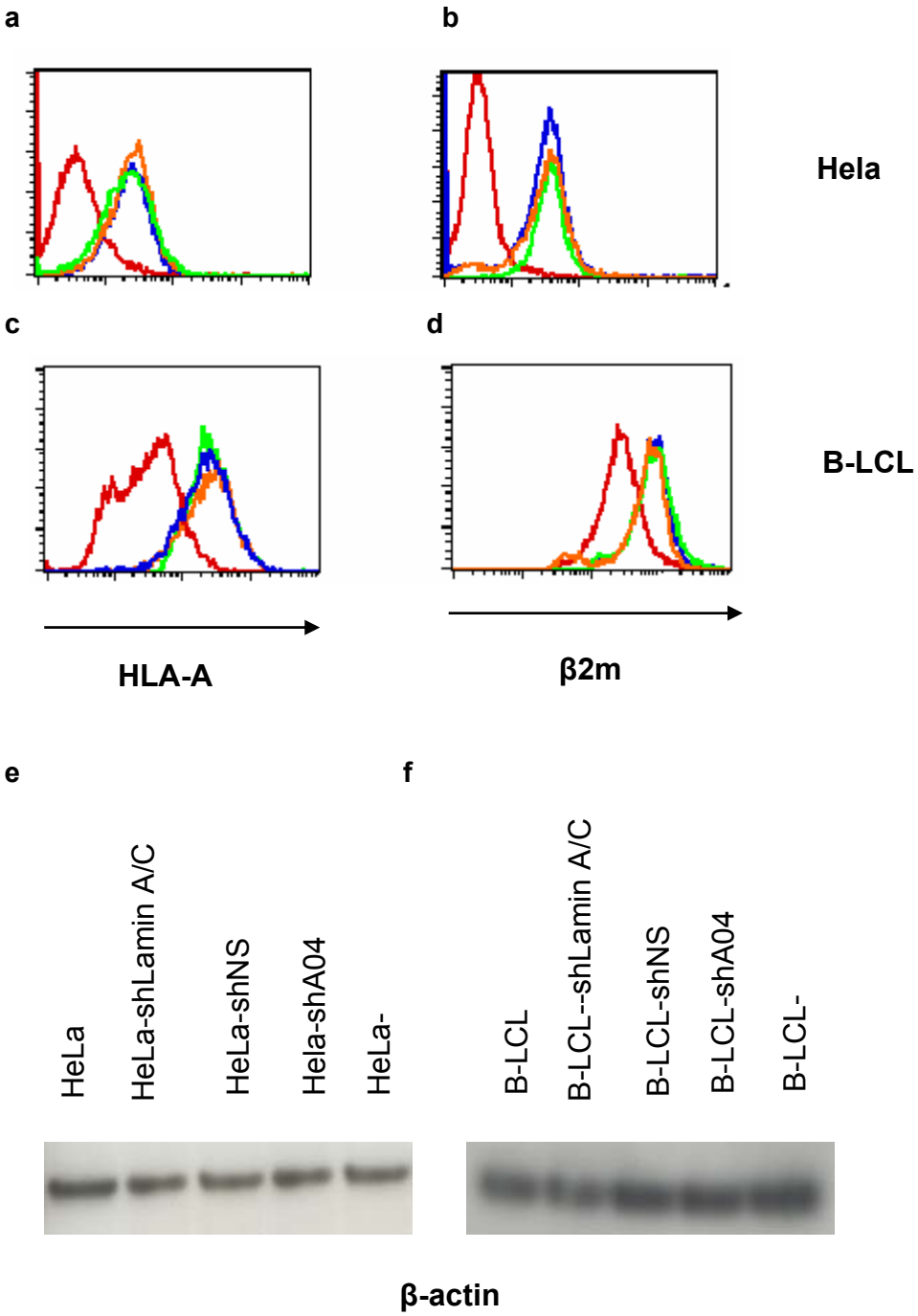


Figure 12. Lentiviral-mediated HLA silencing.

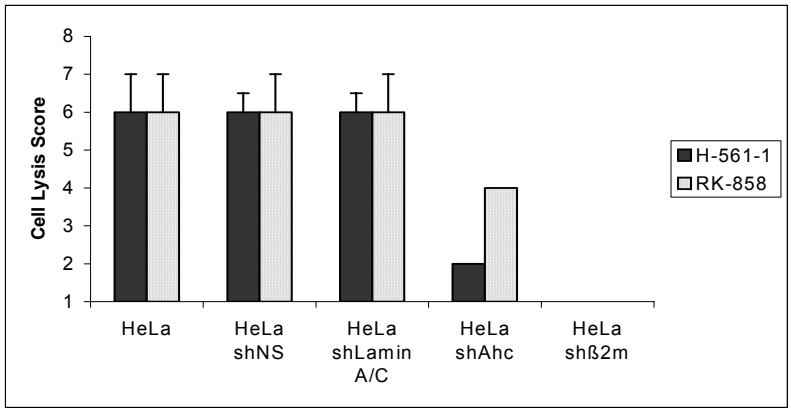
Flow cytometry results obtained with HeLa (a and b) and B-LCL (c and d) cells expressing short-hairpin RNAs (shRNAs) specific for the HLA-A heavy chain (a and c) or ß2-microglobulin (ß2m) (b and d) are indicated in red curves. Cells in figures 12 a and c were stained with a FITC-labeled monoclonal antibody having HLA-A28 specificity to detect HLA-A allele-specific expression. In figure 12 b and d, an anti-ß2m FITC-labeled antibody was used to evaluate ß2m expression. Non-transduced cells (blue curve) and those transduced with vectors encoding nonsense shRNAs (orange

curve) or with shRNAs specific for lamin A/C (green curve) were used as positive and negative controls, respectively. (e and f) In Western blot analysis, β-actin levels did not change in HeLa and B-LCL cells expressing the different shRNAs. HeLa/B-LCL, native cells; shAhc, cells expressing shRNAs specific for the HLA-A heavy chain; shß2m, cells expressing shRNAs specific for ß2m; shLamin A/C, cells expressing shRNAs specific for lamin A/C; shNS, cells expressing non-specific shRNAs.

Microcytotoxicity assay

As HLA antigens of transplanted cells or tissues are targets for humoral rejection, the effect of HLA silencing was determined in a complement-dependent MCT. HeLa and B-LCL cells (both homozygous for HLA-A28) non-transduced or expressing different types of shRNAs were either incubated with polyclonal or monoclonal HLA anti-sera specific for HLA-A2,28. The results are summarized in Figure 13. Cell lysis in non-transduced HeLa and B-LCL cells and cells expressing lamin A/C-specific or nonspecific shRNA was around 80-100% (mean score of 6-8) when polyclonal sera were used and 100% (mean score of 8) when incubated with monoclonal antibodies. However, for HLA-suppressed HeLa and B-LCL cells stably transduced with HLA-A gene-specific shRNAs the cell lysis was only 11 to 40% (mean score of 2-4) when polyclonal sera were used and 40% (mean score of 4) when incubated with monoclonal antibodies. For HeLa and B-LCL cells stably transduced with ß2m-specific shRNAs (indirectly HLA class I specific) the cell lysis was only up to 20% (mean score of up to 2) when polyclonal sera and the monoclonal antibody were used.

a


Cell Lysis Score
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7
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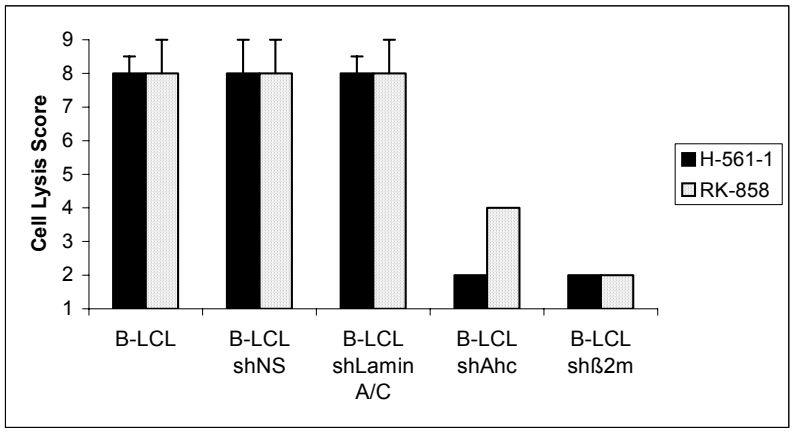
HeLa shNS
HeLa shLamin A/C
HeLa shAhc
HeLa shß2m
H-561-1
RK-858

b


Cell Lysis Score
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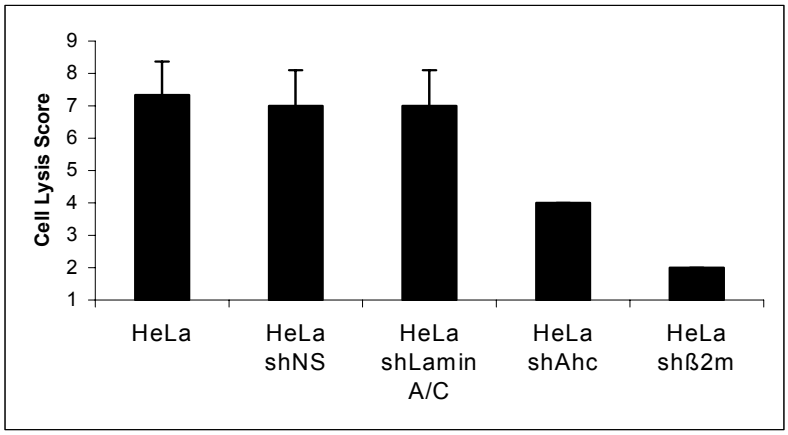
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HeLa shß2m

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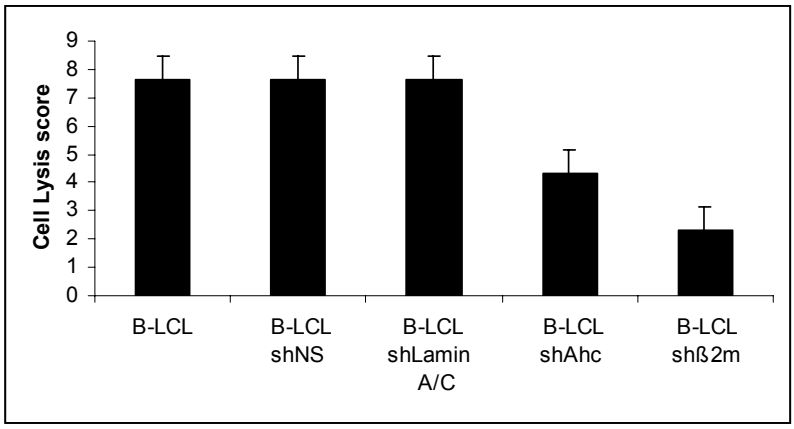
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7
6
5
4
3
2
1
0
B-LCL
B-LCL shNS
B-LCL shLamin A/C
B-LCL shAhc
B-LCL shß2m

Figure 13. Complement-dependent microcytotoxicity assay.
A complement-dependent microcytotoxicity assay was performed with HeLa and B-LCL cells (all homozygous for HLA-A28). Non-transduced cells and cells transduced with lentiviruses encoding nonsense (shNS), HLA-A heavy chain-specific (shAhc), lamin A/C-specific (shLamin A/C) or ß2-microglobulin-specific (shß2m) short-hairpin RNAs were stained with anti-HLA-A28 patient sera (H-561-1, RK-858) (a and b) or a monoclonal IgM antibody having HLA-A2,28 specificity (c and d). The percentages of complement-lysed cells are scored on a scale of 1 to 8 (score 1: 0-10%, 2: 11-20%, 4: 21-40%, 6: 41-80%, 8: 81-100%). The mean values obtained from eight experiments are shown.

NK cell cytotoxicity

NK cell cytotoxicity assays were performed by incubation of NK cells with native K562 cells (NK cell lysis susceptible), K562-A11 cells or K562-A11 cells expressing shß2m06 (90% reduction in HLA-A11 expression, data not shown). Freshly isolated NK cells lysed HLA-deficient K562 and HLA-class I silenced K562-A11 cells by up to 96 and 92%, respectively, whereas a 41% lysis was detectable in HLA-A*11 expressing K562 cells (K562-A11) (Fig. 14 a-c). When pre-stimulated NK cells were used, they lysed up to 90% of HLA-deficient K562 and HLA-class I silenced K562-A11 cells (Fig 14 d, f). In contrast, expression of HLA-A*11 in K562-A11 inhibited cell lysis by pre-stimulated NK cells (Fig 14 e). In the NK cells cytotoxicity assays with B-LCLs only freshly isolated NK cells were used as effector cells. NK cell-mediated cell lysis of up to 95% was observed in HLA-deficient LCL721.221 cells (Fig. 14 g), whereas almost no lysis was detectable when non-transduced B-LCLs were used as target cells (Fig. 14 h). Only a minimal NK cell cytotoxicity of 10% was observed when HLA class I suppressed B-LCLs were used (Fig. 14 i).

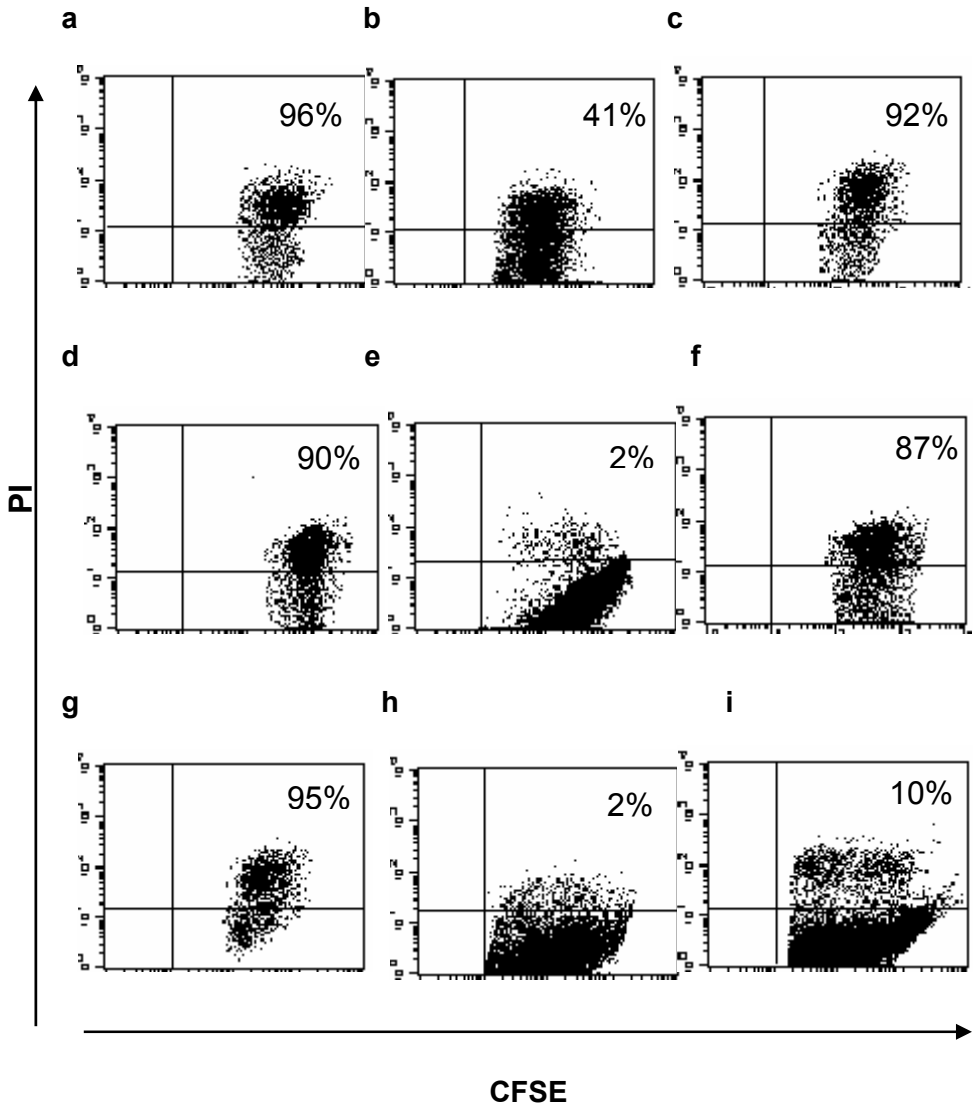


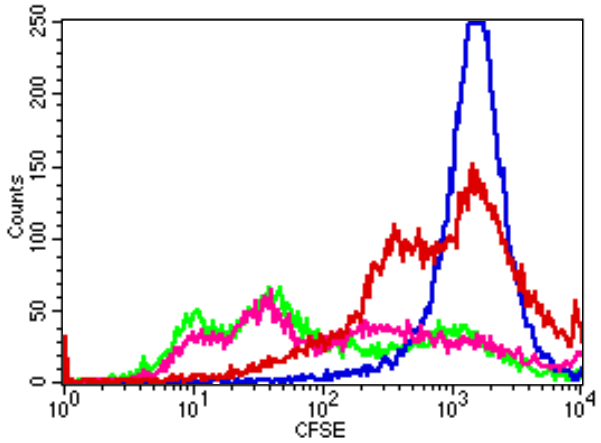
Figure 14. NK cell cytotoxicity assay.

Freshly isolated NK cells were incubated with (a) native K562 cells, (b) K562 expressing HLA-A11, (c) K562-A11 expressing shß2m06, (g) LCL721.221, (h) native B-LCL cells, and (i) B-LCL cells expressing shβ2m06. NK cells pre-stimulated by incubation with HLA-A*11 expressing K562 cells (K562-A11) for 48h were incubated with (d) native K562 cells, (e) K562 expressing HLA-A11, and (f) K562-A11 expressing shß2m06. Target cells were labeled with CFSE. Only the target cells are shown in the dot plot. Cellular cytotoxicity was evaluated by PI staining.

T cell response

T cell proliferation and IFN-γ secretion assays were used to assess $CD8^+$ T cell response against allogeneic cells. Primed $CD8^+$ T cells strongly proliferated in presence of either non-transduced B-LCLs or B-LCLs transduced for nonsense shRNA expression (3 cycles of cell division). $CD8^+$ T cells showed only 1 cycle of cell division when exposed to HLA-class I silenced B-LCL cells (Fig. 15a). In presence of allogeneic B-LCL cells presenting normal levels of HLA class I expression, CD8+ T cells produced IFN-γ levels of up to 8596 pg/ ml compared to levels of 459,3 pg/ ml and 31 pg/ ml when incubated with HLA class I-suppressed and HLA class I-deficient cells, respectively (Fig. 15b).

a



b

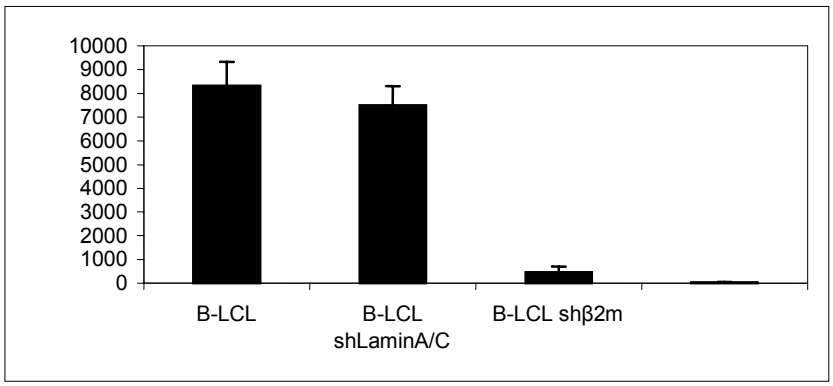


Figure 15. T cells assays.

$CD8^+$ T cell proliferation assay. Pre-stimulated effector T cells were labeled with CFSE and incubated with different target cells: native B-LCLs (green), B-LCLs expressing shLaminA/C (pink), B-LCLs expressing shß2m06 (red) or HLA class I-deficient LCL721.221 cells (blue) (a). Interferon-γ secretion assay. Interferon-γ secretion was quantified by ELISA in culture supernatants (b).

Knockdown of MHC Class I in marmoset fibroblasts CM0203F

As a basis for preclinical studies, establishment of methods in a clinical relevant animal model is unavoidable. Mice are certainly the most commonly used animal model. The transfer of results from a mouse-model to human is difficult because of the phylogenetic distance between mouse and human. The transfer of large animals models to human is much easier. As such a large animal model, non human primates hold tremendous potential. We therefore transferred the approach of shRNA mediated silencing of MHC class I by targeting ß2m to the well established non-human primate model marmoset (*Callithrix jacchus).*

Fibroblasts of marmosets were transduced with lentiviral vectors containing shRNAs against MHC class I. Transduction of fibroblasts was performed to establish the method of MHC silencing in marmoset cells and to test different shRNAs. The results obtained should later be tranferred to silencing MHC in marmoset ESC.

The shRNAs used were designed by comparing sequences of MHC of human and rhesus monkey since the genome of the common marmoset is not described to much detail yet. Human and rhesus monkey are phylogenetically close to marmoset and one can assume a high homology in the sequence of MHC between the species.

To find a highly effective siRNA we designed five different shRNA sequences targeting ß2m. All lentiviral vectors with specific siRNA against MHC class I were tested in marmoset fibroblasts. Two of the five siRNAs did not reduce the MHC class I expression in the transduced cells. The other three siRNA cassettes were effective in silencing MHC class I. Silencing of MHC class I up to 85% was performed with siRNA ß2m02 (Fig. 16+17).

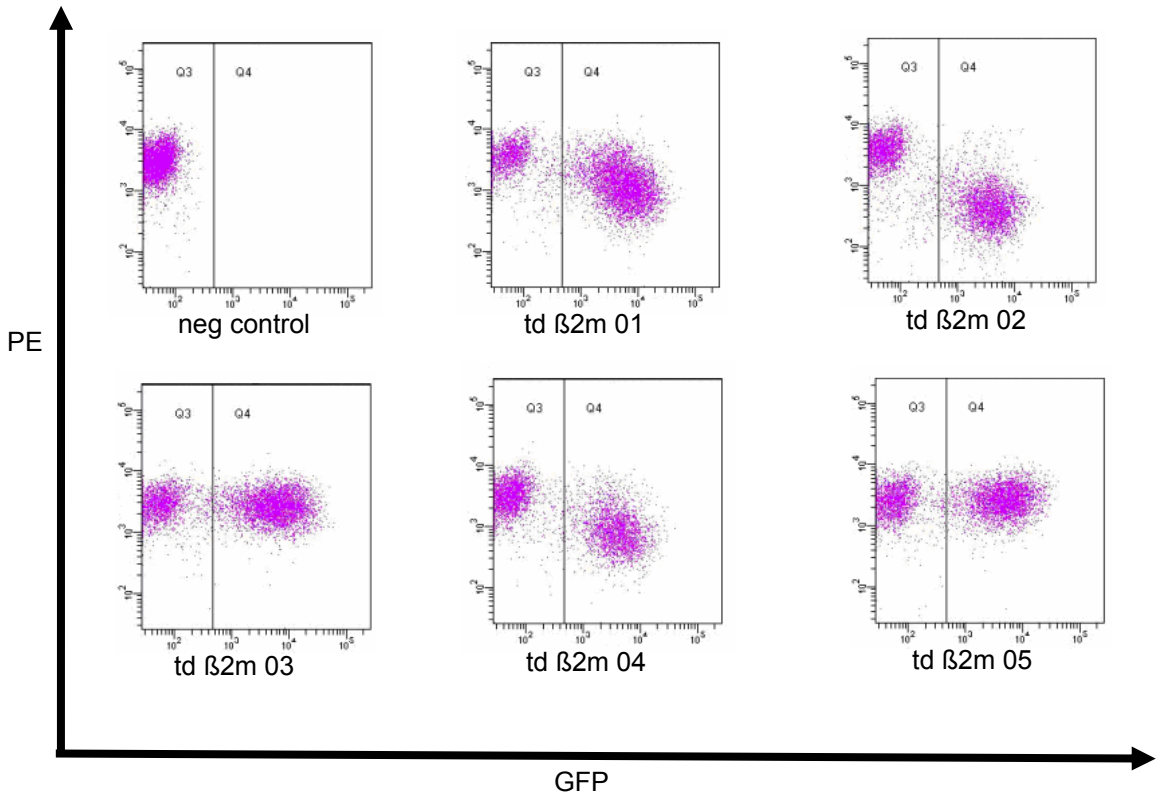


Figure 16. FACS analysis of MHC Class I expression in marmoset fibroblasts. Transduction of RNAi cassettes containing the sequences for shRNAs targeting ß2m suppressed MHC class I protein expression in marmoset fibroblasts. Silencing of MHC class I on protein level is up to 85% with siRNA ß2m02. The two siRNAs ß2m03 and ß2m05 did not result in a detectable decrease in MHC expression. Highest reduction of MHC class I expression could be measured with siRNA ß2m02.

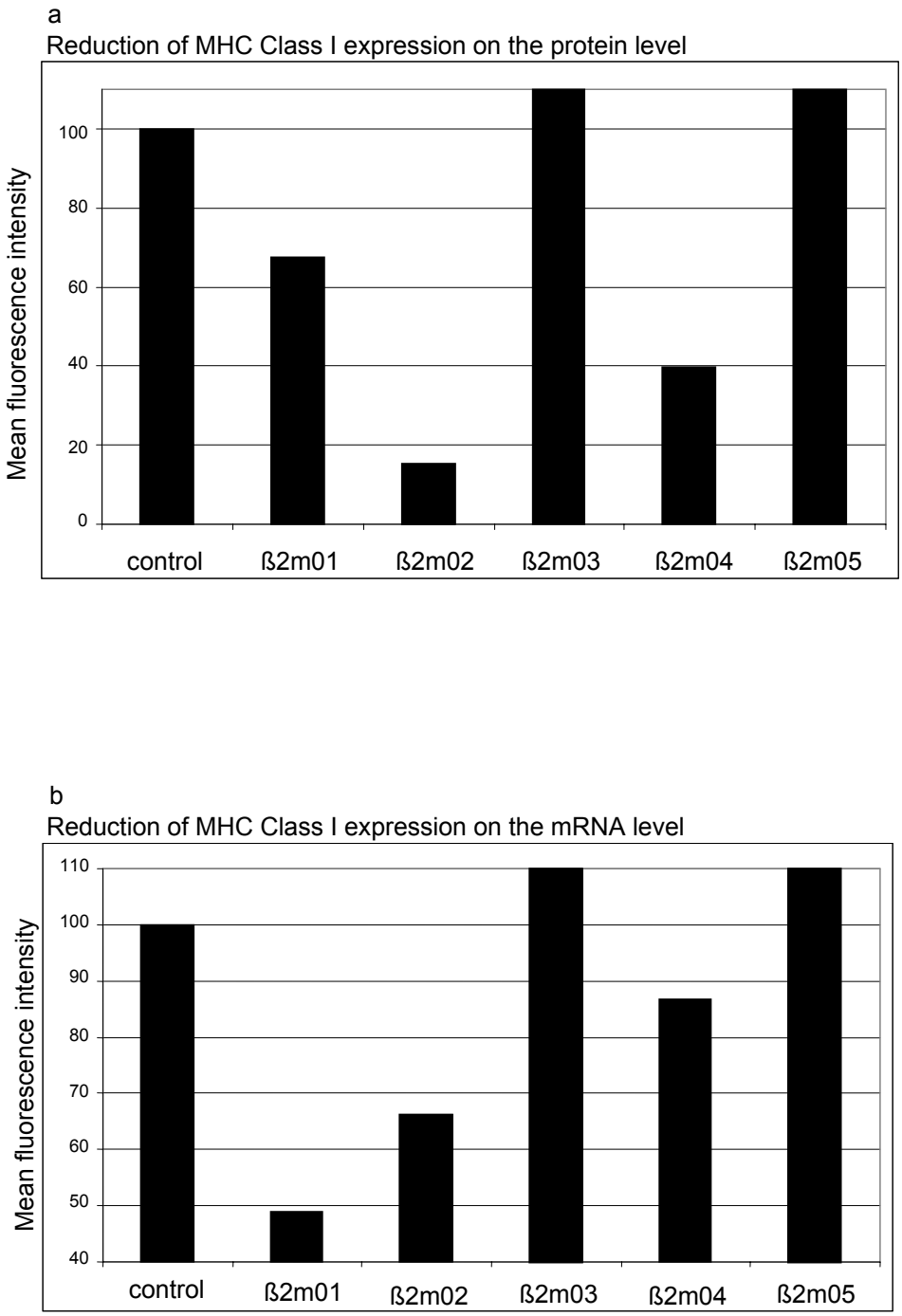


Figure 17. Reduction of MHC Class I expression in marmoset fibroblasts.

On the protein level measured by flow cytometry, MHC class I expression in marmoset fibroblasts could be reduced by up to 85% (a). The reduction was confirmed by real-time RT-PCR on the mRNA level. However, the level of reduction in expression was overall lower (b).

Knockdown of MHC class I in marmoset ESC cjes001

Results obtained of MHC reduction in marmoset fibroblasts were subsequently transferred to marmoset ESC. As described above, ESC hold tremendous potential for regenerative medicine approaches. Marmoset ESC serve as a large animal model to establish methods. shRNA used for silencing MHC class I expression were transduced in marmoset ESC by lentiviral transduction. EGFP was used as a reporter gene to quantify transduction efficiency. Efficient transduction was achieved for all different siRNAs. Three typical examples are shown in figure 18 (a-c).

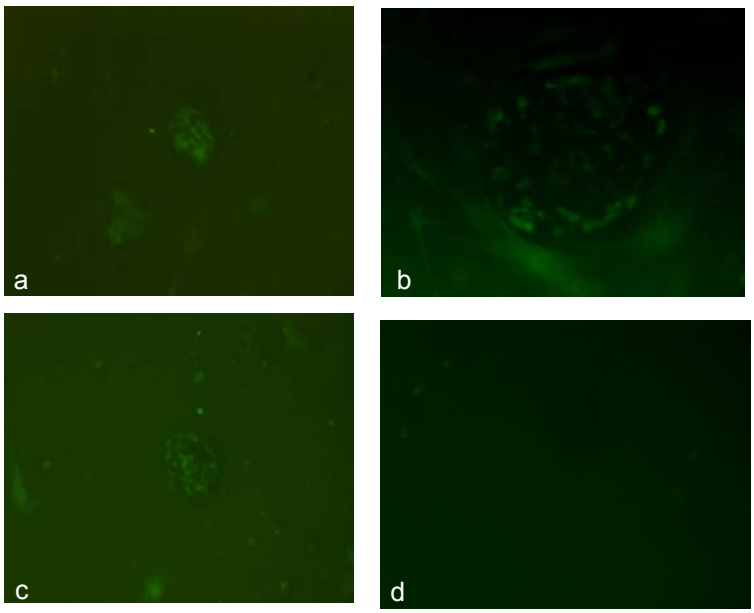


Figure 18. Transduction of marmoset ESC cjes001.
Successful transduction with lentiviral siRNA vectors in marmoset ESC. EGFP was used as a reporter gene. Transduction with the three different siRNA ß2m02, ß2m04 and ß2m05 could be shown by microscopy (a-c). Picture d shows the negative control.

Reduction of MHC class I expression on the protein level was decreased up to 42% with siRNA cassettes ß2m02, 04 and 05 as measured by flow cytometry (Fig. 19). The experiment was performed in two independent approaches. Downregulation of MHC class I expression on mRNA level was decreased to levels between 35-70% with siRNA ß2m 02 and 04 (Fig. 20).

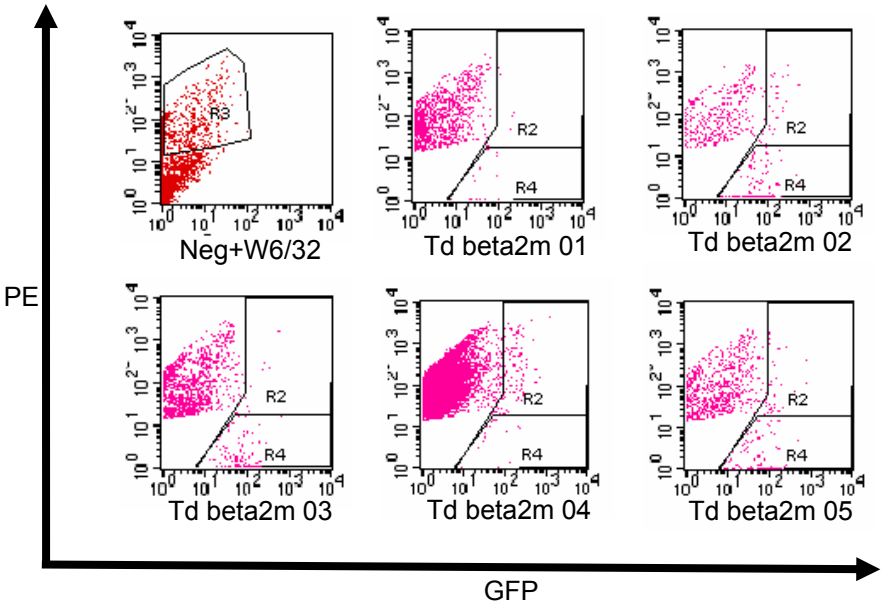


Figure 19. FACS analysis of MHC Class I expression in marmoset ESC cjes001. Successful reduction of MHC Class I expression with siRNA cassettes ß2m02, 04 and 05 in cjes001 up to 42%.

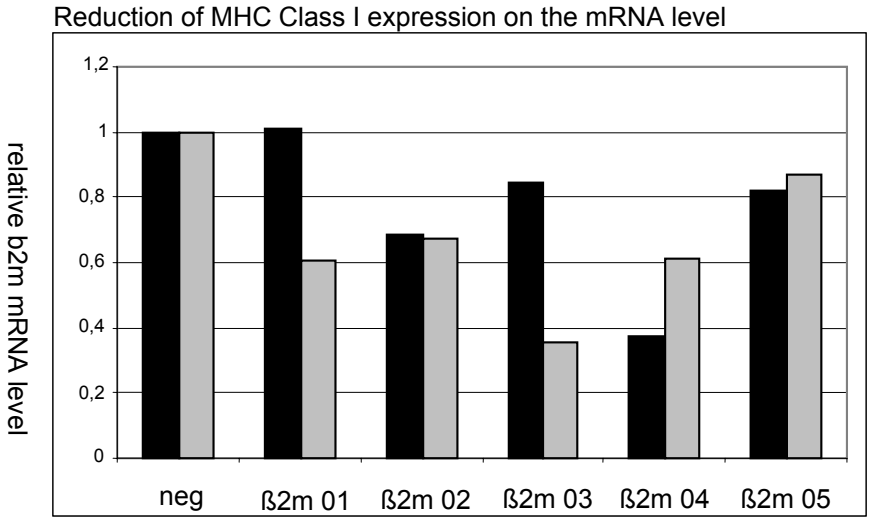


Figure 20. Analysis of MHC Class I expression in marmoset ESC cjes001.
The two different colours shows two independent experiments of silencing MHC Class I in cjes001. Reproducible decrease in MHC expression to levels between 35-70% was observed with siRNA ß2m 02 and 04.

CONCLUSIONS

The emergence of cell-based regenerative medicine as a potential therapy for substitution of diseased or injured tissues is intimately correlated with the necessity to inhibit the host immune response to the modified autologous or transdifferentiated allogeneic cells (Passier 2003). MHC silencing can prevent the immune system from recognizing immunogenic peptides in the genetically modified autologous transplant or optimize the matching of recipient and allogeneic donor cells (Ottinger et al. 2002). The principle of the new approach is that not the recipient's immune system but the transplanted donor cells are modified to induce immunologic tolerance in the recipient. Thus, rejection of transplanted cells or tissue can be prevented without the risk of the hazardous side effects associated with a general impairment of the immune system. This strategy would allow for minimizing post-transplant long-term immunosuppressive therapy. Recently, it was shown that silencing MHC

antigens by transfection of RNAi expression cassettes can inhibit T cell mediated immune recognition (Figueiredo et al. 2004, Gonzalez et al. 2005).

This work presents a new strategy for long-term knockdown of MHC using shRNA-lentiviral delivery. MHC class I suppression was carried out in a class-specific way by targeting ß2m. Finally, it was demonstrated that HLA class I knockdown was effective in preventing CD8+ T cell response and that the residual HLA expression in HLA-silenced cells was effective in inhibiting NK cell-mediated lysis.

The development of alloantibody responses to transplanted tissue is one of the most relevant factors contributing to graft injury and rejection. Hyperacute rejection is the classical example of antibody-mediated rejection. Pre-transplant HLA compatibility testing is routinely done using a complement-dependent lymphocytotoxicity test whereby donor lymphocytes are stained with the recipient's serum and rabbit complement. The finding of lysed donor lymphocytes, representing a positive crossmatch, should be interpreted as a contraindication for transplantation (e.g. renal transplantation) or, at least, as a risk factor for graft failure (HSCT) (Abdelgany et al. 2003).

CD8+ T-cell-mediated rejection presents another significant barrier to allogeneic transplantation. Proliferation and interferon-γ secretion assays indicated a markedly reduced CD8+ T cell response against MHC class I suppressed cells when compared to native cells. These results support the hypothesis that MHC silencing might have a relevant impact on decreasing the risk of graft failure. Interestingly, our in vitro data suggest that complete silencing of non-permissive HLA antigens may not be necessary to induce acceptance of otherwise rejected tissues.

The clinical applicability of MHC-silencing also depends on the susceptibility of transplanted cells to NK cell-mediated cytotoxicity. According to the 'missing self' hypothesis, it is the function of NK cells to recognize and eliminate cells that fail to express certain self HLA class I molecules (Ljunggren and Karre 1990). NK cell specificity is determined by a balance of signals generated by various stimulatory and inhibitory receptors (Brumbaugh et al. 1998). Several inhibitory NK cell receptors have specificity for HLA class I allotypes. Our NK

cell assays strongly suggest that the residual HLA antigen expression on HLA-silenced cells is protective against attack by autologous NK cells. However, in an artificial system using as targets K562 cells transduced to express a single HLA class I specificity we observed a partial inhibition with freshly isolated NK cells and an almost complete inhibition when pre-stimulated NK cells were used as effector cells. It might be interesting to further investigate whether modified cells silenced for particular MHC molecules or presenting recombinant MHC can be used for modulation of NK cell activity.

Target sequences within the coding region of ß2m were selected to design siRNAs to silence all MHC class I molecules on the cell surface (MHC class I-specific silencing). Due to the high degree of MHC heterozygosity, the application of a group-specific silencing strategy might even enable allele-specific MHC suppression in the majority of patients exhibiting heterozygosity at the respective MHC locus. From the therapeutic viewpoint, allele-specific silencing might become an important tool in the treatment or prevention of dominant inherited diseases (Miller et al. 2004). Allele- or group-specific silencing is also an attractive possibility in cellular therapeutics as the suppression of a specific non permissive allele might contribute to better engraftment.

An efficient delivery system is crucial for the development of RNAi-based therapy (Van den Haute et al. 2003). A variety of strategies to express interfering RNAs with the use of virus vector-based cassettes have been explored, including retroviral and lentiviral vectors (Abbas-Terki et al. 2002, Devroe and Silver 2002, Scherr et al. 2003). In this study, a lentiviral vector system was used due to its capability to transduce dividing and non-dividing cells and to mediate stable protein suppression by integrating the RNAi cassette into the genome. Persistent silencing of MHC class I molecules was achieved by transducing cells with lentiviral vectors coding for shRNAs that target ß2m. In addition, the fact that the expression of the housekeeping protein lamin A/C remained unaffected over the whole observation period reflects the specificity of the expressed shRNAs.

Transplants might be more susceptible to post-transplant virus and bacteria infections by downregulation of ß2m, since inhibition of virus-derived peptide, microbial lipids or lipopeptides presentation to Cluster of Differentiation 1 (CD1)-

restricted T cells will be indirectly affected (Beckman et al. 1994). Experiments using inducible expression systems with regulatory promoters will have to show whether RNAi-mediated suppression of HLA can temporarily be reversed if therapeutically required.

In conclusion, our data strongly support the idea that MHC expression can be effectively silenced. The possibility to deliver the siRNAs by viral transduction, which offers the advantages of stable transcript reduction and organ selective applicability, provides the basis for exciting new approaches to cell therapy in the field of regenerative medicine.

PERSPECTIVES

The MHC genomic region comprises the most polymorphic loci of the whole human genome. MHC diversity allows the presentation of virtually all intracellular degraded proteins to the effectors of the immune system. This fact constitutes one of the most important hallmarks of our capacity of defence against foreign pathogens and also permits a stringent distinction between the self and the non-self (Krensky 1997). Although, MHC diversity is a central point to the efficacy of the immune function, it constitutes a disadvantage to the transplantation of allogeneic cells, tissues or organs which are recognized by the host as non-self and consequently destroyed (Newberg et al. 1996, Tran et al. 2003). Nevertheless, transplantation is the only treatment available for several haematological malignant diseases, organ failure or situations of loss of specialized cells (e.g. Parkinson's disease, diabetes mellitus). Several factors including the paucity of organs and the difficulty to find a histocompatible donor, strongly limit the possibility to treat those patients. Recently, regenerative medicine appeared as new field that use healthy cells as repair units to restore injured tissues or organs. The lack of sources constitutes a significant hurdle to the development of cellular therapies. A large number of groups have been working in the *in vitro* generation of tissue-specific cells from embryonic or

somatic stem cells, which can be differentiated and propagated into large batches of terminally differentiated cells, when exposed to certain growth factor cocktails (Shufaro and Reubinoff 2004, Strom et al. 2001). Although these approaches circumvent sourcing problems and allow standardization of protocols, a prerequisite for the widespread application of stem cell therapy, they still have to overcome the barrier of allogeneicity (Barrett et al. 2003, Claas et al. 2003, Thomas 1995).

The application of new immunosuppressive regimens has reduced the incidence of acute rejection and has extended the life expectancy of allograft recipients. However, posttransplant malignancy has become an important cause of mortality. The cause of posttransplant malignancy is thought to be multifactorial and likely involves impaired immunosurveillance of neoplastic cells. Although calcineurin inhibitors and azathioprine have been linked with posttransplant malignancies, newer agents such as mycophenolate mofetil and sirolimus have not and indeed may have antitumor properties. However, long-term data are needed to determine if the use of these agents will ultimately lower the mortality due to malignancy for transplant recipients (Bartynski et al. 2005, Cardoso and Oliveira 2005, Webster et al. 2005).

A significant number of experimental studies on tolerance-inducting strategies have been reported, and they can be divided into three major categories: chimerism induction, T-cell depletion and costimulatory receptor blockade. Moreover, it has been shown that regulatory T cells exert a dominant effect in controlling autoimmunity and maintaining peripheral tolerance and are also involved in preventing allograft rejection and graft versus host disease (Boussiotis et al. 1994, Colovai et al. 1996, Sebille et al. 2001, Sun et al. 2004). Among CD4+ T cells, the best described are the naturally occurring CD4+CD25+ regulatory T cells and type 1 regulatory T cells. Nevertheless, the mechanisms of suppression mediated by regulatory T cells that might enable their use to modulate specific immune responses are still not fully known. In addition, despite the recent development of methods allowing the ex-vivo expansion of regulatory T cells it remains difficult to reach a sufficient number of cells for several in-vivo infusions (Bharat et al. 2005, Waldmann et al. 2006).

Here, we presented a strategy to reduce immunogeneicity of allogeneic cellular

therapeutics based on silencing the expression of MHC class I antigens both in human cells as well as in cells from non-human primates including ESC. The approach to reduce cellular immunogenicity here described involves the modification of the grafted cells and differs from the strategies developed so far, which are based on the manipulation of the recipient's immune system. Cells differentiated to act as repair units of tissues are parenchymal cells and therefore will express essentially MHC class I antigens. In an allogeneic grafting protocol those cells would trigger a $CD8^+$- dependent T cell response. Thus, we used the recently described siRNA and shRNA-based RNAi technology to knockdown the expression MHC class I molecules. The light chain ß2m was chosen as a target for the siRNA sequences to create a situation of immunological blindness. With this approach the modified allogeneic target cells will be almost deficient of MHC class I molecules on the cell surface and, consequently, the presentation of minor histocompatibility antigens suitable to $CD8^+$ T cell recognition is also inhibited.

Glycolipids represent the third type of T-cell stimulatory antigen. Several reports showed that glycolipids bearing a variety of sugars linked of different types of lipids tails are able to stimulate $\alpha\beta$- or $\gamma\delta$-T cells. Glycolipids are presented by CD1 molecules. Similarly to MHC class I molecules, all CD1 molecules expressed in the cell surface are heterodimers formed by the heavy chain encoded by the CD1 gene covalently associated with ß2-microglobulin. The recognition of glycolipids/CD1 complexes by CD1-restricted T cells constitutes an important arm of the immune system in the defense against viral infections (De Libero and Mori 2005). By the constitutive down-regulation of ß2m in a MHC class I silencing approach, the expression of CD1 molecules is indirectly suppressed (because similar to MHC class I molecules, CD1 heavy chains are not corrected folded in the absence of ß2m and are not stable when expressed on the cell surface). This fact would completely block the possibility to mount an immune response in case of viral infection. Moreover, permanent ß2m silencing might be harmful for the recipient because in transplantation situations of stem cells-derived products there is an increased risk of teratoma formation due to the presence of pluripotent cells in the graft cellular pool. Here, antigen presentation via MHC class I molecules is required to trigger an effective

immune response against the tumor cells.

TCR recognition of MHC/peptide complexes, specifically for humas directs many aspects of T cell biology, including thymic selection, survival of naive T cells and differentiation into effectors and memory T cells. In the past years, several studies have shown that T cells are important players in triggering cellular rejection. In an allogeneic grafting situation, the cytotoxic properties of $CD8^+$ T cells might be directly activated by recognition of non-self peptides presented by non-self HLA class I molecules or indirectly as a consequence of $CD4^+$ T cell activation (Batchelor and Lechler 1982, Benichou et al. 1999, Heeger et al. 2000, Matesic et al. 1998). Some characteristics of T cell activation are an increase in the proliferation rate as well as in the secretion of IFN-γ. Thus, to investigate the impact of HLA class I silencing in the adaptive response we performed $CD8^+$ T cells proliferation and IFN-γ secretion assays by using native or HLA class I suppressed EBV-transformed B-LCL cells as a target to primed allogeneic $CD8^+$T cells. As expected, native B-LCLs were strongly able to stimulate $CD8^+$ T cells, but it was observed that shRNA-mediated HLA class I-silenced cells were incapable to elicit $CD8^+$ T cell proliferation as well as secretion of IFN-γ. Therefore we conclude that HLA silencing prevent allogeneic stimulation at the humoral and adaptive immune response levels.

An important arm of the immune system is constituted by NK cells. Their lytic action depends on a balance of activating and inhibitory signals upon recognition of ligands present on the target cell by KIR, LAIRL, LIRL and NCR receptors. Similarly to the TCR, some of those receptors recognize HLA class I molecules. However, in contrast to the TCR they are blind to the HLA polymorphic variability (Brumbaugh et al. 1998, Draghi et al. 2005, Waller 2004). Early in the 90s, Ljunggren and Karre proposed the “missing-self hypothesis”, whereby the major function of NK cells is to kill cell targets with reduced levels of HLA class I molecules, such as in the case of cancer cells or virus-infected cells. In fact, some reports have appeared in the last years showing the potential benefits of using allogeneic NK cells to enhance the graft-versus-leukemia effect. Nevertheless, NK cell activity might be disadvantageous for cells silenced for HLA class I expression, because these cells may lack some inhibitory ligands resulting in a cytolytic NK cell response. To address this

question, NK cell cytotoxic assays were performed in an autologous setting to discard a possible influence of disparities in the KIR/ligand system. The results indicated that the residual expression of HLA class I molecules on B-LCLs cells is sufficient to inhibit NK cell lysis. However, we observed an increase of NK cell mediated cytotoxicity when monocytes were used. This observation may reflect that susceptibility to NK cell lysis may depend on the cell type used and in the amount of HLA class I molecules remaining on the cell surface. It is known that expression of HLA-G confers natural protection against NK cell lysis. To overcome the possible downside of HLA silencing approaches we are currently constructing a recombinant HLA-G molecule. This protein will be co-expressed in the HLA class I silenced cells used for transplantation in order to provide an inhibitory signal to NK cells and, consequently, to protect the silenced cells against NK cell lysis.

The presented strategy strongly supports the feasibility of using MHC class I silencing as a way to escape the destructive effect of an immune response against transplanted allogeneic cells both in human cells and in nonhuman primate cells, including embryonic stem cells. We thus believe to have contributed for the development of a new approach that may facilitate the application of cell-based therapies.

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Reviewed by
Professor Dr. Ulrich Martin, Leibniz Research Labs for Biotechnology and Artificial Organs (LEBAO) Hannover
Professor Dr. Anaclet Ngezahayo, Institute of Biophysics, Leibniz University of Hannover

3.4. Meeting Report: Symposium in Stem Cell Repair and Regeneration

Title

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Symposium in Stem Cell Repair and Regeneration

Authors

Fleischmann G

Published in

Cloning and Stem Cells, 2007

CLONING AND STEM CELLS
Volume 9, Number 2, 2007

DOI: 10.1089/clo.2006.0075

Meeting Report

Symposium in Stem Cell Repair and Regeneration

GESINE FLEISCHMANN

On October 2nd and 3rd I attended the third Symposium in Stem Cell Repair and Regeneration at the Hammersmith Hospital, London, UK. Nagy Habib, Professor of Hepatobiliary Surgery, Division of Surgical Oncology, Reproduction and Anaesthetics, Faculty of Medicine, Imperial College London, Hammersmith Campus, London, UK, invited a number of excellent speakers to give a report of their work.

This meeting summary gives a review about this interesting meeting. I will particularly focus on the immunogenicity of stem cells and a few (pre)clinical studies which were especially impressive.

The presentations comprised a mixture of educational sessions and presentations of novel results, so we could get a good overview about both established and new results. Furthermore, it was a well-balanced mixture of (pre-)clinical studies and basic research.

Janet M. Hock (Aastrom Biotech, USA) presented results on tissue repair cells (TRC) generated from bone marrow stem cells. Her group differentiated these TRCs to osteoblasts. They tested them for their ability to repair and regenerate tissue in nonunion fractures that have failed prior interventions. A total of 29 patients were treated, 6 of these in combination with a ceramic matrix. In 27 patients clinical signs of healing as well as radiographic bone bridging could be documented 6 months posttreatment, suggesting that TRC therapy is associated with osteoinduction, healing, and new bone formation. No adverse events were observed, and she reported an intriguing observation that TRC injection was associated with reduced swelling and inflammation. However, one caveat is that this was not a controlled study.

The working group of Nagy Habib presented their results on a novel type of stem cells derived from the adherent CD34+ fraction of bone marrow called Omnicytes (Guinn *et al*., 2006). Omnicytes have been used in a clinical study for patients with liver failure with promising results, with three of five patients showing improvement in serum bilirubin and four of five in serum albumin (Gordon *et al*., 2006). It was reported that Omnicytes have a low risk of tumorigenesis *in vivo*, which may be explained by their source being adult tissue. Since all applications had been performed in an autologous setting, there was no requirement for immunosuppression after transplantation for these cells. Again, one caveat is that this was an uncontrolled Phase I study.

The importance of conducting well-controlled clinical studies was underscored by Eric Alton (Imperial College, UK), who stressed that initial clinical trials of stem cell therapy for cardiovascular diseases had all worked, but had all been uncontrolled. Furthermore, he pointed out that these clinical trials were based primarily on preclinical studies that had been performed with acute cardiac injury models, not end-stage heart disease as usually seen in humans.

Kathryn Wood (Oxford University, UK) presented her research on insulin-producing cell clusters (IPCCs) generated from mouse ES cells using different protocols. The protocol published by the group of Anna Wobus was reported to be the most efficient one (Blyszczuk *et al*., 2003). Generated IPCCs were transplanted into syngeneic CBA mice in which a diabetic phenotype

Institute for Transfusion Medicine, Hannover Medical School, Hanover, Germany.

was induced using the β-cell toxin streptozotocin. Reversal of hyperglycaemia was initially observed in every recipient immediately after transplantation of IPCCs. However, this was only sustained for more than 14 days in one-third of recipients due to both NK cell and T cell-mediated responses. The potential of IPCC to trigger an immune response was evaluated by examining the expression of major histocompatibility complex (MHC) molecules by PCR and immunohistochemistry. Surprisingly, neither the ESC nor the IPCC expressed a detectable level of MHC class I or II molecules.

There was also a discussion about immunogenicity after the presentation given by Tracy Wong (Kings College London, UK). In her study, she analyzed collagen VII expression at the dermal epidermal junctions of patients who have recessive dystrophic epidermolysis bullosa. She performed a clinical study with these patients by treatment with cultured autologous, parent-derived haploidentical, or unrelated allogeneic human fibroblasts. The injected parent-derived and allogeneic cells were not rejected when analysis was performed 2 weeks later. Tracy Wong explained this by the fact that collagenase VII is present at low, albeit detectable, levels in these patients, and thus does not serve as a target for an immune response. However, this would not explain the lack of alloreactive responses targeting foreign MHC. In fact, in addition to increased overall collagen VII staining, which she hypothesized might be due to a paracrine effect from the injected cells stimulating existing collagen VII production, an increase in local macrophages and T cells was observed.

While the four above-presented working groups sought to avoid the problem of immunogenicity, the following two presentations offered innovative strategies to overcome the problem of immunogenicity of stem cell-derived cellular therapies.

Paul Fairchild (Oxford University, UK) opened his presentation by a more general overview about ES cells and their possible future importance for cell replacement therapy, stressing that the major problem of rapid rejection is the MHC. In his opinion there is the need for development of alternative strategies to match ESC lines with potential recipients. One strategy he suggests is to use the pluripotency of ES cells to provide a source of donor-derived dendritic cells (DC) (Fairchild *et al*., 2005). Those DCs could then be used to establish a dominant tolerance before transplantation of the actual cells or tissues to be replaced. For mouse ES cells this strategy has been tested, and there is an optimized protocol; for humans, there are first data for a successful adaption.

Noriyuki Kasahara (University of California Los Angeles, CA) presented a very different approach by reducing the transplanted cell's immunogenicity instead of trying to establish donor-specific tolerance. His working group suppressed the HLA expression by lentivirus-mediated transfer of siRNA cassettes (Haga *et al*., 2006). The aim of the study was to prevent graft rejection, reduce the level of immunosuppression needed to maintain graft survival, and to minimize the risk of graft-versus-host disease (GVHD). As expected, lentivirus-mediated gene transfer was more efficient than nonviral gene transfer (Horn *et al*., 2006), which has also previously been used to deliver siRNA for HLA silencing (Gonzalez *et al*., 2005). Also, he showed that the HLA suppression is dose dependent, and results in resistance to alloreactive T-cell-mediated killing without incurring increased sensitivity to NK cell-mediated killing. This promising method of HLA suppression has previously been published (Figueiredo *et al*., 2006).

Recapitulating, it was a great meeting with brilliant speakers. I am sure that with the number of innovative strategies presented, next year's meeting will be as exciting as this year's!

ACKNOWLEDGMENTS

I would like to thank my supervisor and mentor Dr. Peter A. Horn, as well as our department head, Prof. Rainer Blasczyk, for ongoing support for my doctoral work. I am also grateful to Prof. Noriyuki Kasahara for his critical review of the manuscript.

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Address reprint requests to:
Gesine Fleischmann
Institute for Transfusion Medicine
Hannover Medical School
Carl-Neuberg-Str. 1
30625 Hannover, Germany

E-mail: Fleischmann.Gesine@mh-hannover.de

4. Anhang

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Erklärung zur Dissertation

Hierdurch erkläre ich, dass die Dissertation

„Charakterisierung und Manipulation der Immunogenität embryonaler Stammzellen des Neuweltaffen *Callithrix jacchus*“

selbstständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden.

Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

Hannover, den 01.10.2008

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(Unterschrift)

Name: Gesine Fleischmann

Lebenslauf

Name:		Gesine Fleischmann
Geburtsdatum:		27.04.1981
Geburtsort:		Goslar
Schulbildung:	1987-1991	Grundschule Sudmerberg, Goslar
	1991-1993	Orientierungsstufe „Bei der Eiche“, Goslar
	1993-2000	Christian-von-Dohm-Gymnasium, Goslar
Beruflicher Werdegang:	2000-2001	Freiwilliges Soziales Jahr, Malteser Hilfsdienst, Hannover
	2001-2006	Studium der Biologie, Carolo-Wilhelmina Universität, Braunschweig, Abschluss Dipl.Biol.
	ab 2006	Institut für Tansfusionsmedizin, Medizinische Hochschule Hannover

Publikationen

Original Artikel

1. **Fleischmann G** (2007): Symposium in Stem Cell Repair and Regeneration, Cloning and Stem Cells 9(2): 141-143

2. Müller T, **Fleischmann G**, Eildermann K, Mätz-Rensing K, Horn PA, Sasaki E, Behr R (2008) A novel stem cell line derived from the common marmoset monkey (*Callithrix jacchus*) exhibiting germ cell-like characteristics, Human Reproduction (in review)

3. **Fleischmann G**, Müller T, Behr R, Blasczyk R, Sasaki E, Horn PA (2008) Growth characteristics of the non-human primate embryonic stem cell line cjes001 depending on feeder cell treatment, Cloning and stem cells (submitted)

4. **Fleischmann G**, Figueiredo C, Seltsam A, Blasczyk R, Horn PA (2008) Embryonic Stem Cells: MHC Expression and Immunogenicity of Stem Cell-Derived Cellular Therapeutics, Stem Cell Applications in Diseases, Nova Science Publishers, Inc. (Editor F. Columbus) (Preliminary accepted)

Abstracts

1. **Fleischmann G**, Müller T, Behr R, Blasczyk R, Sasaki E, Horn PA :
   Nonhuman primate embryonic stem cells: Culture on γ-irradiated or Mitomycin-treated feeder cells?
   Experimental Hematology 2007; (suppl.1): S. 118 (Abstract P228)
   Poster, 36 th Annual Scientific Meeting ISEH Society for Hematology and Stem Cell, Hamburg, Germany, 28.-30. September 2007

2. Horn P.A., **Fleischmann G**, Elger K, Wurm M, Hanenberg H, Blasczyk R.:
   MGMT-mediated chemoprotection allows for efficient selection of PIG-A transduced cells with the PNH phenotype
   Experimental Hematology 2007; (suppl.1): S. 82 (Abstract P122)
   36th Annual Scientific Meeting of the International Society for Experimental Hematology (ISEH), 2007 Hamburg, Germany, September 28-30

3. **Fleischmann G**, Müller T, Behr R, Blasczyk R, Sasaki E, Horn PA:
   Optimization of culture conditions for nonhuman primate embryonic stem cells
   Transfus Med Hemother 2007; (suppl. 1): S.32
   Poster, 40th Annual Meeting of the German Society for Transfusion Medicine and Immunohematology (DGTI), Friedrichshafen, Germany, 2007
   ausgezeichnet als „Best of Abstracts“ der DGTI Tagung 2007

4. **Fleischmann G**, Figueiredo C, Elger K, Wurm M, Blasczyk R, Horn PA:
   Silencing of MHC class I expression in non-human primate cells by lentiviral expression of shRNA
   Poster, Mechanisms of early differentiation: embryogenesis, myogenesis, Barsinghausen, Germany, 01.-05. September 2008

5. **Fleischmann G**, Figueiredo C, Elger K., Blasczyk R., Horn PA.:
   shRNA-mediated silencing of MHC expression in fibroblasts, B-LCLs and ESCs of non-human primates
   Tissue and Antigens 2008, in press;
   Vortrag, 15th International Histcompatibility and Immunogenetics Workshop, Rio de Janeiro, Brasilien, 08.-20. September 2008
   ausgezeichnet mit „Travel Grant“ der Summer School Immunogenetics 2008, ASHI/EFI