Differenzierung muriner embryonaler Stammzellen in insulinproduzierende Zellen

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Kurzzusammenfassung

Die Transplantation von humanen Spenderpankreata oder isolierten Pankreasinseln zur Therapie eines Typ I Diabetes mellitus ist durch die limitierte Anzahl geeigneter Spenderorgane nur in wenigen Fällen möglich. Daher ist ein artifizielles Organsystem aus Surrogatzellen mit den Charakteristika endokriner β-Zellen von großem medizinischem Interesse. Die Implantation von insulinproduzierenden Surrogatzellen, die aus embryonalen Stammzellen (ES-Zellen) generiert werden, ist eine vielversprechende Strategie zur Therapie des insulinpflichtigen Diabetes mellitus. Das Ziel dieser Dissertation war die Entwicklung eines Differenzierungsprotokolls, das verlässlich insulinproduzierende Zellen aus ES-Zellen generiert. Dieses Protokoll sollte mit einem Sortierungsverfahren kombiniert werden, um unerwünschte Zellen abzutrennen und insulinproduzierende Zellen aufzureinigen. Dazu wurde ein zuvor veröffentlichtes Differenzierungsprotokoll von Lumelsky *et al.* (2001) reproduziert und als Referenz verwendet. Die Differenzierung mit dem Referenzprotokoll erzeugte Zellen mit einem neuronalen Geno- und Phänotyp. Darüber hinaus zeigte sich, dass die differenzierten Zellen Insulin aus dem Kultivierungsmedium passiv aufgenommen hatten, was zu einer deutlichen Überschätzung des Insulingehalts führen kann. Ein neu entwickeltes 4-Stadien Differenzierungsprotokoll konnte im Gegensatz zum Referenzprotokoll ES-Zellen in annähernd monohormonale Zellen differenzieren. Parallel zu dieser Verbesserung konnte der endokrine, β-zellähnliche Charakter der differenzierten ES-Zellen durch eine erhöhte Expression des GLUT2 Glucose Transporters, Glucokinase, Pdx1 und Sur1 belegt werden. Die Implantation von differenzierten Stammzellen in das durch Streptozotocin-Behandlung induzierte diabetische Mausmodell führte nur bei Zellen des neuen Protokolls zu einer signifikanten Reduktion der Blutglucosekonzentration in den Bereich nicht-diabetischer Tiere. Ein weiteres Ziel dieser Dissertation war die Etablierung eines Sortierungsverfahrens für $CK19^+$ Zellen. Es konnte gezeigt werden, dass aufgereinigte $CK19^+$ Zellen das Insulingen exprimierten, das Prohormon Proinsulin in Insulin und C-Peptid prozessierten und in der Lage waren, Insulin auf den Stimulus Glucose hin freizusetzen. Darüber hinaus zeigten $CK19^+$ Zellen den typischen Phänotyp einer pankreatischen β-Zelle mit einem ausgeprägten Differenzierungsstatus und Insulin-Granula im Zytoplasma. Daher repräsentieren diese Zellen einen endokrinen Progenitorzelltyp, mit phänotypischen und genetischen Merkmalen einer insulinsezernierenden Zelle, die als mögliche Surrogatzellen in der Zellersatztherapie des Diabetes mellitus genutzt werden könnten.

Schlagwörter: Diabetes mellitus, Embryonale Stammzellen, Surrogatzellen

Abstract

Transplantation of human pancreata or isolated islets for the therapy of type 1 diabetes mellitus is restricted by a limited availability of donor organs. Therefore, the generation of an artificial organ system comprising surrogate cells with characteristics of endocrine beta cells is of great medical interest. Implantation of insulin-producing surrogate cells, generated from embryonic stem (ES) cells, is a promising strategy for the therapy of insulin-dependent diabetes mellitus. It was therefore the aim of this study to develop a differentiation protocol, which drives the differentiation of ES cells preferentially towards insulin-producing cells and to combine this with a technique for separation of these insulin-producing cells. Therefore, we reproduced a previously published protocol by Lumelsky *et al.* (2001) and used it as a reference. Differentiation with the reference protocol generated cells with a neuronal geno- and phenotype. These cells were particularly prone to the uptake of insulin from the extracellular space, which can lead to an overestimation of the efficiency of a differentiation protocol. The differentiation of ES cells according to a newly developed optimized 4 stage differentiation protocol yielded in contrast to the reference protocol monohormonal insulin-producing cells. At the same time the endocrine beta cell like character of these differentiated ES cells was reinforced by the high gene expression level of the GLUT2 glucose transporter, glucokinase, Pdx1 and Sur1. Implantation of differentiated ES cells from the new differentiation protocol prevented the rise of blood glucose in STZ diabetic mice, and caused a significant reduction of the blood glucose concentrations towards a range typical for non-diabetic mice. Another aim of this work was to establish a cell sorting technique for $CK19^+$ cells. Sorted $CK19^+$ cells expressed the prohormone proinsulin, processed it into mature insulin and C-peptide and released it in response to glucose. Moreover, $CK19^+$ showed the typical phenotype of pancreatic beta cells with a distinct degree of differentiation and insulin granules in the cytoplasm. Thus, these cells represent an endocrine precursor cell type with the geno- and phenotype of an insulin secretory cell, potentially suitable for insulin replacement therapy in diabetes.

Keywords: Diabetes mellitus, embryonic stem cells, surrogate cells

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1 Einleitung

1.1 Definition und Klassifikation des Typ 1 und Typ 2 Diabetes mellitus

Der Typ 1 Diabetes mellitus (T1DM) ist durch die progrediente Zerstörung der insulinproduzierenden ß-Zellen in den Langerhansschen Inseln des Pankreas gekennzeichnet (Waldhäusl & Lenzen 2007). Der Insulinmangel führt zu den klassischen Symptomen Hyperglykämie, Gewichtsverlust, Polydipsie, Polyurie und Ketoazidose (Kerner 2001).

Der T1DM wird durch eine Autoimmunreaktion ausgelöst, welche die insulinproduzierenden ß-Zellen des Pankreas zerstört. Der T1DM tritt beim Menschen vom Kindsalter bis zum jungen Erwachsenenalter auf, später wird das Auftreten seltener (Bach 1994; Eisenbarth & Stegall 1996; Benoist & Mathis 1997). Ein T1DM wird nicht in den ersten sechs Lebensmonaten beobachtet (Hürter 1997), danach steigt die Inzidenz jedoch kontinuierlich an und erreicht ein kurzes Zwischenplateau im Alter von ca. fünf bis sieben Jahren. Der Inzidenzgipfel des T1DM liegt in der Pubertät, bei den Mädchen zwischen 12 und 13 Jahren, bei den Jungen ein bis zwei Jahre später (Hürter 1997).

Da es sich beim T1DM um eine multifaktorielle Erkrankung handelt, wird Umweltfaktoren wie Chemikalien, Viren und Nahrungsbestandteilen (Akerblom & Knip 1998; Baldeon 2000; Jun & Yoon 2001; Lenzen 2008a) ein Einfluss auf die Diabetesentwicklung zugeschrieben. Andererseits ist eine deutliche genetische Komponente allgemein als Faktor der Diabetesentstehung akzeptiert, wobei insbesondere der MHC-Locus von zentraler Bedeutung ist und auch diagnostische Relevanz besitzt (Nerup *et al.* 1974).

Die Autoimmunzerstörung im T1DM kann in verschiedene Phasen eingeteilt werden (Eisenbarth 2008), an deren Ende ein kompletter Verlust der ß-Zellen des Pankreas steht. Nach einer unauffälligen Phase mit normaler Stoffwechselfunktion kommt es zu so genannten „Triggerereignissen“, welche den Autoimmunprozess einleiten. Diese Ereignisse sind vielfältiger Natur. Es können Ernährungs- und Umweltfaktoren, wie z.B. gluten- und kuhmilchhaltige Nahrungsbestandteile sowie Infektionen (Enteroviren, Coxsackieviren, Rötelviren) eine Rolle spielen (Akerblom & Knip 1998; Baldeon 2000; Jun & Yoon 2001). Diese Triggerereignisse führen zu einer Aktivierung von autoaggressiven Immunzellen, welche die ß-Zellen des Pankreas gezielt attackieren. Dabei wird angenommen, dass Umweltfaktoren durch molekulare Mimikry als Superantigene mit ß-Zellproteinen agieren und so die Selektion von autoaggressiven Zellen positiv beeinflussen (Williams 2003).

Mit Beginn des Autoimmunprozesses kommt es zu einem progredienten Verlust der ß-Zellmasse. In diesem Stadium sind die Patienten mit der Ausnahme von Autoantikörpern gegen ß-Zellbestandteile (ICA/IAA), die im Blut nachgewiesen werden können, klinisch unauffällig (Knip 2002; Williams 2003). Nach einem Verlust von mehr als 50 % der ß-Zellmasse wird eine gestörte Glucosetoleranz beobachtet, nach einer Zerstörung von mehr als 80 % dann auch eine erhöhte Nüchternblutglucosekonzentration (Knip 2002; Eisenbarth 2008). Erst zu diesem Zeitpunkt werden die meisten Patienten klinisch diagnostiziert, da die ausgeprägte Hyperglykämie zum typischen diabetischen Syndrom mit Polyurie, Polydipsie, Ketoazidose und allgemeiner körperlicher Schwäche führt. Sind durch die verbleibende ß-Zellmasse zum Zeitpunkt der Diagnose noch C-Peptidspiegel im Blut nachweisbar, so führt die progrediente Zerstörung der ß-Zellen zum kompletten Verlust der Produktion körpereigenen Insulins und des C-Peptids. Aus diesem Verlauf heraus wird deutlich, dass zum Zeitpunkt der Diagnose eines T1DM der größte Teil der insulinproduzierenden ß-Zellen des Autoimmunprozesses bereits zerstört ist und die Chancen therapeutischer Interventionen auf die lebenslange Substitution von Insulin begrenzt sind.

Die Zerstörung der ß-Zellen des Pankreas wird durch zelluläre Elemente des Immunsystems sowie durch proinflammatorische Zytokine vermittelt, die eine zur Apoptose der ß-Zellen führende Signalkaskade, bestehend aus Caspasen und anderen proapoptotischen Enzymen, auslösen (Bach 1994; Mandrup-Poulsen 2001; Jörns *et al.* 2005). Als Konsensusmodell der ß-Zellzerstörung hat sich das am Steno Diabetes Center (Gentofte, Dänemark) entwickelte „Kopenhagener“ Modell etabliert (Abb. 1.1), das das Zusammenspiel von zellulären und humoralen Immunmediatoren bei der ß-Zellzerstörung beschreibt (Nerup *et al.* 1994). Die Autoimmunzerstörung der ß-Zelle beginnt mit einem initialen Angriff der Immunzellen, die zu einer Freisetzung von ß-Zellproteinen führt. Diese Proteinfreisetzung kann z.B. direkt durch Viren und Chemikalien, aber auch indirekt durch Nahrungsbestandteile oder Zytokine initialisiert werden. In diesem Zusammenhang spielt die genetische Prädisposition der Patienten eine wichtige Rolle (Hürter 1997). So hat der Nachweis der engen Beziehung zwischen MHC Antigenen und dem T1DM (Cudworth & Woodrow 1974; McDevitt & Bodmer 1974; Nerup *et al.* 1974) sowohl das Konzept des multifaktoriellen Erbmodus als auch die These der genetischen Heterogenität bestätigt (Cudworth 1978; Rotter & Rimoin 1978).

Die freigesetzten ß-Zellproteine werden von Makrophagen, Monozyten oder dendritischen Zellen aufgenommen und als Antigen präsentiert (sogenannte antigenpräsentierende Zellen, APC). Diese Immunzellen führen zu einer Aktivierung von ß-zellspezifischen,

autoaggressiven Zellen. An diesem Prozess sind proinflammatorische Zytokine und Chemokine wesentlich beteiligt (Cnop *et al.* 2005; Eisenbarth 2008). In der nächsten Phase kommt es durch die Verstärkung der Immunmechanismen zur progredienten Zerstörung der ß-Zellen. Die Präsentation der ß-Zellantigene führt zu einer Aktivierung spezifischer T-Helfer-Zellen, die das Antigen über MHC-Klasse II Moleküle präsentieren. Auch die T-Helferzellen sezernieren Zytokine, die zum einen die Zytokinproduktion der anderen antigenpräsentierenden Zellen weiter anregen; zum anderen führt diese hohe Konzentration von Zytokinen zur Produktion von freien Radikalen wie Stickstoffmonoxid (NO), das durch das Enzym (induzierbare) Nitroxidsynthase (iNOS) synthetisiert wird, und Superoxidanionen in den ß-Zellen des Pankreas (Lenzen 2008b). Dieser *Circulus vitiosus* aus Antigenfreisetzung und Antigenpräsentation verstärkt sich selbst, wird jedoch durch die ß-Zellmasse letztlich limitiert.

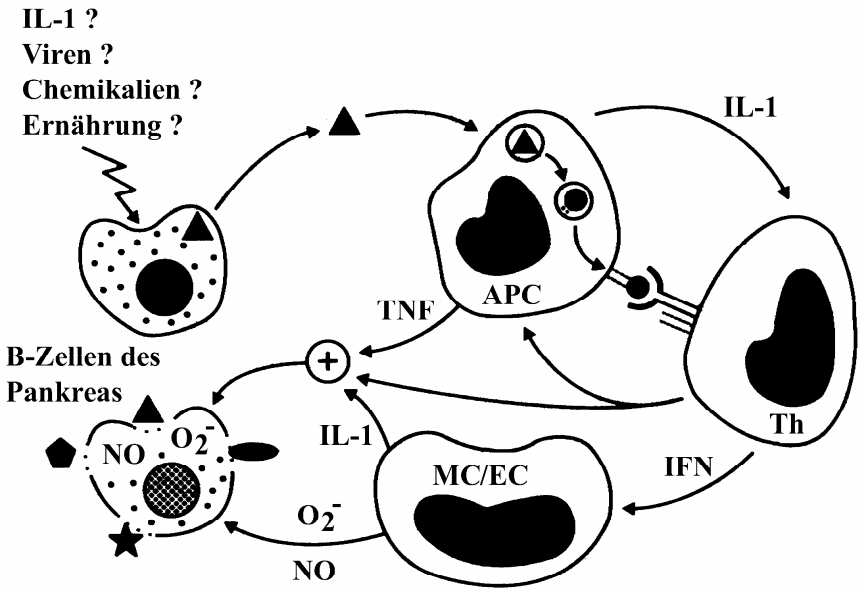

Abb. 1.1 Das Kopenhagenmodell nach Nerup *et al.* (1994) zeigt schematisch die Zerstörung der ß-Zellen durch Autoimmunprozesse.

Eine genetische Prädisposition kombiniert mit exogenen Triggern wie Infektionen oder Nahrungsbestandteilen führt zu einer Schädigung der ß-Zelle und zur Initiierung der ß-Zellzerstörung. Die dabei freigesetzten ß-zellspezifischen Proteine werden durch Antigen-präsentierende Zellen (APCs) aufgenommen, prozessiert und präsentiert. In einer zweiten Phase (Verstärkungsphase) werden durch die aktivierten APCs Zytokine (IL-1ß, TNF-α) sezerniert. Gleichzeitig werden die von APCs präsentierten Antigene durch spezifische T-Helferzellen (Th) erkannt. Die Aktivierung der T-Helferzellen stimuliert die Sekretion von Zytokinen, von denen insbesondere das IFN-γ die Expression von MHC II-Molekülen induziert. Zusätzlich wird die Sekretion von IL-1ß und TNF-α durch die APCs in einer IFN-γ-bedingten Feedback-Stimulierung verstärkt. Als Folge der hohen lokalen Konzentrationen der Zytokine IL-1ß, TNF-α und IFN-γ wird die Zerstörung von ß-Zellen des Pankreas durch die Freisetzung von Sauerstoffradikalen und NO aus Makrophagen (MC), Endothelzellen (EC) und T-Lymphozyten potenziert. Das Ergebnis dieser Vorgänge ist die Selbstverstärkung des eingeleiteten Autoimmunprozesses bis zur völligen Zerstörung der insulinproduzierenden Zellen.

Die freigesetzten Zytokine initiieren apoptotische und nekrotische Signalwege, die zur Zerstörung der ß-Zellen führen. Das Ergebnis dieses Prozesses sind Langerhanssche Inseln, die keine ß-Zellen mehr enthalten und als sogenannte „End-Stage Islets“ bezeichnet werden (Nerup *et al.* 1994). Der daraus resultierende Diabetes wird auch als insulinpflichtiger Diabetes mellitus vom Typ 1 bezeichnet. Der Anteil von Patienten mit einem absoluten Insulinmangel liegt in Deutschland bei ca. 0,7 % der Gesamtbevölkerung (Hauner *et al.* 2003). Als Folge der Erkrankung entstehen mikrovaskuläre Gefäßschädigungen, die wiederum zu einer Niereninsuffizienz, Retinopathie und Neuropathie führen können. Größere Blutgefäße, deren Zellen in Folge der chronisch überhöhten Blutglucosewerte glyciert werden, bilden in der Folge massive kardiovaskuläre Komplikationen aus, sowie ischämische Insulte.

Beim nichtinsulinpflichtigen Typ 2 Diabetes mellitus (T2DM) handelt es sich um einen Insulinsekretionsdefekt der β-Zellen auf Glucosestimulation, der zusammen mit einer Insulinresistenz der peripheren Gewebe über das Stadium der gestörten Glucosetoleranz ebenfalls zu einer chronischen Hyperglykämie führt (Pickup & Williams 2003). Als Ursache für diesen Defekt werden Funktionsstörungen von β-zelleigenen Strukturen angenommen, die an der Signalweiterleitung und Aktivierung der Insulinsekretion beteiligt sind.

Der Gesamtanteil der Patienten mit einem T2DM liegt in Deutschland bei ca. 90 % und macht somit den größeren Teil der Diabetiker aus (Hauner *et al.* 2003). Die meisten Patienten sind über 45 Jahre und im Zusammenhang mit dem Alter ist auch Adiposität als Risikofaktor anzusehen. Im Gegensatz zu den meist jugendlichen Typ-1-Diabetikern ist die Beeinträchtigung durch die veränderte Stoffwechselfunktion geringer, da eine Restfunktion der vorhandenen β-Zellen das Auftreten lebensbedrohlicher Ketoazidosen verhindert.

1.2 Gentherapie des Diabetes mellitus

Die Stoffwechsellage eines Typ-1-Diabetikers kann durch eine Insulinsubstitution behandelt werden. Allerdings ist eine Wiederherstellung der β-Zellmasse nach heutigem Kenntnisstand nicht möglich. Daraus resultiert für den Typ-1-Diabetiker eine lebenslange Abhängigkeit von regelmäßigen Insulingaben, die in Form von Injektionen erfolgt. In der Regel ist keines der gegenwärtigen Substitutionsverfahren mit Insulin, Insulinanaloga und Insulinpumpen-systemen in der Lage, die Stoffwechsellage dauerhaft zu normalisieren, so dass die Entwicklung von Spätkomplikationen verhindert werden kann. Hinzu kommt die Problematik, dass die Insulintherapie stringente, lebenslange Anforderungen an den Patienten stellt, die Substitution zeitgerecht und in entsprechender Dosierung vorzunehmen. Eine

disziplinierte Ernährung ist ebenso unabdingbar. Daher bedarf diese Form der Insulinsubstitution eines hohen Maßes an Konsequenz und Motivation des Patienten, was verständlicherweise im Verlaufe seiner lebenslangen Krankheit nur schwerlich durchzuhalten ist. Besonders im Hinblick auf die zu erwartenden Spätschäden eines behandelten T1DM, der hohen Prävalenz innerhalb der Gesamtbevölkerung und der finanziellen Belastung der Gesellschaft durch diese chronische Erkrankung besitzen Therapiekonzepte des T1DM mittels Gentherapie enorme Attraktion (Tiedge & Lenzen 2005). Die Therapie des T1DM kann nicht ausschließlich durch Transplantation von humanen Spenderpankreata durchgeführt werden, da die Anzahl geeigneter Spenderorgane limitiert ist. Daher ist ein artifizielles Organsystem aus so genannten Surrogatzellen, die über die wesentlichen Charakteristika endokrinerer β-Zellen verfügen, von großem wissenschaftlichem und medizinischem Interesse.

Unter einer Surrogatzelle versteht man eine Zelle, die möglichst alle Eigenschaften der Zelle haben soll, die ersetzt werden muss. Die ideale Surrogatzelle wäre demnach eine Zelle, welche die wichtigsten Funktionen der β-Zelle aufweist. Das entscheidende Kriterium ist die bedarfsgerechte Freisetzung von reifem Insulin. Im Optimalfall sollten Surrogatzellen nur auf einen physiologischen Stimulus hin Insulin sezernieren. Es sind aber auch Therapiekonzepte im Gespräch, die einen basalen Bedarf an Insulin sicherstellen sollen, um lebensbedrohliche ketoazidotische Entgleisungen des Metabolismus beim Typ-1-Diabetiker zu verhindern (Elsner *et al.* 2008). Auch andere Kriterien, wie die Frage der Infektiösität und Toxizität der Surrogatzellen spielen eine entscheidende Rolle. Außerdem sollten Surrogatzellen unbegrenzt verfügbar sein und nach der Implantation nicht durch das Immunsystem des Empfängers abgestoßen werden.

Die Differenzierung von embryonalen Stammzellen in insulinproduzierende Surrogatzellen könnte die Probleme der begrenzten Verfügbarkeit von Spenderpankreata beheben. Die Voraussetzung ist, dass es *in vitro* gelingt, aus Stammzellen Surrogatzellen zu erzeugen, welche die entscheidenden Charakteristika von β-Zellen aufweisen. Dafür ist es notwendig, dass diese Surrogatzellen das Insulingen exprimieren und das Prohormon Proinsulin in reifes Insulin und C-Peptid spalten und in Granula speichern. Außerdem sollten die Surrogatzellen über die Signalerkennungsstrukturen der β-Zelle verfügen, welche für die Auslösung der Insulinsekretion verantwortlich sind (Waldhäusl & Lenzen 2007).

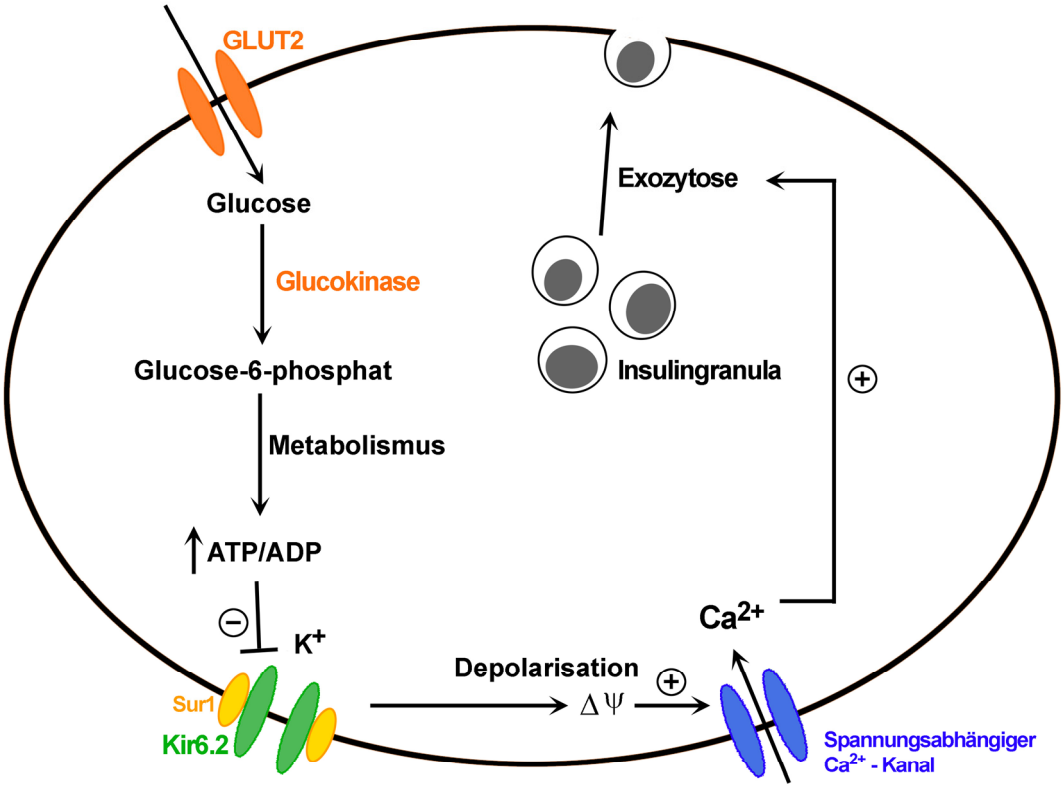

Abb. 1.2 Modell des Mechanismus der glucoseinduzierten Insulinsekretion in β-Zellen des Pankreas

Die Insulinsekretion der β-Zelle wird über eine Erhöhung des ATP/ADP-Quotienten im Zytoplasma gesteuert (Abb. 1.2) Für die Auslösung der Sekretion sind insgesamt vier Strukturproteine verantwortlich. Das Glucosemolekül, welches nach Nahrungsaufnahme im Blut in erhöhter Konzentration vorliegt, wird durch den niedrigaffinen Glucosetransporter GLUT2 über erleichterte Diffusion aus dem Blut in die β-Zelle transportiert. In der β-Zelle findet im ersten Schritt der Glykolyse eine Phosphorylierung der Glucose durch die Glucokinase statt. Das phosphorylierte Glucosemolekül wird dann in den weiteren Umwandlungsreaktionen der Glykolyse und dem zellulären Metabolismus umgebaut, wodurch sich der intrazelluläre ATP-Spiegel erhöht. Der Glucosetransporter GLUT2 und das glucosephosphorylierende Enzym Glucokinase bilden eine Tandem-Struktur bei der Erkennung erhöhter Blutglucosekonzentrationen im Blut. Beide Proteine sind niedrigaffin und haben ihre höchste Aktivität im millimolaren Bereich, so dass die β-Zelle sehr genau auf Änderungen der Blutglucosekonzentration reagieren kann. Die Glucokinase ist dabei der geschwindigkeitsbestimmende Schritt der Phosphorylierung und der Einschleusung der Glucose in den Metabolismus (Lenzen & Panten 1988; Matschinsky 1990). Durch die

Erhöhung des ATP/ADP-Quotienten wird ein Proteinkomplex, bestehend aus dem ATP-sensitiven Kaliumkanal Kir6.2 und dem Sulfonylharnstoffrezeptor Sur1, gehemmt. Die Hemmung führt zum Verschluss des Kanals und in der Folge zur Depolarisation der Zelle. Durch die Depolarisation der Zelle öffnet sich ein spannungsabhängiger Calciumkanal und ermöglicht den Einfluss von Calcium in die β-Zelle. Hierdurch wird die Exozytose des Insulins eingeleitet, welches in Granula im Zytoplasma gespeichert vorliegt. Die optimale Surrogatzelle, die man aus embryonalen Stammzellen gewinnen müsste, sollte daher diese Signalerkennungsstrukturen der β-Zelle aufweisen.

Mit Hinblick auf eine mögliche Therapie mit Surrogatzellen gilt es aber auch noch andere Sachverhalte aufzuklären. Differenzierte Stammzellen verfügen im Tierversuch nach Implantation über ein erhebliches kanzerogenes Potential, das von einer möglichen ungehemmten Proliferation bis zur Ausbildung eines Teratoms reicht (Takahashi *et al.* 2003). Außerdem ist zu klären, inwieweit differenzierte Stammzellen vom Immunsystem des Empfängers angenommen werden oder ob es zu einer Abstoßung des Implantats kommt. Eine Möglichkeit, eine Autoimmunattacke auf das Implantat zu verhindern, stellt die Verkapselung des Implantats dar (Cotton 1996). Die Verkapselung differenzierter embryonaler Stammzellen als mechanische Barriere gegen Zellen des Immunsystems könnte das Problem der Abstoßungsreaktion aufheben. Außerdem hat es auf dem Gebiet der Reprogrammierung humaner adulter Fibroblasten in patientenspezifische embryonale Stammzellen erhebliche Fortschritte gegeben (Takahashi *et al.* 2007; Wernig *et al.* 2007). Durch die Etablierung dieser syngenen Stammzelllinien (iPS Zellen) ließe sich das Problem der Immuntoleranz beheben, da mit solchen Zellen eine Implantation durchgeführt werden könnte, ohne eine Abstoßungsreaktion zu befürchten.

1.3 Generierung von insulinproduzierenden Surrogatzellen aus embryonalen Stammzellen

Embryonale Stammzellen (ES-Zellen) sind pluripotente Zellen, die aus dem Embryoblast der Blastozyste gewonnen werden (Evans & Kaufman 1981). Ihre Pluripotenz verleiht ihnen die Fähigkeit, *in vivo* und *in vitro* in die drei Keimblätter Endoderm, Ektoderm und Mesoderm zu differenzieren. Außerdem können ES-Zellen in der Gewebekultur unter Erhaltung ihrer pluripotenten Eigenschaften kultiviert werden. Diese unbegrenzte Selbsterneuerung von ES-Zellen, verbunden mit der Fähigkeit durch Differenzierung adulte, funktionsfähige Körperzellen hervorzubringen, setzt daher der Verfügbarkeit von ES-Zellen keine technischen Grenzen. Folglich gelten ES-Zellen als vielversprechendes Ausgangsmaterial für die Generierung von insulinproduzierenden Surrogatzellen.

Die erste Arbeit zur Differenzierung von murinen ES-Zellen in insulinproduzierende Zellen wurde von einer Arbeitsgruppe in Spanien veröffentlicht (Soria *et al.* 2000). Eine weitere Arbeit einer US-amerikanischen Gruppe wurde ein Jahr später publiziert (Lumelsky *et al.* 2001). Der Fortschritt, welcher in dieser Arbeit dokumentiert wurde, erschien damals von großer wissenschaftlicher Bedeutung, so dass diese Arbeit zu einer der am meisten zitierten Veröffentlichungen über dieses Thema geworden ist. Das verwendete Differenzierungsprotokoll, welches seitdem unter dem Begriff Lumelsky-Protokoll eine gängige Bezeichnung gefunden hat, ist in seiner ursprünglichen Form eine Weiterentwicklung eines neuronalen Differenzierungsprotokolls (Lee *et al.* 2000). Eine Reihe von weiteren Arbeitsgruppen hat sich um die Reproduktion dieser Arbeit bemüht und ist dabei zu kontroversen Ergebnissen gekommen (Hori *et al.* 2002; Blyszczuk *et al.* 2003; Moritoh *et al.* 2003; Rajagopal *et al.* 2003; Hansson *et al.* 2004; Sipione *et al.* 2004; Bai *et al.* 2005; Paek *et al.* 2005). Daher wurden berechtigte Zweifel laut, ob das angewandte Protokoll überhaupt zur Differenzierung von ES-Zellen in insulinproduzierende Zellen geeignet sei, oder ob es nicht vielmehr zu einer Population von neuronalen Zellen führen würde, die nur über eine geringe Insulingenexpression verfügen (Sipione *et al.* 2004). Weitere Arbeiten an diesem Protokoll ergaben, dass ein erheblicher Anteil der Zellen durch Apoptose zugrunde gegangen war. Andere Veröffentlichungen belegten außerdem, dass ein großer Teil des gemessenen Insulingehalts der ausdifferenzierten Stammzellen auf die Aufnahme von exogenem Insulin aus dem Differenzierungsmedium, das mit 25 µg Insulin pro ml Medium supplementiert wurde, zurückzuführen war (Rajagopal *et al.* 2003; Hansson *et al.* 2004; Paek *et al.* 2005).

Die bisherigen Strategien zur Differenzierung von ES-Zellen in insulinproduzierende Zellen beruhten überwiegend auf der Veränderung von Kultur- bzw. Differenzierungsbedingungen

(Lumelsky *et al.* 2001; Hori *et al.* 2002; Moritoh *et al.* 2003; Bai *et al.* 2005; Shi *et al.* 2005) oder der konstitutiven Überexpression von Transkriptionsfaktoren, die in der Organogenese des Pankreas eine herausragende Rolle spielen (Blyszczuk *et al.* 2003; Miyazaki *et al.* 2004; Shiroi *et al.* 2005). Bislang ist es aber nicht gelungen, ein Differenzierungssystem zu etablieren und dieses mit einem Selektionsverfahren zu kombinieren, mit dem Ziel, insulinproduzierende Zellen anzureichern und von anderen Zelltypen abzutrennen, die unerwünschterweise im Verlaufe der *in vitro* Differenzierung entstehen.

Das Lumelsky-Protokoll ging grundsätzlich von der Annahme aus, dass die *in vitro* Differenzierung von ES-Zellen einen temporären Zelltyp benötigt, der durch den Marker Nestin gekennzeichnet ist. Nestin ist ein Neurofilamentprotein, welches in der Ontogenese des zentralen Nervensystems eine große Rolle spielt. Zellen, die das Nestinprotein exprimieren, wurden im Pankreas im exocrinen Mesenchym, aber auch innerhalb der Langerhansschen Inseln identifiziert und erfolgreich in insulinproduzierende Zellen differenziert (Hunziker & Stein 2000; Zulewski *et al.* 2001). Daher wurde die These entworfen, dass die Neogenese der ß-Zellen auf die Differenzierung von Nestin-positiven Vorläuferzellen im endokrinen Teil des Pankreas zurückzuführen sei (Zulewski *et al.* 2001). Diese These wurde seitdem kontrovers diskutiert und gilt als sehr umstritten (Selander & Edlund 2002; Treutelaar *et al.* 2003). Im Gegensatz zu dieser These stand die Annahme, dass sowohl die Organogenese als auch die Neogenese der endokrinen Zellen des Pankreas auf die Differenzierung epithelialer Zellen der Duktusgänge zurückzuführen ist (Bouwens & Pipeleers 1998; Bonner-Weir *et al.* 2004; Bouwens 2004; Bouwens & Rooman 2005). Diese epithelialen Zellen waren positiv für Cytokeratin 19 (CK19) und konnten *in vitro* bereits in insulinproduzierende Zellen differenziert werden (Bonner-Weir *et al.* 2000; Gao *et al.* 2003; Gao *et al.* 2005). Aufgereinigte Cytokeratin 19 positive Zellen ($CK19^+$) exprimierten *in vitro* den Transkriptionsfaktor Pdx1, der neben Insulin als wichtigster Marker für insulinproduzierende Zellen gilt (Gmyr *et al.* 2000). Eine Arbeit an embryonalen Stammzellen der Maus belegte eine Beteiligung von CK19 an der Differenzierung in insulinproduzierende Zellen (Blyszczuk *et al.* 2004). Einer kalifornischen Arbeitsgruppe ist es mittlerweile gelungen, humane ES-Zellen mit einem sehr eleganten Differenzierungsprotokoll in β-zellähnliche Vorläuferzellen zu differenzieren (D'Amour *et al.* 2006). Diese Arbeit beruhte auf der zuvor veröffentlichten These, dass durch Behandlung mit Activin A in den Frühstadien einer Differenzierung der Anteil an Zellen mit Charakteristika des definitiven Endoderms drastisch erhöht werden könnte (D'Amour *et al.* 2005). Eine ähnliche Schlussfolgerung wurde bereits für Stammzellen der Maus veröffentlicht (Kubo *et al.* 2004). Durch den erhöhten Anteil der endodermalen Zellen sollte die Basis für eine größere

Ausbeute an insulinproduzierenden Zellen geschaffen werden. Eine neuere Arbeit an humanen embryonalen Stammzellen konnte die Beteiligung von CK19 an der Differenzierung in insulinproduzierende Zellen klar belegen (Jiang *et al.* 2007). Daher könnte das Auftreten von $CK19^+$ Zellen im Verlaufe der Differenzierung von ES-Zellen eine analoge Erscheinung zu der beschriebenen Transdifferenzierung von duktalen Zellen in ß-Zellen darstellen.

1.4 Fragestellung und Ziel der Dissertation

Embryonale Stammzellen sind in der Lage jeden adulten Zelltyp durch Differenzierung hervorzubringen. Der Einsatz von embryonalen Stammzellen in der Zellersatztherapie des Diabetes mellitus stellt eine vielversprechende Alternative zur Insulinsubstitution dar. Bislang fehlen aber effiziente Differenzierungsprotokolle, um aus ES Zellen spezifisch monohormonale, insulinproduzierende Surrogatzellen zu erzeugen.

Daher war es das Ziel dieser Arbeit, ein verlässliches Differenzierungsprotokoll für embryonale Stammzellen der Maus zu entwickeln und dieses mit einem Sortierungsverfahren zu kombinieren, um insulinproduzierende Zellen von anderen, unerwünschten Zellen abzutrennen.

1. Zunächst sollte das Lumelsky-Protokoll reproduziert und als Referenzprotokoll etabliert werden.
2. Durch die Entwicklung eines neuen optimierten 4-Stadien Differenzierungsprotokolls sollten die Nachteile des Referenzprotokolls überwunden werden. Die Funktionalität der Zellen aus beiden Differenzierungsprotokollen sollte durch Implantationsversuche im Streptozotocin-diabetischen Mausmodell geklärt werden.
3. Zellklone, die mit einem Plasmid stabil transfiziert wurden, in welchem der Cytokeratin 19 Promoter die Expression des fluoreszierenden Reportergens eYFP kontrolliert, sollten mit dem neuen optimierten 4-Stadien Differenzierungsprotokoll differenziert werden. Gleichzeitig sollten im Verlauf der Differenzierung fluoreszierende Zellen ($CK19^+$) und nicht fluoreszierende Zellen ($CK19^-$) mit einem FACS-Sorter aufgereinigt werden.
4. Um die Beteiligung von CK19 an der Differenzierung in insulinproduzierende Surrogatzellen aufzuklären, sollten beide Zellfraktionen einer detaillierten biochemischen, molekularbiologischen und morphologischen Analyse unterzogen werden.

2 Publikationen

Titel:

A new experimental protocol for preferential differentiation of mouse embryonic stem cells into insulin-producing cells

Zeitschrift:

Cell Transplantation

Zur Publikation angenommen am 30.05.2008.

Titel:

Changes in gene expression and morphology of mouse embryonic stem cells upon differentiation into insulin-producing cells *in vitro* and *in vivo*

Zeitschrift:

American Journal of Pathology

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Titel:

An efficient experimental strategy for mouse ES cell differentiation and separation of a cytokeratin-19-positive population of insulin-producing cells

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A New Experimental Protocol for Preferential Differentiation of Mouse Embryonic Stem Cells Into Insulin-Producing Cells

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Mouse embryonic stem (ES) cells have the potential to differentiate into insulin-producing cells, but efficient protocols for in vitro differentiation have not been established. Here we have developed a new optimized four-stage differentiation protocol and compared this with an established reference protocol. The new protocol minimized differentiation towards neuronal progeny, resulting in a population of insulin-producing cells with β-cell characteristics but lacking neuronal features. The yield of glucagon and somatostatin cells was negligible. Crucial for this improved yield was the removal of a nestin selection step as well as removal of culture supplements that promote differentiation towards the neuronal lineage. Supplementation of the differentiation medium with insulin and fetal calf serum was beneficial for differentiation towards monohormonal insulin-positive cells. After implantation into diabetic mice these insulin-producing cells produced a time-dependent improvement of the diabetic metabolic state, in contrast to cells differentiated according to the reference protocol. Using a spinner culture instead of an adherent culture of ES cells prevented the differentiation towards insulin-producing cells. Thus, prevention of cell attachment in a spinner culture represents a means to keep ES cells in an undifferentiated state and to inhibit differentiation. In conclusion, this study describes a new optimized four-stage protocol for differentiating ES cells to insulin-producing cells with minimal neuronal cell formation.

Key words: Embryonic stem cells; Differentiation; Insulin; Diabetes

INTRODUCTION

Embryonic stem cells are pluripotent cells purified from the inner cell mass of the blastocyst-stage embryo (11). They can be permanently cultured and represent an unlimited source of cells with the potential to differentiate into any kind of adult tissue. Because these cells possess the potential for cell replacement therapy they could substitute for the shortage of pancreatic islets required for implantation therapy of type 1 diabetes mellitus. Indeed, the generation of insulin-producing surrogate cells from ES cells with characteristics comparable to those of natural pancreatic β-cells has been attempted from mouse (16,26) and human embryonic stem cells (1,6). However, controversial results have emerged.

The differentiation protocol by Lumelsky et al. (16), originally developed from a culture protocol for neuronal differentiation (15), also has been used for differentiation towards insulin-producing cells. This protocol has been modified by several groups, but with limited success and divergent results (2,3,14,17). Cells differentiated according to the Lumelsky protocol are prone to cell death and may take up insulin from the differentiation medium, which is supplemented with very high concentrations of insulin (13,21,23). The neuronal orientation of the protocol has cast doubt upon its suitability for differentiation into insulin-producing cells, because the cell type obtained is neuronal with very limited constitutive insulin gene expression, low insulin content, and poor insulin release compared with a true insulin-secreting cell type (25).

Thus, the aim of this study was to develop an improved differentiation protocol suitable for generation of insulin-producing surrogate cells. We compared four protocols for ES cell differentiation towards insulin-producing cells. We reproduced the five-stage protocol by Lumelsky et al. (16) and compared this with a slightly modified protocol, in which fetal calf serum (FCS) was added to the final cultivation step of the differentiation procedure. We also designed a new optimized four-stage

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differentiation protocol. This protocol relies on the development of two-layered spherical clusters (embryoid bodies) from single cells in suspension, and on the outgrowth of these embryoid bodies (EBs) in serum-free adherent cell culture. We supplemented the differentiation medium during the final phase with FCS and nicotinamide. Our new optimized four-stage differentiation protocol minimized neuronal differentiation and increased expression of β-cell characteristic genes. Through removal of the nestin selection step and through removal of cell culture supplements that would promote survival of cells undergoing differentiation towards the neuronal lineage, we were able to generate cells with a typical β-cell phenotype.

It has been reported that cultivation of ES cells in a histotypic spinner culture improves maturation and differentiation (3). We therefore also differentiated ES cells in a spinner culture using our optimized four-stage differentiation protocol in combination with a magnetic glass ball steering system. Interestingly, the results show that in contrast to previous findings, cells in spinner culture retained an embryonic phenotype and showed no signs of differentiation towards insulin-producing cells.

MATERIALS AND METHODS

Materials

DMEM and DMEM/F-12 tissue culture media, glutamine, nonessential amino acids, bFGF, and Pluronic F-68 were obtained from Invitrogen (Karlsruhe, Germany). Fetal calf serum (FCS) embryonic stem cell grade and gentamicin were purchased from PAA (Vienna, Austria) and leukemia inhibitory factor (LIF) from Chemicon (Temecula, CA, USA). Insulin, transferrin, sodium selenite, putrescine, and progesterone were from Sigma (St. Louis, MO, USA). All primer pairs, including random hexamer primers, were synthesized by MWG (Munich, Germany). The RevertAid™ H-M-MuLV reverse transcriptase was from Fermentas (St. Leon-Rot, Germany). The Biotherm™ Taq-polymerase as well as the dNTPs were from Genecraft (Münster, Germany). SybrGreen I was from Biozym (Hess. Oldendorf, Germany) and the plastic ware for the real-time-PCR reaction was from Abgene (Hamburg, Germany). Ac-DEVD-AMC was obtained from Biosource International (Camarillo, CA, USA) and AMC from Merck (Darmstadt, Germany). The ultrasensitive insulin ELISA was purchased from Mercodia (Uppsala, Sweden). Unless otherwise mentioned chemicals of analytical grade were obtained from Sigma or Merck (Darmstadt, Germany).

Cell Lines and Culture Conditions

The mouse embryonic stem (ES) cell line ES-D3 (10), which allows feeder-free culturing as well as co-culture with inactivated primary mouse embryonic fibroblasts (feeder layer), was purchased from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). In order to maintain the cells in an embryonic state, they were cultured on a feeder layer of mouse embryonic fibroblasts in stem cell medium (DMEM) containing 25 mM glucose and supplemented with 15% (v/v) FCS, 2 mM L-glutamine, 100 μM nonessential amino acids, 0.1 mM β-mercaptoethanol, 50 μg/ml gentamicin, and 1000 U/ml LIF in a humidified atmosphere at 37°C and 5% CO_2. Medium was changed daily and the cells were transferred for two passages on gelatin-coated tissue culture dishes to remove the feeder layer.

For differentiation the cells were cultured either according to a reference protocol (16), to a modified reference protocol, to a new optimized four-stage differentiation protocol, or to the new optimized four-stage differentiation protocol conducted in a histotypic spinner culture (Fig. 1). Detailed information about the reference protocol can be found elsewhere (16). To modify the reference protocol, the final differentiation medium was supplemented with different concentrations of FCS (1–10%). For differentiation with the new optimized four-stage differentiation protocol the cells were trypsinized and counted with a hemocytometer. One million cells were transferred onto a bacterial culture dish in medium as described above but devoid of LIF. Cells were then grown for up to 5 days in suspension. During this time cells formed EBs, which were allowed to settle down on gelatin-coated dishes in serum-free DMEM/F-12 medium supplemented with 25 μg/ml insulin, 50 μg/ml transferrin, 30 nM sodium selenite, 20 nM progesterone, 100 μM putrescine, 2 mM L-glutamine, 100 μM nonessential amino acids, and 10 ng/ml bFGF for 14 days. Thereafter the cells were cultured for 7 days in DMEM/F-12 medium supplemented with 25 μg/ml insulin, 50 μg/ml transferrin, 30 nM sodium selenite, 20 nM progesterone, 100 μM putrescine, 5% FCS, 2 mM L-glutamine, 100 mM nonessential amino acids, and 10 mM nicotinamide.

For differentiation in a spinner culture embryonic stem cells were trypsinized and counted with a hemocytometer. Subsequently 2×10^7 cells were resuspended in 100 ml DMEM medium containing 25 mM glucose, 15% (v/v) FCS, 2 mM L-glutamine, 100 μM nonessential amino acids, 0.1 mM β-mercaptoethanol, and 50 μg/ml gentamicin. The cells were then transferred into 125-ml glass ball spinner flasks (Techne, Jahnsdorf, Germany) and put under constant rotation at about 25 revolutions per minute on a Biosystem 4 remote-controlled stirrer connected to a Variomag Biomodul 40B (H+P Labortechnik, Oberschleissheim, Germany). After 5 days of differentiation the medium was changed to serum-free DMEM/F-12 medium supplemented with 25 μg/ml

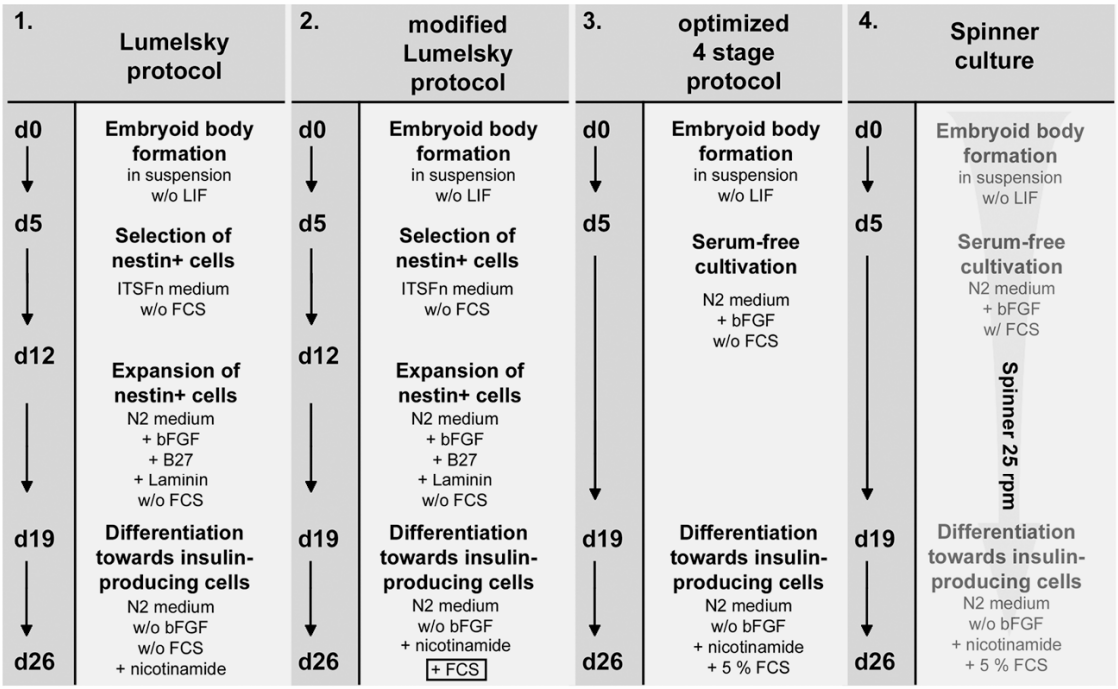

Figure 1. Schematic presentation of the four different culture protocols used for differentiation of mouse embryonic stem (ES) cells into insulin-producing cells.

insulin, 50 µg/ml transferrin, 30 nM sodium selenite, 20 nM progesterone, 100 µM putrescine, 2 mM L-glutamine, 100 µM nonessential amino acids, 10 ng/ml bFGF, and 0.1% Pluronic F-68 for 14 days. Thereafter the cells were cultured for 7 days in DMEM/F-12 medium supplemented with 25 µg/ml insulin, 50 µg/ml transferrin, 30 nM sodium selenite, 20 nM progesterone, 100 µM putrescine, 5% FCS, 2 mM L-glutamine, 1× nonessential amino acids, 0.1% Pluronic F-68, and 10 mM nicotinamide.

Molecular Biology

Total RNA was isolated from ES cells using the Chomczynski protocol (4). RNA was quantified photometrically and analyzed on a denaturating agarose gel. For cDNA synthesis random hexamers were used to prime the reaction of the RevertAid™ H⁻M-MuLV reverse transcriptase. The QuantiTect SYBR Green™ technology (Qiagen, Hilden, Germany), which uses a fluorescent dye that binds only double-stranded DNA, was employed. The reactions were performed using the DNA Engine Opticon™ Sequence Detection System (Biozym Diagnostik, Hess. Oldendorf, Germany). Samples were first denatured at 94°C for 3 min followed by 40 PCR cycles. Each cycle comprised a melting step at 94°C for 30 s, an annealing step at 62°C for 30 s, and an extension step at 72°C for 30 s. Primers used for qRT-PCR were designed exon-spanning to avoid amplification of genomic DNA (Table 1). All amplicons were in a size ranging from 100 to 300 base pairs. Each PCR amplification was performed in triplicate. The optimal parameters for the PCR reactions were empirically defined. The purity of the amplified PCR product was verified by melting curves. Data are expressed as relative gene expression after normalization to the β-actin housekeeping gene using the Qgene96 and LineRegPCR software (18,24).

Caspase 3 Enzyme Activity

The caspase 3 enzyme activity in ES cells was determined by specific cleavage of the fluorescent substrate Ac-DEVD-AMC (27). Cells (1×10^7) were lysed in cell lysis buffer (50 mM HEPES, pH 7.4, 0.1% Chaps, 5 mM DTT, 0.1 mM EDTA) for 5 min at 4°C and centrifuged for 10 min at $10{,}000 \times g$. The protein content was subsequently determined with the Bradford assay. Total protein (10 µg) was added to 80 µl assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% Chaps, 10 mM DTT, 1 mM EDTA, 10% glycerol) and to 10 µl of the caspase-3 substrate Ac-DEVD-AMC, providing a final

Table 1. Primers Used for qRT-PCR.

Gene	Primer Sequence
Insulin	F: 5′-CCCACCCAGGCTTTTGTCAAACAGC-3′ R: 5′-TCCAGCTGGTAGAGGGAGCAGATG-3′
Glucagon	F: 5′-CAGGGCACATTCACCAGCGACTAC-3′ R: 5′-TCAGAGAAGGAGCCATCAGCGTG-3′
Somatostatin	F: 5′-ATGCTGTCCTGCCGTCTCCA-3′ R: 5′-TGCAGCTCCAGCCTCATCTCG-3′
IAPP	F: 5′-TGCAGCTCCAGCCTCATCTCG-3′ R: 5′-CTCTCTGTGGCACTGAACCA-3′
Glut2	F: 5′-GAAGACAAGATCACCGGAACCTTGG-3′ R: 5′-GGTCATCCAGTGGAACACCCAAAA-3′
Glucokinase	F: 5′-GAGGTCGGCATGATTGTGGGCA-3′ R: 5′-GCGCCCCACTCTGTGTTGACACAC-3′
Kir6.2	F: 5′-TGCTGTCCCGAAAGGGCATTATC-3′ R: 5′-TGCAGTTGCCTTTCTTGGACACG-3′
Sur1	F: 5′-ACCAAGGTGTCCTCAACAACGGCT-3′ R: 5′-TGGAGCCAGGTGCTATGGTGAATG-3′
Nestin	F: 5′-GAGAGTCGCTTAGAGGTGCA-3′ R: 5′-CCACTTCCAGACTAAGGGAC-3′
NCAM	F: 5′-CGACGAGGCCGAATACGTCTG-3′ R: 5′-GCTCCTCTAGTTCCATGGCCGTC-3′
Pdx1	F: 5′-ACCGCGTCCAGCTCCCTTTC-3′ R: 5′-CAACATCACTGCCAGCTCCACC-3′
Nkx6.1	F: 5′-AGAACCGCAGGACCAAGTGGAGAA-3′ R: 5′-TCGTCATCCTCCTCATTCTCCGAAG-3′
Oct4	F: 5′-AGGCCCGGAAGAGAAAGCGAACTA-3′ R: 5′-TGGGGGCAGAGGAAAGGATACAGC-3′
Cytokeratin 19	F: 5′-GGTGCCACCATTGACAACTC-3′ R: 5′-CTGCATCTCCAGGTCAGTCC-3′
Carbonic anhydrase 2	F: 5′-CCACCACTGGGGATACAGCAAGC-3′ R: 5′-GTCCTCCTTTCAGCACTGCATTGTC-3′
Amylase 2	F: 5′-CTGTGAACACAGATGGCGTCAAATC-3′ R: 5′-GCAGGAAGACCAGTCTGTAAAGTGGC-3′
Albumin	F: 5′-CCTCCTCTTCGTCTCCGGCTCTG-3′ R: 5′-GGGATTTGTCACAGTTGGCGGC-3′
β-Actin	F: 5′-AGAGGGAAATCGTGCGTGAC-3′ R: 5′-CAATAGTGATGACCTGGCCGT-3′

F: forward (sense) primer; R: reverse (antisense) primer.

substrate concentration of 0.03 mM. The increase of free fluorescence was quantified fluorimetrically at 360 nm (excitation) and 460 nm (emission) for 3 h at 37°C. Caspase-3 activity was measured throughout the increase of fluorescence in 60 min. The units were calculated against a standard dilution curve of free AMC. The caspase-3 enzyme activity is expressed as one unit = cleavage of 1 nmol AMC × h^{-1}.

Implantation of ES Cells

CD-1 albino mice (Charles River, Margate, UK) were housed in an air-conditioned room at 21 ± 1°C and 50%

humidity with a 12:12 h light/dark cycle. Drinking water and a standard breeding diet (RM3, Special Diet Services, Witham, UK) were freely available. Seventeen male mice at 10–15 weeks of age were used in the experiments. Diabetes was induced by IP administration of streptozotocin (120 mg/kg) to 4-h fasted mice. Blood glucose was monitored (Glucotrend, Roche Diagnostics, Lewes, UK) using nonfasted tail-tip blood samples, and implants were undertaken after 7–12 days when blood glucose was typically 270–360 mg/dl. Food was withheld 2–4 h before surgery under isoflurane anesthesia. Implants of approximately 2×10^7 cells, corresponding to 66 ng insulin/2×10^7 cells for implants from the reference protocol and 96 ng insulin/2×10^7 cells for implants from the new optimized four-stage differentiation protocol, were introduced beneath the left kidney capsule. Blood glucose and body weight were monitored until the study was terminated. All animal procedures were conducted in accordance with the British Animals Scientific Procedures Act.

Alkaline Phosphatase Staining

For alkaline phosphatase staining, undifferentiated stem cells and cells differentiated in a spinner culture were trypsinized and seeded on a feeder layer of inactivated mouse embryonic fibroblasts and cultured for 4–5 days in stem cell medium. Subsequently the cells were stained for alkaline phosphatase with the alkaline phosphatase detection kit from Chemicon according to the manufacturer's instructions (Chemicon, Temecula, CA, USA). For light microscopy, stained colonies were observed on a Nikon TMS microscope using phase-contrast filters and documented with a Nikon Coolpix 4500 digital camera.

Insulin Content

For measurement of cellular insulin content, cells were sonicated in Krebs-Ringer buffer. Insulin was determined by ultrasensitive ELISA according to the manufacturer's instructions.

Ultrastructural Characterization

For electron microscopy, cell pellets were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, postfixed in 1% OsO_4, and finally embedded in Epon. Thin sections were contrast stained with saturated solutions of lead citrate and uranyl acetate and viewed in an electron microscope (12).

Statistical Analyses

Data are expressed as mean values ± SEM. Unless stated otherwise statistical analyses were performed using one-way ANOVA followed by Dunnett's test for multiple comparisons or *t*-test for correlations using the Prism analysis program (Graphpad, San Diego, CA, USA).

RESULTS

Comparison of Four Different Protocols for Differentiation of Embryonic Stem Cells Into Insulin-Producing Cells

Islet Hormones. Mouse embryonic stem (ES) cells did not express pancreatic islet hormones when maintained in an undifferentiated, pluripotent state cultured in the presence of LIF (Table 2). When the cells were differentiated according to the protocol by Lumelsky and coworkers (16) quantitative analysis of gene expression confirmed expression of the four endocrine hormones insulin, glucagon, somatostatin, and IAPP (Table 2). When the cells were differentiated according to the Lumelsky protocol in the additional presence of 5% FCS during the last 7 days of the 26-day protocol, the expression levels of the three major hormones insulin, glucagon, and somatostatin were reduced by around 50% (Table 2). Only the expression of IAPP was increased (Table 2). Thus, the addition of FCS, though it preserved the ultrastructure (data not shown) of the differentiated cells, neither reduced the polyhormonal character nor affected the expression level of the hormones positively (Table 2). This, however, was achieved through the new optimized four-stage differentiation protocol. The cells expressed insulin, while glucagon expression was not detectable and somatostatin expression was lower than the Lumelsky reference protocol (Table 2). Even the expression of IAPP was significantly reduced (Table 2).

Structural Markers. Mouse ES cells did not express the Glut2 glucose transporter gene and ES cells showed virtually no expression of the neuronal cell markers nestin and NCAM. The genes for glucokinase and the genes for Kir6.2 and Sur1, which are characteristic for insulin-secreting β-cells as well as neuronal cells, were expressed in ES cells, but to a lesser extent than in cells differentiated according to the Lumelsky reference protocol (Table 2).

Addition of 5% FCS during the last 7 days of differentiation culture of the Lumelsky protocol significantly increased expression of the Glut2, Sur1, and nestin genes in particular (Table 2). In the new optimized four-stage differentiation protocol the expression level of the three structural genes typical for β-cells, namely glucokinase, Kir6.2, and Sur1, were maintained at a high level while the expression of the neuronal markers nestin and NCAM decreased very significantly (Table 2). The expression level of the Glut2 glucose transporter was significantly increased. In addition, expression levels of

Table 2. Comparison of Four Protocols for Differentiation of Mouse Embryonic Stem (ES) Cells Into Insulin-Producing Cells

	ES Cells (%)	Lumelsky Protocol (%)	Lumelsky Protocol + FCS (%)	Optimized Four-Stage Protocol (%)	Spinner Culture (%)
Islet hormones					
Insulin	n.d. (4)*	100 ± 8 (16)	57 ± 3 (8)	131 ± 14 (30)	11 ± 3 (3)
Glucagon	n.d. (4)*	100 ± 23 (8)	41 ± 16 (3)	n.d. (4)*	n.d. (4)*
Somatostatin	n.d. (4)†	100 ± 20 (8)	42 ± 16 (4)†	11 ± 3 (21)†	2 ± 1 (3)†
IAPP	n.d. (4)†	100 ± 15 (10)	147 ± 31 (6)	66 ± 14 (16)	3 ± 0 (4)*
Structural markers					
Glut2	n.d. (4)	100 ± 21 (11)	239 ± 52 (5)	270 ± 53 (18)*	45 ± 15 (3)
Glucokinase	44 ± 7 (4)	100 ± 10 (10)	86 ± 7 (5)	105 ± 18 (21)	38 ± 3 (5)
Kir6.2	32 ± 3 (4)	100 ± 6 (8)	149 ± 28 (5)	103 ± 18 (18)	39 ± 19 (4)
Sur1	28 ± 4 (5)	100 ± 11 (11)	226 ± 32 (4)	175 ± 30 (21)	51 ± 3 (4)
Nestin	2 ± 0 (5)†	100 ± 16 (9)	357 ± 39 (3)†	47 ± 8 (21)†	30 ± 8 (4)*
NCAM	1 ± 0 (4)†	100 ± 15 (10)	54 ± 10 (5)*	17 ± 2 (17)†	51 ± 9 (3)*
CK19	146 ± 72 (4)	100 ± 22 (5)	275 ± 80 (3)	895 ± 81 (10)†	103 ± 18 (5)
CA2	30 ± 9 (4)	100 ± 29 (4)	20 ± 8 (3)	561 ± 103 (11)*	236 ± 77 (4)
Transcription factors					
Pdx1	554 ± 93 (4)	100 ± 21 (7)	140 ± 79 (3)	1246 ± 274 (19)*	335 ± 60 (3)
Nkx6.1	1 ± 0 (4)*	100 ± 13 (9)	253 ± 36 (4)†	71 ± 17 (19)	8 ± 2 (3)
Oct4	264 ± 34 (4)†	100 ± 10 (7)	265 ± 80 (4)†	138 ± 17 (21)	432 ± 25 (3)†

Data shown are gene expression values determined by qPCR on day 26 of differentiation according to the Lumelsky reference protocol, the Lumelsky protocol plus 5% fetal calf serum (FCS), the new optimized four-stage protocol, a spinner culture protocol, and, for comparison, the undifferentiated ES cells. Depicted are changes in relative gene expression in percent normalized to the reference protocol by Lumelsky. Values shown are means ± SEM of the relative gene expression with the numbers of experiments given in parentheses. n.d. = not detectable.
*$p < 0.05$, compared with differentiated cells according to the Lumelsky reference protocol (second column).
†$p < 0.01$, compared with differentiated cells according to the Lumelsky reference protocol (second column).

carbonic anhydrase 2 (CA2) and cytokeratin 19 (CK19), both markers for pancreatic duct cells, were increased significantly by ninefold and sixfold, respectively (Table 2). Gene expression analysis of amylase revealed only traces of mRNA expression in cells from the reference protocol, while in cells from the new optimized four-stage protocol and in stem cells amylase expression remained undetectable (data not shown). Albumin expression, a marker for the differentiation towards hepatic progeny, was also negligible in all protocols with levels typically at or below the detection limit of the qRT-PCR (data not shown).

Transcription Factors. In contrast to ES cells differentiated according to the Lumelsky protocol, undifferentiated ES cells did not express Nkx6.1, which is a transcription factor characteristic for developing CNS and insulin-producing cells (Table 2). However, ES cells showed expression of the embryonic transcription factor Oct4 and the β-cell transcription factor Pdx1 (Table 2). Addition of 5% FCS to the culture medium of the Lumelsky protocol increased expression in particular of Oct4 and Nkx6.1 (Table 2). Differentiation according to the new optimized four-stage protocol did not significantly affect Nkx6.1 and Oct4 expression when compared to the Lumelsky reference protocol but increased expression of Pdx1 very significantly (Table 2).

Spinner Culture. ES cells, which were differentiated in a spinner culture flask, exhibited negligible signs of expression of endocrine marker genes (Table 2). The gene with the highest expression was Oct4, which is known to be increased at the beginning of a differentiation process for embryonic stem cells (Table 2). Nkx6.1 expression remained marginal and Pdx1 expression was also not increased when compared to undifferentiated pluripotent ES cells (Table 2). A significant expression of nestin and NCAM during spinner culture when compared to undifferentiated pluripotent ES cells indicated a differentiation towards neuronal progeny (Table 2), but not towards endocrine lineage as illustrated by the low expression levels of insulin, somatostatin and IAPP (Table 2). Indeed, the expression of glucagon could not be detected in cells from the spinner culture. There was low-level expression of other structural genes, such as Glut2, glucokinase, Kir6.2, and Sur1, after spinner culture, generally similar to undifferentiated pluripotent ES cells (Table 2). Only the expression of CA2 was elevated around twofold when compared with ES cells, but this increase was not significant (Table 2).

Effect of Serum Supplementation on Insulin Gene Expression in Mouse ES Cells After Differentiation According to the Lumelsky Reference Protocol

Insulin gene expression of ES cells after differentiation with increasing amounts of fetal calf serum (1–10%) during the last 7 days of the differentiation protocol caused a significant decrease in the expression of insulin (Fig. 2A). Insulin gene expression decreased to 40% compared to the Lumelsky reference protocol, when the culture medium had been supplemented with 5% FCS (Fig. 2A). Higher FCS concentrations up to 10% did not further decrease insulin gene expression (Fig. 2A).

Effect of Insulin Removal From the Culture Medium on Insulin Gene Expression in ES Cells After Differentiation According to the Lumelsky Reference Protocol

When the medium was deprived of insulin, expression of the insulin gene decreased to 74% compared to the reference protocol, which was supplemented with 25 µg/ml insulin (Fig. 2B).

Changes of the Ultrastructure of ES Cells Undergoing Differentiation

ES cells that were differentiated according to the Lumelsky reference protocol possessed typical features of neuroendocrine cells. These cells contain a large number of small dark peptidergic vesicles presumably containing neurotransmitters and neuropeptide hormones. These cells were often detected adjacent to nerve fibers (Fig. 3A). In contrast, ES cells that were differentiated according to the new optimized four-stage protocol exhibited clear signs of endocrine differentiation (Fig. 3B). These cells showed both a well-developed rough endoplasmic reticulum and Golgi apparatus, well suited for insulin synthesis, together with a large number of insulin-containing secretory granules, some located in the close vicinity of the plasma membrane appropriate for exocytosis (Fig. 3B).

Comparison of the Caspase-3 Activity of ES Eells, Embryoid Bodies, and Cells at Days 12, 19, and 26 of Differentiation According to the Lumelsky Reference Protocol or to the New Optimized Four-Stage Protocol

Undifferentiated ES cells exhibited low caspase-3 activity (Fig. 4). Upon differentiation and formation of embryoid bodies (EBs) the enzyme activity increased around fourfold. When the cells were selected according to the Lumelsky reference protocol towards nestin-positive cells, the caspase-3 activity increased around ninefold at day 12 compared with undifferentiated ES cells. After a similar period cells differentiated by the new optimized four-stage protocol showed a significantly lower caspase-3 activity (Fig. 4). During the nestin expansion step of the Lumelsky protocol caspase-3 activity decreased but was still about twofold higher than in cells differentiated with the new optimized four-stage protocol (Fig. 4). After 26 days of differentiation cells from both protocols possessed comparable caspase-3 activity levels (Fig. 4).

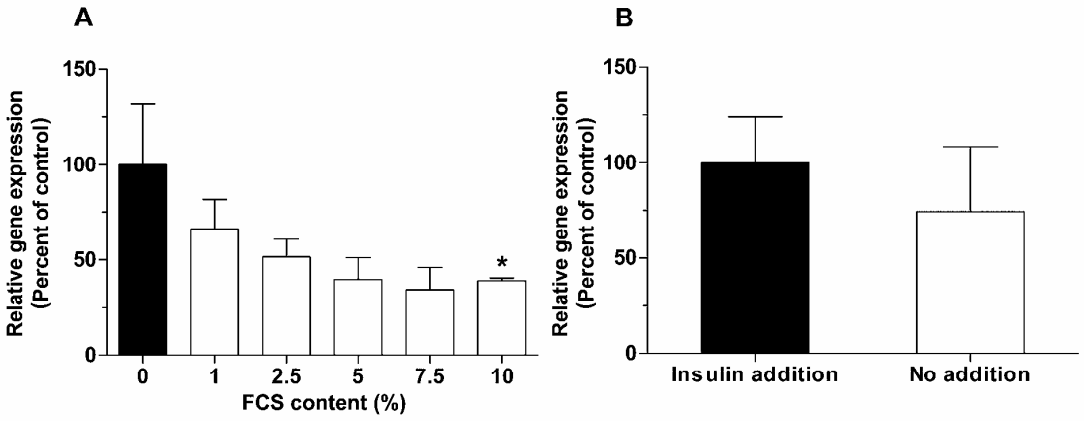

Figure 2. (A) Effect of serum supplementation on insulin gene expression in mouse embryonic stem (ES) cells after differentiation according to the Lumelsky reference protocol. (B) Effect of insulin removal from the differentiation medium on the insulin gene expression in ES cells after differentiation with the Lumelsky reference protocol. Data shown are insulin gene expression values determined by qPCR on day 26 of differentiation. Depicted are the changes in relative expression in percent normalized to the Lumelsky reference protocol. Values shown are means ± SEM of the relative gene expression of five to seven experiments. $*p < 0.05$ compared with cells cultured with 0% FCS.

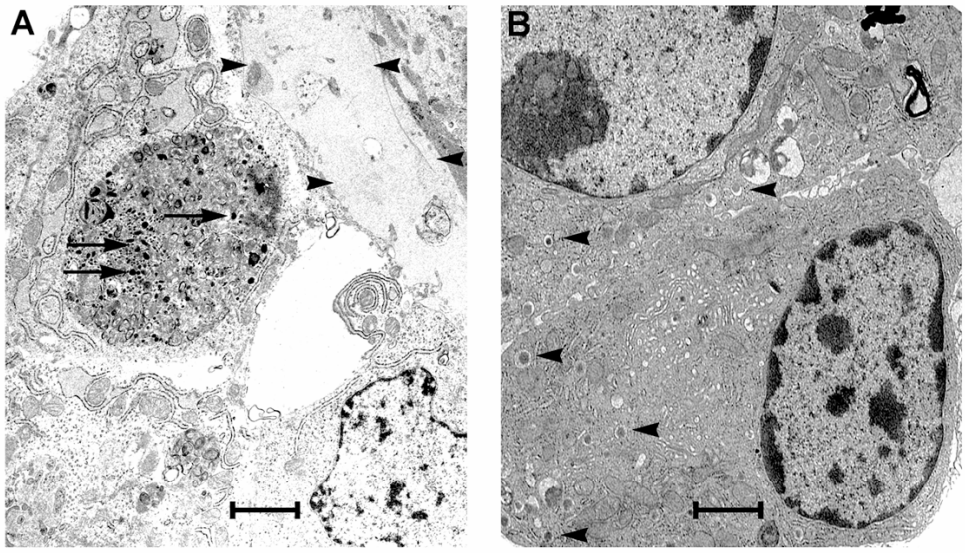

Figure 3. Changes of the ultrastructure of mouse embryonic stem (ES) cells undergoing differentiation. (A) Ultrastructure of a cell with neuroendocrine characteristics differentiated according to the Lumelsky reference protocol. The electron micrograph shows a neuroendocrine cell with peptidergic vesicles (arrow) in close proximity to a nerve fiber (arrowhead). Scale bar: 1 µm. (B) Ultrastructure of an insulin-producing cell differentiated according to the new optimized four-stage protocol. The electron micrograph shows an endocrine cell with insulin secretory granules (arrowhead) with a halo and dense core region. Scale bar: 1 µm.

Ultrastructure and Alkaline Phosphatase Staining of Undifferentiated Mouse ES Cells and ES Cells After 26 Days in a Spinner Culture

Analysis of ES cells by electron microscopy showed typical structural features of undifferentiated ES cells. The large nucleus typically appeared irregularly shaped with a high number of dense nucleoli (Fig. 5A). The cells exhibited a low ratio of cytoplasm to nucleus and a modest development of cellular organelles such as rough endoplasmatic reticulum (ER) and Golgi apparatus, although the cytoplasm was rich in free ribosomes and mitochondria. The cells frequently displayed microvilli leaving an obvious extracellular space between adjacent cells. Cell contacts between neighboring cells such as gap junctions were not observed (Fig. 5A).

ES cells, which were differentiated in a spinner culture, generally displayed the same phenotype as undifferentiated ES cells (Fig. 5B). The ratio of cytoplasm to nucleus was low. In addition the cytoplasm was rich in free ribosomes and mitochondria, as shown by undifferentiated ES cells (Fig. 5B). The cells formed cell aggregates with 50–70 cells in one section without gap junctions or other cell contacts (Fig. 5B). Morphological signs of neuroendocrine and especially endocrine differentiation could not be observed in cells from the spinner culture.

Undifferentiated ES cells grown on a layer of mitotically inactivated fibroblasts appeared upon light microscopical examination as typically round-shaped colonies. The colonies consisted of several hundred cells with uniform red staining of alkaline phosphatase that was slightly stronger at the periphery of a single colony than the core (Fig. 5C).

When cells from the spinner culture were replated on an inactivated feeder layer, they underwent continuous growth in distinct colonies. The colonies increased in size without any changes in the phenotype or signs of differentiation (Fig. 5D). The outgrowth retained the appearance of round-shaped colonies with smooth edges. Upon alkaline phosphatase staining these colonies showed the same red staining pattern as undifferentiated ES cells (Fig. 5D).

Implantation of Mouse Embryonic Stem (ES) Cells Differentiated According to the Lumelsky Reference Protocol and the New Optimized Four-Stage Protocol Under the Kidney Capsule of Streptozotocin (STZ) Diabetic Mice

The streptozotocin (STZ)-induced diabetes was associated with a progressive increase of hyperglycemia (Fig. 6). Without treatment (control), STZ-diabetic mice developed severe hyperglycemia (>400 mg/dl) and health status deteriorated, requiring this group to be terminated at day 14. Pseudoislets comprising about 2 ×

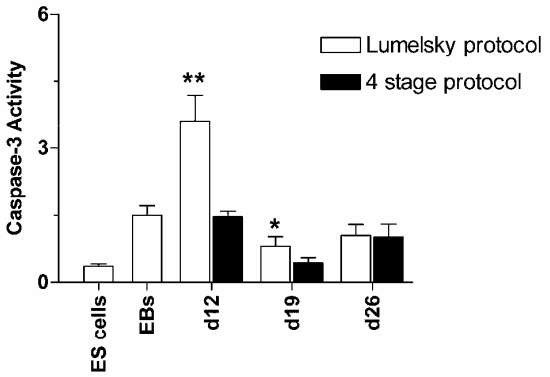

Figure 4. Comparison of the caspase-3 activity of mouse embryonic stem (ES) cells, EBs and cells at days 12, 19, and 26 of differentiation with the Lumelsky protocol or the new optimized four-stage protocol. Values shown are means ± SEM of the caspase-3 activity (μmol DEVD × h^{-1}) of 6–14 experiments. *$p < 0.05$, **$p < 0.01$ comparison between the Lumelsky and the new optimized four-stage protocol.

10^7 cells differentiated by the Lumelsky reference protocol were implanted under the kidney capsule. These pseudoislets were transferred into insulin-free buffered saline prior to implantation to avoid any acute glucose-lowering effect of the high insulin concentration in the differentiation medium. The pseudoislet implant did not prevent the rise in hyperglycemia, but limited the blood glucose concentration to about 400 mg/dl for 3–16 days after implantation. In contrast to the control and Lumelsky reference groups, mice implanted with 2×10^7 cells differentiated by the optimized four-stage differentiation protocol prevented the rise in hyperglycemia, and reduced blood glucose concentrations to <200 mg/dl by day 16. Cells were implanted using insulin-free buffered saline to avoid any artifactual effect of insulin in the medium.

Insulin Content

Content of insulin protein, determined by ELISA, was in the same range when cells had been differentiated in the presence of exogenous insulin according to the Lumelsky reference protocol (3.08 ± 0.09 pg insulin/μg DNA) and according to the new optimized four-stage protocol (4.68 ± 1.10 pg insulin/μg DNA). Removal of exogenous insulin during the last 7 days of differentiation culture reduced the cellular insulin content. However, this reduction was significantly more with the Lumelsky reference protocol (0.33 ± 0.24 pg insulin/μg DNA) than with the new optimized four-stage protocol (2.67 ± 0.74 pg insulin/μg DNA). In undifferentiated ES cells and cells subjected to 26 days of spinner culture insulin content values were below the detection limit.

DISCUSSION

In this study we developed a new optimized four-stage protocol for differentiation of mouse ES cells towards insulin-producing cells and compared this new differentiation protocol with a reference protocol originally developed by Lumelsky and collaborators (16). In addition we studied the effect of FCS supplementation to the culture medium and analyzed the potential suitability of a spinner culture technique for differentiation of ES cells towards insulin-producing cells. We reproduced the protocol by Lumelsky and coworkers and, in contrast to earlier work (25), we obtained evidence for insulin gene expression along with the expression of other pancreatic islet hormones. However, analysis of neuronal markers exhibited a high expression level of the nestin and NCAM genes, while expression of Pdx1, which is required for transactivation of the insulin gene, was lower than the Pdx1 expression detected in undifferentiated ES cells (17). Furthermore, withdrawal of exogenous insulin from the differentiation medium markedly lowered the insulin content of these cells, even though insulin gene expression was not decreased significantly. This could in part explain the confusion in previous studies regarding the source of the insulin accumulated by the cells, and explain why the original Lumelsky reference protocol has created a false impression of effectiveness (13,20,23,25).

The lack of Pdx1 expression and the inability to store significant quantities of insulin despite clearly detectable insulin expression indicate a nonregulated activation of the insulin gene, such as reported for developing tissue of the central nervous system (7–9,22). Indeed, ultrastructural analyses revealed that the differentiated cells exhibited typical signs of neuronal development. Not surprisingly, therefore, these cells failed to significantly reduce blood glucose concentrations in the STZ-diabetic mouse model. Thus, the gene expression pattern found in cells of the Lumelsky protocol (16) together with the ultrastructural findings and the in vivo results provide clear proof for differentiation towards a neuronal cell type with a moderate insulin expression but without typical characteristics of insulin-producing β-cells.

Because the original protocol proposed the idea of nestin positivity as a crucial feature of a putative β-cell precursor cell, the nestin selection step was introduced into the Lumelsky protocol. We, however, observed that during the nestin selection within the Lumelsky differentiation protocol the activity of the apoptosis-inducing protein caspase-3 was prominent. Thus, the nestin selection step during differentiation of ES cells is apparently a means to enrich neuronal cells or those cells that are undergoing differentiation towards neuronal development, while differentiation towards cell types of the en-

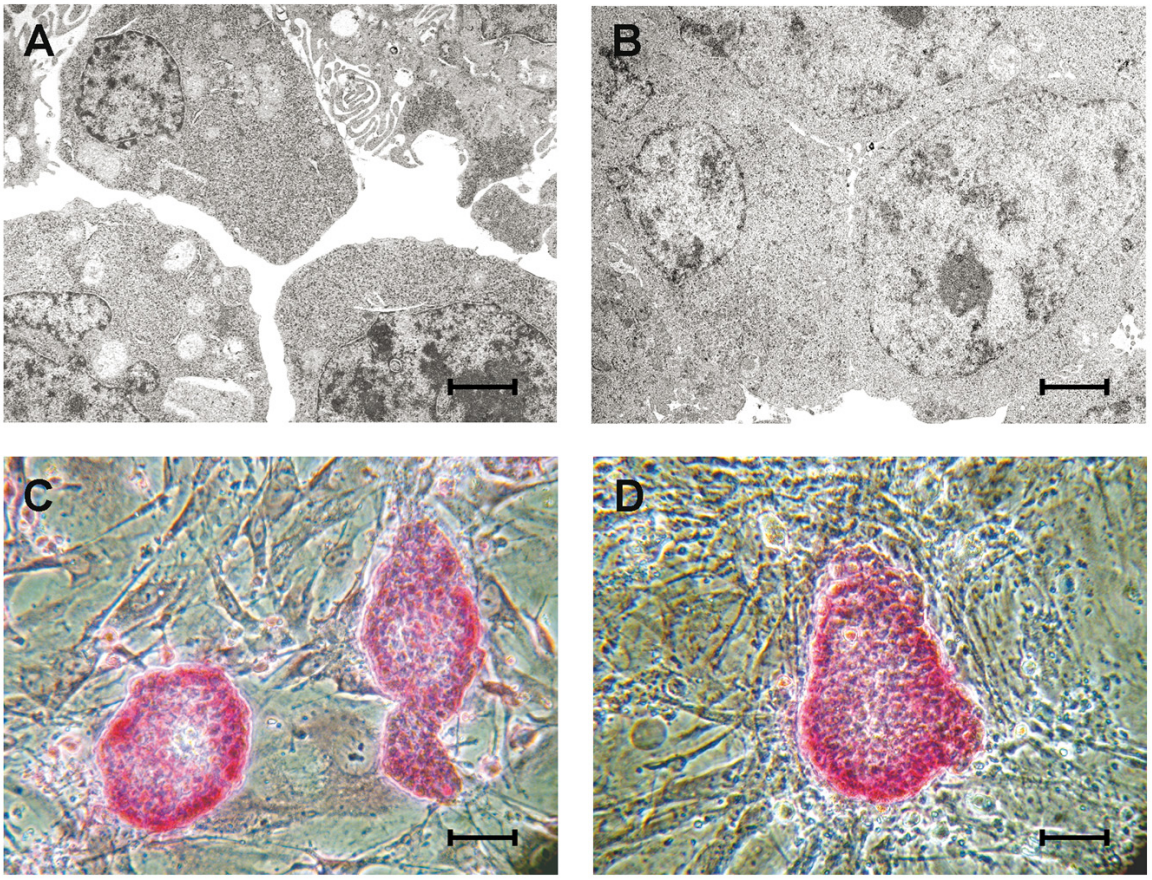

Figure 5. Ultrastructure (A, B) and alkaline phosphatase (C, D) staining of undifferentiated mouse embryonic stem (ES) cells (A, C) and ES cells after 26 days in a spinner culture (B, D). (A) Ultrastructure of undifferentiated ES cells showing free ribosomes and mitochondria but no other higher cell organelles. Scale bar: 1 µm. (B) Ultrastructure of undifferentiated cells after 26 days in a spinner culture with comparable morphology. Scale bar: 1 µm. (C) Alkaline phosphatase staining (red) in colonies of undifferentiated ES cells plated on a layer of inactivated feeder cells. Scale bar: 100 µm. (D) Alkaline phosphatase staining (red) in colonies of replated undifferentiated cells on a layer of inactivated feeder cells after prior spinner culture for 26 days. Scale bar: 100 µm.

docrine lineage may be suppressed. FCS supplementation to the original Lumelsky protocol even enhanced the neuronal character of the cells obtained with this differentiation protocol.

To overcome the drawbacks of the reference protocol we developed an improved differentiation protocol in order to obtain preferentially insulin-producing cells. We removed the nestin selection step and composed differentiation media lacking cell culture supplements that promote differentiation towards neuronal cells. In addition, the differentiation medium was supplemented with nutrients to avoid deprivation during the final cultivation step. We observed that cells that were differentiated according to this new optimized four-stage differentiation protocol expressed nearly exclusively insulin. Glucagon expression disappeared and somatostatin was negligible compared with the reference protocol. The reduced expression of nestin and NCAM reflects a shift away from neuronal differentiation towards a monohormonal population of insulin-producing cells. Interestingly, the removal of the nestin selection step also significantly reduced the activity of caspase-3, providing evidence for improved survival conditions of differentiated cells. At the same time the β-cell-like character of these insulin-producing cells was reinforced by the high expression of the Glut2 glucose transporter gene and other β-cell characteristic structural genes such as glucokinase, Kir6.2, and Sur1. Markers for differentiation towards ductal cells, namely CK19 and CA2, were significantly increased and the lack of expression of amylase and albumin excludes significant differentiation towards exocrine pancreas and hepatic progeny.

It is widely accepted that β-cells arise from ductal cells during organogenesis of the pancreas. Not surpris-

ingly, therefore, the strong expression of CK19 in our cells appears in parallel to the reinforced levels of insulin expression and other structural markers for endocrine cells. This reflects an in vitro differentiation mechanism analogous to that described for the differentiation of ductal tissue into endocrine insulin-producing cells during fetal development of the pancreas (19). Pdx1 is required for a regulated insulin expression in insulin-producing cells. In addition, we achieved a very high level of Pdx1 gene expression in cells differentiated according to this new optimized differentiation protocol.

We further investigated whether the changes in the expression pattern of these genes contributed to a phenotype with closer resemblance of a pancreatic β-cell. Indeed, the removal of the nestin selection step yielded cells with a clear endocrine phenotype as documented by electron micrographs of insulin-producing cells that contained insulin secretory granules. Addition of insulin to the differentiation medium of our new optimized differentiation protocol enabled the cells to produce and store more insulin independent of uptake of exogenous insulin from the differentiation medium. These findings were further supported by the outcome of the in vivo experiments, where implantation of cells from the new differentiation protocol prevented the rise of blood glucose in STZ-diabetic mice, and caused a significant reduction of the glucose values well below 200 mg/dl towards a range typical for nondiabetic mice.

It has recently been reported that a differentiation procedure based on the protocol by Lumelsky et al. (16) followed by a differentiation stage where adherent cells were transferred to a histotypic spinner culture produced a 14-fold increase in insulin content (3). The spinner culture keeps the cells in suspension during the whole differentiation process whereas the new optimized four-stage protocol presented in this study and other differentiation protocols developed earlier depend on adherent cell culture. In contrast to the previous report, we found that the spinner culture was not suitable for differentiation of ES cells towards insulin-producing cells, but rather maintained the embryonic character of the cells as documented ultrastructurally and by alkaline phosphatase staining of the cells (5). Moreover, we confirmed that the gene expression pattern in these cells demonstrated no significant changes when compared with undifferentiated ES cells.

This lack of differentiation during the spinner culture may be due to the inability of the cells to develop cell–cell contacts and gap junctions, which we confirmed by ultrastructural studies, because these are apparently required for differentiation (28). This prevention of differentiation caused by the prevention of adherence may represent an attractive method for standard tissue culture of embryonic stem cells, especially of those cell lines that normally require a layer of inactivated primary embryonic fibroblasts such as human embryonic stem cells. In conclusion, therefore, the present study has provided a new protocol for steering ES cells away from a neuronal progeny in order to facilitate differentiation to insulin-producing cells with β-cell-like features. The new protocol produced cells that can prevent the rising hyperglycemia after implantation in an animal model of

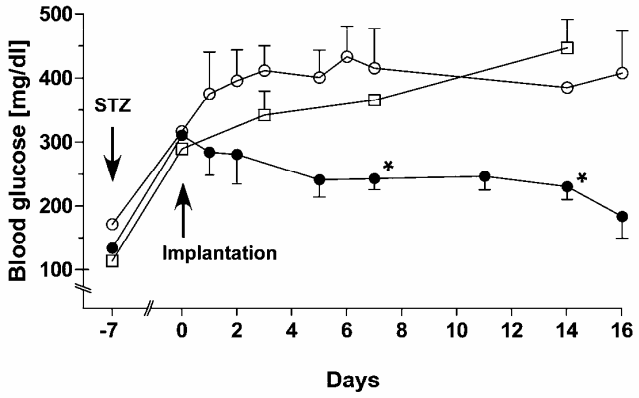

Figure 6. Implantation of mouse embryonic stem (ES) cells differentiated according to the Lumelsky reference protocol and the new optimized four-stage protocol under the kidney capsule of streptozotocin (STZ) diabetic mice. Shown are blood glucose concentrations of diabetic mice injected with streptozotocin (STZ) (120 mg/kg) 7 days prior to implantation of cells differentiated according to the Lumelsky reference protocol (open circles) or to the new optimized four-stage protocol (filled circles) and for comparison of control STZ diabetic mice that received no cells (open boxes). Values shown are means ± SEM from four to seven animals. $*p < 0.05$ compared with cells from the Lumelsky reference protocol.

insulinopenic diabetes. We also present evidence that preventing cell adherence can prolong the pluripotent embryonic state.

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Changes in gene expression and morphology of mouse embryonic stem cells upon differentiation into insulin-producing cells *in vitro* and *in vivo*

Running title: *in vitro* and *in vivo* morphology of implanted ES cells

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ABSTRACT

Implantation of mouse embryonic stem (ES) cells properly differentiated in vitro into insulin-producing cells according to an efficient differentiation protocol can have a further beneficial effect due to a final differentiation in the in vivo environment. In order to analyse this effect, a morphological study with respect to changes of beta cell characteristics after implantation of ES cells which were differentiated *in vitro* according to a reference protocol or a new 4 stage differentiation protocol was performed. The putative beneficial effect of an *in vivo* environment on ES cells developed into insulin-producing endocrine cells was evaluated before and after implantation. ES cells from two differentiation protocols were analysed for gene expression by *in situ* RT-PCR and by immunohistochemistry for protein expression. The signs of differentiation were additionally compared by ultrastructural analysis. In comparison with undifferentiated and nestin positive ES cells developed according the reference protocol, the number of differentiated ES cells from the new 4 stage protocol significantly increased under *in vivo* conditions. The cells, grown in a tissue-like structure, expressed on the gene and protein level the transcription factor Pdx1, insulin, IAPP, the GLUT2 glucose transporter and glucokinase, which are functional markers for glucose-induced insulin secretion of pancreatic beta cells. The comparative analysis revealed the maturation of in vitro differentiated ES cells after implantation *in vivo*. The study shows that ES cells with a higher degree of differentiation after *in vitro* differentiation according to the new 4 stage protocol further maturated in vivo when compared to those of the reference protocol.

INTRODUCTION

Implantation of insulin-producing cells into a diabetic organism is a promising therapeutic option for the treatment of diabetes [1]. The limited availability of donor islets for transplantation has drawn the attention to embryonic stem cells, which are available in unlimited numbers and have the potential to differentiate into most somatic cell types, including insulin-producing cells [2]. While different groups have recently published advances in this field [3, 4], the morphology of ES cells differentiated *in vitro* towards insulin-producing cells has not been analysed in detail before and after implantation. In particular the impact of the *in vivo* environment upon differentiation and maturation of differentiated ES cells after implantation has not been analysed yet. Therefore, it was the aim of this study to examine the maturation process and the benefit of the *in vivo* environment including a 3-dimensional growth in a tissue-like manner. We therefore performed a morphological study on ES cells which were differentiated *in vitro* according to a reference protocol previously published by Lumelsky and collaborators [5] and to a new efficient 4 stage differentiation protocol [2, 6]. Differentiated cells from both protocols were implanted into CD-1 albino mice and grafts were explanted 14 days after implantation.

The *in vitro* transition from ES cells to insulin-producing cells during differentiation was documented by a detailed analysis of protein expression of beta cell markers and embryonic transcription factors. Moreover, the cells from both protocols were subjected to an ultrastructural analysis of cell organelles to assess the differentiation status of the cells. Grafts of implanted ES cells were in parallel analyzed on gene and protein expression using the same beta cell markers and embryonic transcription factors as under *in vitro* conditions.

Pdx1 is an important marker for beta cell development and is expressed from the early stage of pancreas development initially when the epithelial duct forms out. Later on Pdx1 is selectively expressed in mature beta-cells. Since Pdx1 is the major transactivator of the insulin gene its presence is decisive for the transcriptional regulation of the insulin gene [7].

Downstream of the transcription and translation of the insulin gene occurs the processing of the proinsulin protein into mature insulin by cleavage of the internal C-peptide and its storage in secretory granules. This maturation process is associated with the storage of insulin in the core and C-peptide into in the halo region of secretory granules. Thus, C-peptide is a suitable marker to show not only the processing of proinsulin into mature insulin but also to verify *de novo* synthesis in contrast to insulin uptake from an exogenous source [8]. IAPP was used as a second islet hormone because it is specifically expressed in pancreatic beta cells without the possibility of an uptake from culture medium [9].

Two additional structures, which are essential for signal recognition in the process of glucose-induced insulin secretion of beta cells, are the GLUT2 glucose transporter and the glucose phosphorylating enzyme glucokinase. Glucokinase is the glucose sensor of the beta cell coupling glucose recognition to the exocytosis of insulin [10 11, 12]. The GLUT2 glucose transporter allows unlimited intracellular access of glucose to glucokinase, which is transactivated by Pdx1 [13]. Therefore the expression of Pdx1 together with insulin and C-peptide, the co-stored IAPP as well as the GLUT2 glucose transporter and glucokinase, were analysed to monitor the benefit of the *in vivo* environment on differentiated stem cells. In order to analyze the loss of stemness during the course of differentiation and subsequent implantation the gene and protein expression of the embryonic marker genes Oct4 and Nanog were monitored additionally. The expression of the intermediate neurofilament protein nestin was assessed to address the question whether neuronal progeny can be reduced with the new protocol in comparison to the reference protocol. This study shows that ES cells differentiated in vitro with the new 4 stage protocol comprised a higher degree of differentiation and further maturated *in vivo* when compared to those of the reference protocol.

MATERIALS AND METHODS

Materials

DMEM and DMEM/F-12 tissue culture media, glutamine, non-essential amino acids and bFGF were obtained from Invitrogen (Karlsruhe, Germany). Fetal calf serum (FCS) embryonic stem cell grade and gentamicin were purchased from PAA (Vienna, Austria) and leukemia inhibitory factor (LIF) from Chemicon (Temecula, USA). Insulin, transferrin, sodium selenite, putrescine and progesterone were from Sigma (St. Louis, MO, USA). All primer pairs, including random hexamer primers, were synthesised by MWG (Munich, Germany). The RevertAid™ H⁻M-MuLV reverse transcriptase was from Fermentas (St. Leon-Rot, Germany). The Biotherm™ Taq-polymerase as well as the dNTP's were from Genecraft (Münster, Germany). Unless otherwise mentioned chemicals of analytical grade were obtained from Sigma or Merck (Darmstadt, Germany).

Cell culture conditions

The mouse embryonic stem (ES) cell line ES-D3 [14] was purchased from the American Tissue Culture Collection (ATCC, Manassas, USA). In order to maintain the cells in an embryonic state, they were cultured on a feeder layer of mouse embryonic fibroblasts in stem cell medium (DMEM) containing 25 mM glucose and supplemented with 15 % (v/v) FCS, 2 mM L-glutamine, 100 µM non-essential amino acids, 0.1 mM β-mercaptoethanol, 50 µg/ml gentamicin and 1000 U/ml LIF in a humidified atmosphere at 37°C and 5 % CO2. Medium was changed daily and the cells were transferred for two passages on gelatin-coated tissue culture dishes to remove the feeder layer. For differentiation the cells were cultured according to a reference protocol [5] or to a new optimized 4 stage differentiation protocol [2,6]. For differentiation with the new optimized 4 stage differentiation protocol the cells were trypsinized and counted with a hemocytometer. 10^6 cells were transferred onto a bacterial culture dish in medium as described above but devoid of LIF. Cells were then grown for up to 5 days in suspension. During this time cells formed embryoid bodies which were allowed to

settle down on gelatin-coated dishes in serum-free DMEM/F-12 medium supplemented with 25 µg/ml insulin, 50 µg/ml transferrin, 30 nM sodium selenite, 20 nM progesterone, 100 µM putrescine, 2 mM L-glutamine, 100 µM non-essential amino acids and 10 ng/ml bFGF for 14 days. Thereafter the cells were cultured for 7 days in DMEM/F-12 medium supplemented with 25 µg/ml insulin, 50 µg/ml transferrin, 30 nM sodium selenite, 20 nM progesterone, 100 µM putrescine, 5 % FCS, 2 mM L-glutamine, 100 µM non-essential amino acids and 10 mM nicotinamide.

Implantation of ES cells

CD-1 albino mice (Charles River, Margate, UK) were housed in an air-conditioned room at 21 ± 1°C and 50 % humidity with a 12:12 h light/dark cycle. Drinking water and a standard breeding diet (RM3, Special Diet Services, Witham, UK) were freely available. Male mice were used at 10-15 weeks of age. Diabetes was induced by IP administration of streptozotocin (120 mg/kg) to 4h fasted mice. Blood glucose was monitored (GlucotrendR, Roche Diagnostics, Lewes, UK) using non-fasted tail-tip blood samples, and implants were undertaken after 7-12 days when blood glucose was typically 270 – 360 mg/dl. Food was withheld 2-4 h before surgery under isoflurane anaesthesia. Implants of approximately 2 x 10^7 cells were introduced beneath the left kidney capsule. Mice were killed by cervical dislocation after 14 days and the implants were removed for histological and immunocytochemical analyses. All animal procedures were conducted in accordance with the British Animals Scientific Procedures Act.

Tissue processing

For light microscopy, ES cells immobilized on slides, ES cell pellets and transplanted ES cells under the kidney capsule were fixed in 4 % para-formaldehyde in 0.15 M phosphate buffered saline (PBS), pH 7.3, and embedded in paraffin. Additionally, small ES cell pellets were fixed in 2 % para-formaldehyde and 2.5 % glutaraldehyde in 0.1 M cacodylate buffer,

pH 7.3, postfixed in 1 % OsO_4 and finally embedded in Epon for electron microscopical analysis [15].

Immunocytochemical staining of slides

For immunocytochemical staining of ES cells 1 x 10^6 differentiated cells were seeded overnight on glass slides and subsequently fixed with ice cold methanol. After a washing step the cells were permeabilized and blocked with PBS plus 0.2 % Triton X-100 and 5 % donkey serum. The slides were incubated with primary antibodies (Table 2) diluted in PBS with 0.1 % Triton X-100 and 0.1 % BSA at room temperature for 30 min or overnight at 4°C. Following this step the cells were washed with PBS and incubated with secondary antibodies for 30 min (1:500). Secondary antibodies from donkey were conjugated with Cy2, Texas Red or Cy5 and were obtained from Dianova (Hamburg, Germany). For nuclear counterstain 300 nM DAPI was used for 5 min at room temperature. Slides were thereafter mounted with Mowiol (Merck, Darmstadt, Germany) plus 0.06 % Dabco (St. Louis, USA).

Immunohistochemical staining of sections

Serial paraffin sections of the ES cell pellets from the differentiation protocols and after transplantation under the kidney capsule were stained either by the avidin-biotin-complex (ABC) method or by an immunofluorescence staining [16]. The slides were incubated overnight with the specific antibodies (Table 2), followed by a 30 min incubation with either biotinylated or fluorescence labelled goat anti-rabbit IgG, goat anti guinea pig or rabbit anti-mouse IgG (1:100, Santa Cruz Biotechnology, Santa Cruz, USA). In the ABC method the slides were incubated with a mixture of streptavidin (1:100) and biotin-peroxidase (1:1000) (Jackson ImmunoResearch, Suffolk, UK) and developed with 0.7 mM diaminobenzidine in 0.002 % hydrogen peroxidase containing phosphate buffered saline (PBS), pH 7.3. The same primary antibodies were used as for the immunofluorescence staining of the slides (Table 2). All antibodies were certified for immunohistochemistry and showed specific immunostaining in tissue sections from normal mouse pancreas or ES cells in early differentiation stages.

***In situ* RT-PCR**

Transplanted ES cells were fixed on 3-Chamber SuperFrost Plus™ slides on a heating block at 100°C for 2 min. Subsequently the slides were treated with proteinase K (20 µg/ml) for 20 min. at 37°C. Proteinase K was inactivated thereafter at 95°C for 2 min followed by an overnight incubation with a RNase-free DNase solution (1 U/ml) at 37°C. The *in situ* RT-PCR procedure was a modification according to different protocols [17, 18]. After rinsing with DEPC water and air drying reverse transcription was performed in a buffer containing a mixture of all nucleotides (1 mM dATP, dGTP, dCTP and dTTP) (Genecraft, Germany), oligo-dT primer (Invitrogen, UK), M-MLV reverse transcriptase (0.5 U/µl) (Invitrogen, UK), RNasin (Promega, USA) and ddH_2O. Slides were incubated at 37°C for 1 h in a moist chamber and thereafter reverse transcriptase was inactivated at 92°C for 2 min. PCR amplification was performed in a buffer containing a nucleotide mix (10 µM digoxigenin (DIG) 11-dUTP, 190 µM dTTP, 200 µM each of dATP, dCTP, dGTP), the specific forward and reverse primer (1.25 µM) and Taq polymerase (0.1 U/µl) (Biotherm, Genecraft, Germany), self-seal reagent (MJ Research, Waltham, USA) and ddH_2O. 15 µl of the PCR reaction mix was applied to each chamber of the slide and sealed by cover slips. Thereafter the slides were placed in a thermal cycler suitable for *in situ* amplifications (MJ Research, Waltham, USA). PCR amplification was performed according to the following protocol: initial denaturation at 95°C for 3 min, followed by 35 – 40 cycles with a denaturation at 95°C for 45 seconds, annealing at 57°C for 45 seconds and extension at 72°C for 45 seconds. A final extension was performed for 10 min at 72°C. After removal of the cover slips the slides were incubated with a blocking reagent for 1 h. Thereafter the incorporated DIG-labeled nucleotides were detected by an anti-digoxigenin antibody labeled with alkaline phosphatase (1:500) or rhodamine (1:300) at room temperature for 1 h. Alkaline phosphatase activity was detected by the NBT/BCIP (nitro blue tetrazolium chloride/ 5-bromo-4-chloro-3-indolyl phosphate toluidine salt) colour reaction (three hours incubation at room temperature). The

primer sequences for mouse preproinsulin, IAPP, glucagon, somatostatin, Pdx1, Oct4, nestin, GLUT2, GK, ß-actin were listed in Table 1.

Analysis of ES cells

Immunostained ES cells mounted on cover slips were analysed with an IX81 inverted microscope (Olympus Optical, Tokyo, Japan). Sections of ES cell pellets or ES cell grafts stained for gene or protein expression were viewed using bright field and fluorescence illumination with the BX61 upright microscope (Olympus Optical). Both microscopes were equipped with specific excitation filter systems for rhodamine, Cy2, fluorescein isothiocyanate (FITC), Texas Red, Cy5 and DAPI dyes (AHF Analysentechnik, Tübingen, Germany). Ultrathin sections for ultrastructural analysis of ES cells were viewed by an electron microscope 10 (Carl Zeiss AG, Oberkochen, Germany). To identify growing behaviour under culture conditions the cells were observed on a Nikon TMS microscope using phase-contrast filters and documented with a Nikon Coolpix 4500 digital camera.

RESULTS

Phase contrast analysis of undifferentiated ES cells and differentiated ES cells after differentiation with the reference protocol or the new 4 stage differentiation protocol *in vitro*

Undifferentiated mouse ES cells, when observed under light microscopy, grew in typical round-shaped and smooth colonies of variable size (Figure 1A, D). Individual cells were difficult to distinguish, as it is typical for embryonic stem cell colonies, with the exception of the nuclei comprising normally one or more nucleoli. Stem cell colonies were thickened in the center but thinned out towards the periphery of the colony. Some ES ells in the periphery were stretched and formed bulky pseudopodia (Figure 1A, D).

Upon differentiation and nestin selection according to the reference protocol, after the intermediate embryonic body formation step, the typical stem cell colony morphology disappeared and the majority of cells acquired a distinct neuronal-like cell appearance (Fig 1. B, E). These cells formed complex networks through branches with axonal characteristics. Those colony-forming networks were often attached to a basis of a fibroblast-like cell layer (Figure 1B, E).

When embryonic stem cells were cultured according to the new 4 stage differentiation protocol, which is devoid of the nestin selection step, the cells grew out to form a monolayer without any signs of neuronal-like differentiation and with cuboid formed as well as fibroblast-like cell types. At the end of the differentiation process three-dimensional buds branched out of these monolayer cells and were evenly distributed (Figure 1 C, F). These cell clusters were composed of cells with distinguishable cell borders and without any neuronal branches as observed in the reference protocol. On the other hand, colonies as described for undifferentiated stem cells were virtually absent from this time point.

In situ* PCR of ES cells after differentiation with the reference protocol or the new 4 stage differentiation protocol *in vitro

Using the *in situ* RT-PCR technique, gene expression of peptides and transcription factors was analysed in ES cells differentiated according either to the reference protocol or to the new 4 stage protocol. In ES cells differentiated according to the reference protocol mRNA transcripts of preproinsulin were completely lacking in intact cells but were detected in the cytoplasm of intact ES cells developed according to the new 4 stage protocol (Table3). The majority of these ES cells revealed gene expression of the peptide IAPP which is naturally co-stored in mature beta cell insulin secretory granules. The gene expression of other islet hormones was detectable to a minor degree in non-insulin positive cells from both differentiation protocols (Table 3). Pdx1 mRNA expression was not detectable in the cytoplasm of ES cells differentiated according to the reference protocol, while a significant Pdx1 gene expression was found in the cytoplasm of intact ES cells differentiated according to the new 4 stage protocol (Table 3). The mRNA transcripts of the glucose-recognition structures, namely the GLUT2 glucose transporter and the glucokinase were found in single ES cells of the new 4 stage protocol but were undetectable in cells from the reference protocol (Table 3). On the other hand, the mRNA expression of the intermediate neurofilamentous protein nestin was visible in nearly all ES cells from the reference protocol (Table 3) but drastically decreased in ES cells from the new 4 stage protocol (Table 3). A strong reduction of nestin mRNA expression in ES cells of the new 4 stage protocol was observed in parallel to a reduction of the transcription factors Nanog and Oct4 proving the higher degree of differentiation in comparison to ES cells generated according to the reference protocol.

Immunocytochemical staining of ES cells after differentiation according to the reference protocol or to the new 4 stage differentiation protocol *in vitro*

ES cells cultured according to the reference protocol showed a very dense staining for insulin in apoptotic cells lacking an intact nucleus (Figure 2A). Mostly, these ES cells expressed no marker for insulin biosynthesis. In contrast, ES cells differentiated according to the new 4 stage differentiation protocol showed positive immunostaining for C-peptide and insulin in the cytoplasm in a dot-like appearance both when analysed as single cells on slides or in pseudoislet aggregates in cell pellets after 26 days of culture (Figure 2B). The staining for a special differentiation marker, the neuroectodermal marker nestin, was present in the majority of the cells differentiated towards the neuronal fate in the Lumelsky protocol. These cells showed a strong filamentous immunostaining in the cytoplasm (Figure 2C), whereas the ES cells developed according to the new protocol revealed a reduced number of nestin positive cells with faint immunoreactivity (Figure 2D). Remarkably, a distinct population of densely immunostained nestin positive cells from the reference protocol showed inclusion bodies immunoreactive for insulin in the cytoplasm (see also the ultrastructural analysis results). This staining was different from the dot-like appearance found in cells from the new 4 stage differentiation protocol. The immunostaining for the embryonic transcription factors Oct4 and Nanog revealed a small clustered subpopulation of undifferentiated stem cells which showed a nuclear co-staining for both transcription factors in cells from both differentiation protocols (Figure 2 E and F).

Ultrastructure of undifferentiated and differentiated mouse ES cells *in vitro*

Ultrastructural analysis of ES cells revealed the typical morphology of undifferentiated cells. The nuclei were large and appeared unevenly shaped. Typically, they comprised a high number of dense nucleoli (Figure 3A). The ratio of cytoplasm to nucleus was low and higher cell organelles, namely rough endoplasmatic reticulum (ER) and Golgi apparatus, were developed only to a minor degree. The cytoplasm was rich in free ribosomes and

mitochondria. Despite the densely packed morphology of the stem cell colonies, cell contacts such as gap junctions were not observed. Stem cells in the outer periphery showed in many cases microvilli thereby defining extracellular space between proximate colonies (Figure 3A). Differentiated ES cells, which were developed according to the reference protocol, showed neuroectodermal characteristics as depicted in Figure 3B. Some cells displayed a fiber phenotype with a high amount of neurofilaments and a small number of other cell organelles such as mitochondria characterizing the axonal region. Other well differentiated cells contained high numbers of different peptidergic vesicles concentrated in dense body formations, sometimes surrounded by a cellular membrane. Other regions of these cells showed well developed rough endoplasmic reticulum and mitochondria in the cytoplasm (Figure 3B).

In contrast to the neuronal differentiation of cells from the reference protocol, ES cells from the new protocol exhibited clear characteristics of endocrine differentiation with respect to subcellular organelles for synthesis, processing, storage and release of preproinsulin without concomitant signs of neuronal differentiation (Figure 3C). The cells showed both a well developed rough endoplasmic reticulum and Golgi apparatus well suited for protein synthesis and the formation of secretory granules, together with a high number of mitochondria providing the basis for sufficient energy supply for these processes. A distinct portion of these granules demonstrated typical features of beta cell insulin granules with a clear distinct dark core region and a pale halo region (Figure 3C).

***In situ* PCR of implanted ES cells after differentiation with the reference protocol or the new 4 stage differentiation protocol after implantation in a diabetic mouse model**

Using the *in situ* RT-PCR technique, gene expression of peptides and transcription factors was analyzed in differentiated ES cells grown in a graft with a tissue-like character after implantation. In ES cell grafts differentiated according to the reference protocol mRNA transcripts of preproinsulin were rarely seen in the cytoplasm of intact cells. A significant

level of preproinsulin gene expression was observed only in apoptotic cells (Fig 4A). After differentiation according to the new 4 stage protocol, preproinsulin mRNA was expressed in cytoplasmic regions in the ES cell graft. Most importantly these mRNA transcribing ES cells showed no signs of apoptosis (Fig 4B). Additionally, these ES cells revealed also a high mRNA expression of the peptide IAPP which is naturally co-stored in mature beta cell secretory granules (Fig 4D). In contrast, ES cell grafts from cells differentiated according to the reference protocol expressed mRNA for IAPP only in apoptotic cells, in which expression of preproinsulin could also be detected (Fig 4C). Pdx1 mRNA expression was not detectable in the cytoplasm of the ES cell graft differentiated according to the reference protocol (Figure 4E), while abundant Pdx1 gene expression was found in the cytoplasm of intact ES cell grafts differentiated according to the new 4 stage protocol (Fig 4F). On the other hand the intermediate neurofilamentous protein nestin was homogeneously expressed in nearly all ES cells from the reference protocol (Figure 4G). However, gene expression of nestin was drastically decreased in ES cell grafts of the new 4 stage protocol in the cytoplasm in the majority of the cells (Figure 4H).

Protein expression of embryonic transcription factors in ES cells after differentiation according to the reference protocol and to the new 4 stage differentiation protocol after implantation in a diabetic mouse model

In order to analyze changes in the degree of differentiation in the *in vivo* situation after implantation of differentiated ES cells the embryonic transcription factors Oct4 and Nanog were immunohistochemically analysed in cells from both protocols. Only ES cells from the reference protocol showed a distinct nuclear staining (Fig 5A). Positive cells were mostly clustered and located in highly apoptotic areas. Interestingly, immunostaining of Oct4 in cells after implantation into mice from the new 4 stage protocol was only found in the cytoplasm with a very faint density and in a minor portion of the cells (Fig5B). The immunostaining pattern of Nanog was similar as described for Oct4 (Figure 5C, D and Table 4).

Protein expression of beta cell markers in ES cells after differentiation according to the reference protocol and to the new 4 stage differentiation protocol after implantation in a diabetic mouse model

In order to verify the endocrine character of differentiated implanted cells, especially of the insulin-producing cells, ES cell grafts were stained for islet hormones and beta cell markers. Only a small number of implanted ES cells from the reference protocol showed both a very faint C-peptide (Figure 6A and Table 4) and insulin staining (data not shown). ES cells with a dense immunoreactivity in the cytoplasm showed signs of apoptosis. Glucagon and somatostatin immunostaining were rarely observed in ES cells after implantation. ES cells differentiated according to the new 4 stage protocol showed a higher degree of endocrine differentiation and were in the majority viable cells with distinct C-peptide and insulin positive staining (Figure 6B). Other islet hormones could not be traced immunohistochemically in the cells (Table 4). Glucokinase protein expression was not detectable in the cytoplasm of ES cells in the reference protocol (Figure 6C) but was abundantly present in typical heterogeneous density in the cytoplasm of intact cells differentiated according to the new 4 stage protocol (Figure 6D). Only apoptotic ES cells from the reference protocol stained positive for the GLUT2 glucose transporter (Figure 6E). The expression of the GLUT2 glucose transporter protein in ES cells from the new 4 stage protocol showed two different staining patterns. The majority of cells expressed GLUT2 in a faint intensity all over the cytoplasm while a distinct subpopulation showed a dense and mostly to the plasma membrane restricted GLUT2 glucose transporter expression. All cells appeared functionally intact without signs of cellular alteration (Figure 6F). The results from the *in vivo* experiments are summarized in Table 4.

DISCUSSION

In this study we compared the morphological changes of mouse embryonic stem cells upon differentiation into insulin-producing cells under *in vitro* and *in vivo* conditions. The cultured ES cells were analysed immunohistochemically and ultrastructurally. Grafts of implanted ES cells were analyzed for expression of transcription factors and beta cell typical marker genes by *in situ* RT-PCR and immunohistochemistry on the gene and protein level. The aim of this study was to assess the effect of implantation into a diabetic animal and document the beneficial effect of this environment on the differentiation efficiency using two different protocols. We developed a new optimized 4 stage protocol for differentiation of mouse ES cells towards insulin-producing cells [2, 6] and compared this protocol with a reference protocol originally designed for neuronal differentiation but later adopted for differentiation of ES cells towards endocrine cell types [5].

When ES cells were differentiated under *in vitro* conditions with either the reference or the new protocol the cells lost the typical stem cell phenotype characterized by a smooth surface and non-subdivision of cells as well as the characteristic syncytium-like cell formation and colony growth [19]. Throughout the final days of differentiation the cells from the reference protocol developed axonal branches to a high degree thereby confirming earlier reports [20]. In contrast the cells from the new 4 stage protocol lacked this axonal appearance, thus providing no indication for differentiation towards the neuronal lineage. The cells from the new 4 stage protocol formed a two layered tissue with a basal monolayer from which buds of cell clusters grew out. Recent work in the field of adult stem cell differentiation has provided evidence that those cell clusters might serve as a pool for endocrine progenitor cells under *in vitro* conditions [21].

The suppression of neuronal differentiation was verified by the loss of nestin immunoreactivity in cells from the new protocol, whereas nestin positive cells formed the major population of differentiated cells in the reference protocol. Remarkably, the reduction

of nestin expression was paralleled by an increase of cells positive for insulin and C-peptide when compared to the reference protocol. The beneficial reduction of nestin was in agreement with recent findings from a new differentiation protocol for mouse ES cells [22] but in contrast to previous reports from the same group [23] and other investigators [24]. The immunohistochemical results with respect to nestin expression were confirmed in the present study by ultrastructural analyses showing distinct organelles for protein biosynthesis in cells from the new protocol. Thereby, we could document at the same time that those cells were viable and showed no signs of apoptotic or necrotic cell death. In agreement with the results of the present study other groups have recently shown, that such a reduction of neuronal cell fate is a prerequisite for a proper differentiation into endocrine or other somatic cell types [3, 25, 26].

Nonetheless, it has so far not been shown to which extent the *in vivo* situation can foster the maturation process of mouse ES cell derived endocrine cells, in particular with respect to the loss of embryonic transcription factor expression and functional characteristics of the implanted insulin-producing cells. Other groups have shown, that blood glucose concentrations of diabetic animals could be lowered by implantation of differentiated insulin-producing ES cells. In those studies the morphology of the ES cell graft was only analysed by conventional HE staining or immunostaining for insulin [22, 24, 25, 27-29]. The present study documents a decrease of embryonic transcription factors and in parallel an increase of crucial beta cell markers on the gene and protein expression level.

After implantation of ES cells from both protocols the beneficial effect of the *in vivo* environment was visible by the distinct reduction of Oct4 and Nanog expressing cells, which were associated with clear signs of apoptosis. This apoptosis permitted the removal of undifferentiated cells in the grafts of the implanted ES cells. Nonetheless, the maturation of implanted ES cells remained restricted to nestin positive cells without expression of typical beta cell markers in cells differentiated *in vitro* with the reference protocol. In contrast, ES

cells differentiated *in vitro* according to the new 4stage protocol showed a high degree of expression of the Pdx1 transcription factor. Pdx1 is the major regulator of beta cell development during pancreas organogenesis and acts later on in adult cells as the regulative element for the insulin gene expression [7, 30, 31]. Thus, an important milestone in the differentiation of mouse ES cells towards the lineage of endocrine insulin-producing cells is the presence of the gene and protein expression of Pdx1, which has been documented so far only in cultures of *in vitro* differentiated human ES cells [3, 4]. In cells from the reference protocol Pdx1 expression remained virtually undetectable even after implantation, indicating the failure of differentiation towards an insulin-producing cell [8, 20, 32]. With Pdx1 expression as an insulin transactivator, the mRNA transcription of the preproinsulin gene seems plausible because the mRNA encoding proinsulin was detectable in differentiated ES cells after implantation. In addition this conclusion is supported by the observation of the expression of IAPP, which is normally co-stored together with insulin in mature secretory granules of beta cells and thereby excludes the possibility of an uptake of insulin from the extracellular space [9].

In grafts of ES cells differentiated prior to implantation according to the reference protocol, the expression of the GLUT2 glucose transporter and the glucose phosphorylating enzyme glucokinase, which are both prerequisites for regulated release of insulin, was detected only in single cells in a very faint manner. The higher differentiation level of implanted insulin-producing cells originating from the new 4 stage protocol comprised the gene and protein expression of both glucose recognition structures. Moreover, from a morphological point of view, these proteins were detected in those cell compartments, where they typically reside also in mature pancreatic beta cells [12].

Taken together, the gene and protein expression of the hormones, as well as of the glucose recognition structures and of Pdx1 clearly demonstrate the beneficial effect of the *in vivo* environment for a directed differentiation towards endocrine insulin-producing cells with

characteristics comparable to those of pancreatic beta cells. However, a proper and efficient protocol for differention of ES cells towards insulin-producing cells is a crucial prerequisite for further maturation after *in vivo* implantation. This benefit of a favourable *in vivo* environment is negligible, however, unless the basis for a proper differentiation towards insulin-producing cells is set during the preceding *in vitro* phase of differention, as demonstrated in the present study for the reference protocol.

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TABLES

Table 1 Primer sequences used for *in situ* PCR

Gene		Primer sequence
Insulin	Fw	5'-CCCACCCAGGCTTTTGTCAAACAGC-3'
	Rv	5'-TCCAGCTGGTAGAGGGAGCAGATG-3'
IAPP	Fw	5'-TGCAGCTCCAGCCTCATCTCG-3'
	Rv	5'-CTCTCTGTGGCACTGAACCA-3'
Glucagon	Fw	5'-CAGGGCACATTCACCAGCGACTAC-3'
	Rv	5'-TCAGAGAAGGAGCCATCAGCGTG-3'
Somatostatin	Fw	5'-ATGCTGTCCTGCCGTCTCCA-3'
	Rv	5'-TGCAGCTCCAGCCTCATCTCG -3'
Nestin	Fw	5'-GAGAGTCGCTTAGAGGTGCA-3'
	Rv	5'-CCACTTCCAGACTAAGGGAC-3'
GLUT2	Fw	5'-GAAGACAAGATCACCGGAACCTTGG-3'
	Rv	5'-GGTCATCCAGTGGAACACCCAAAA-3'
Pdx1	Fw	5'-ACCGCGTCCAGCTCCCTTTC-3'
	Rv	5'-CAACATCACTGCCAGCTCCACC-3'
Glucokinase	Fw	5'-GAGGTCGGCATGATTGTGGGCA-3'
	Rv	5'-GCGCCCCACTCTGTGTTGACACAC-3'
Oct4	Fw	5'-AGGCCCGGAAGAGAAAGCGAACTA-3'
	Rv	5'-TGGGGGCAGAGGAAAGGATACAGC-3'
Beta actin	Fw	5'-AGAGGGAAATCGTGCGTGAC-3'
	Rv	5'-CAATAGTGATGACCTGGCCGT-3'

Fw – forward (sense) primer; Rv – reverse (antisense) primer. If possible amplicons were designed intron-spanning and were in a size ranging from 100-300 bp. Beta actin was used as housekeeping gene.

Table 2 Antibodies for immunohistochemical staining

Antibody	Working Dilution	Source
Rat insulin, guinea pig	1:200	Abcam, Cambridge, UK
Rat insulin, guinea pig	1:600	Dako, Hamburg, Germany
Human IAPP, rabbit	1:500	Peninsula, San Diego, USA
Rat C-peptide, goat	1:1000	Linco Research, St. Charles, USA
Human glucagon, rabbit	1:2000	Dako, Hamburg, Germany
Human somatostatin, rabbit	1:1000	Abcam, Cambridge, UK
Mouse nestin, mouse	1:200	Chemicon, Temecula, USA
Mouse GLUT2, goat	1:500	Santa Cruz Biotechnology, Santa Cruz, USA
Mouse, Pdx1, mouse	1:200	R&D Systems, Minneapollis, USA
Rat glucokinase, rabbit	1:50	Own production, affinity purified
Mouse Oct4, mouse	1:200	R&D Systems, Minneapollis, USA
Mouse Nanog, rabbit	1:200	Abcam, Cambridge, UK

Table 3 Changes in gene and protein expression of transcription factors, endocrine cell and beta cell specific markers in ES cells differentiated either with the reference protocol or the new 4 stage differentiation protocol under *in vitro* conditions.

Parameter	**Reference protocol**		**4 stage protocol**	
	Gene expression	Protein expression	Gene expression	Protein expression
Insulin	0*	0	(+)	(+)
Glucagon	+	+	0	0
Somatostatin	+	+	(+)	(+)
IAPP	0	0	(+)	(+)
Pdx1	+	+	+	(+)
GLUT2	0	0	(+)	(+)
Glucokinase	0	0	(+)	(+)
Nestin	+++	++	+	(+)
Nanog	+++	++	+	(+)
Oct4	+++	++	+	(+)

The score represents the following stages: 0 = no expression, (+) = in single cells, + = in up to 5-10 % of the intact cells, ++ = 11-25 % of the cells, +++= in more than 50 % of cells. * gene expression found by *in situ* PCR.

Table 4 Changes in gene and protein expression of transcription factors, endocrine cell and beta cell specific markers in ES cells differentiated either with the reference protocol or the new 4 stage differentiation protocol under *in vivo* conditions.

Parameter	**Reference protocol**		**4 stage protocol**	
	Gene expression	Protein expression	Gene expression	*Protein expression*
Insulin	(+)	(+)	++	+
Glucagon	+	++	(+)	(+)
Somatostatin	+	+	0	0
IAPP	(+)	(+)	++	+
Pdx1	+	+	+++	+
GLUT2	+	+	++	++
Glucokinase	(+)	(+)	++	++
Nestin	+++	++	(+)	0
Nanog	+++	++	(+)	(+)
Oct4	+++	++	(+)	(+)

The score represents the following stages: 0 = no expression, (+) = in single cells, mostly with apoptotic or necrotic signs, + = in up to 5-10 % of the intact cells, ++ = in 11-25 % of the cells, +++ = in more than 50 % of cells.

FIGURES

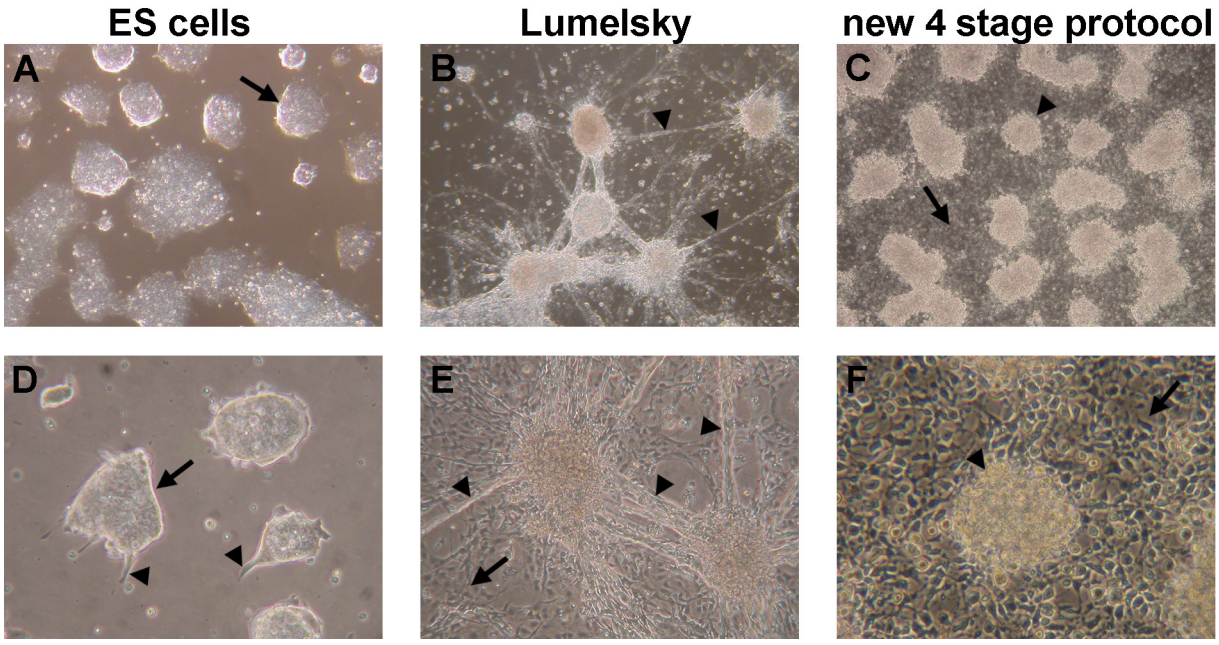

Figure 1 Phase contrast analysis of undifferentiated ES cells and differentiated ES cells after differentiation with the reference protocol or the new 4 stage differentiation protocol in vitro. Morphology of undifferentiated ES cells with typical colony appearance (*arrows*) and pseudopodia (*arrowhead*) (**A**, **D**), ES cells differentiated according to the reference protocol with axonal-like branches (*arrowheads*) on a monolayer of fibroblasts (*arrows*) (**B**, **E**) and to the new 4 stage differentiation protocol with formation of a monolayer (*arrows*) and buds of cell clusters (*arrowheads*) (**C**, **F**) in two different magnifications.

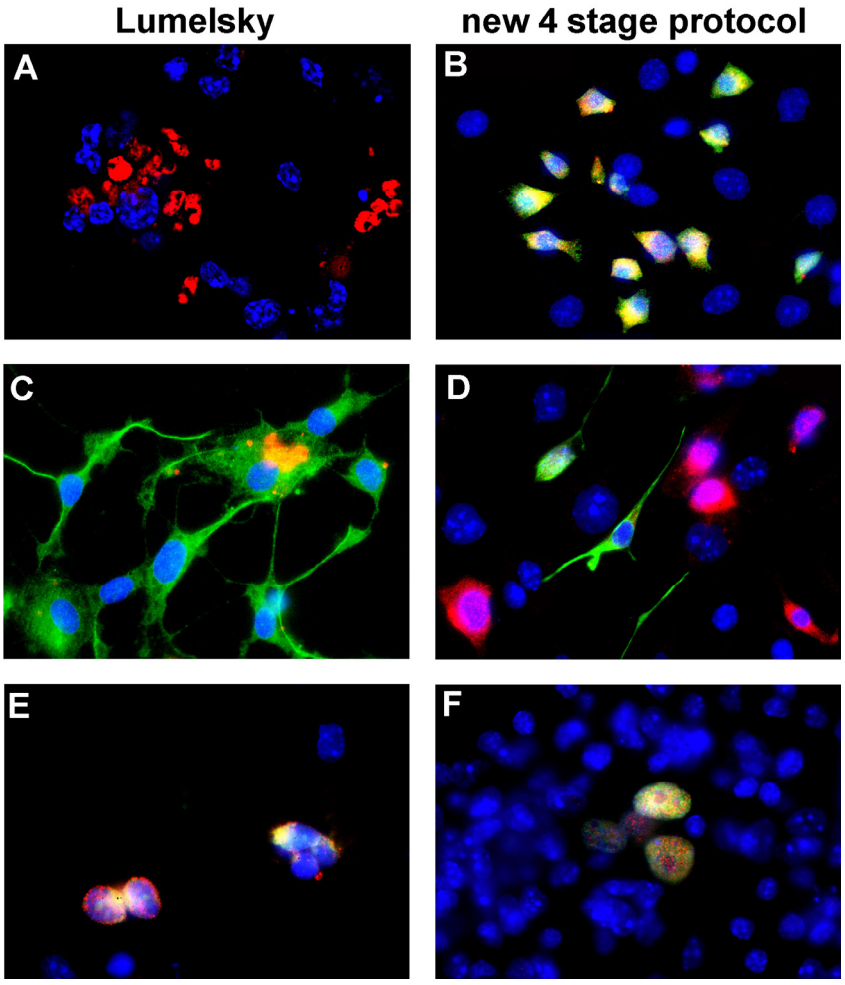

Figure 2 Immunocytochemical staining of ES cells after differentiation according to the reference protocol or to the new 4 stage differentiation protocol. ES cells differentiated according to the reference protocol (**A**, **C**, **E**) and to our new differentiation protocol the (**B**, **D**, **F**) were fixed on glass slides and co-stained for C-peptide (green) and insulin (red) (**A**, **B**), nestin (green) and insulin (red) (**C**, **D**) and Oct4 (green) and Nanog (red) (**E**, **F**). The merge is shown in yellow. Original magnification x400.

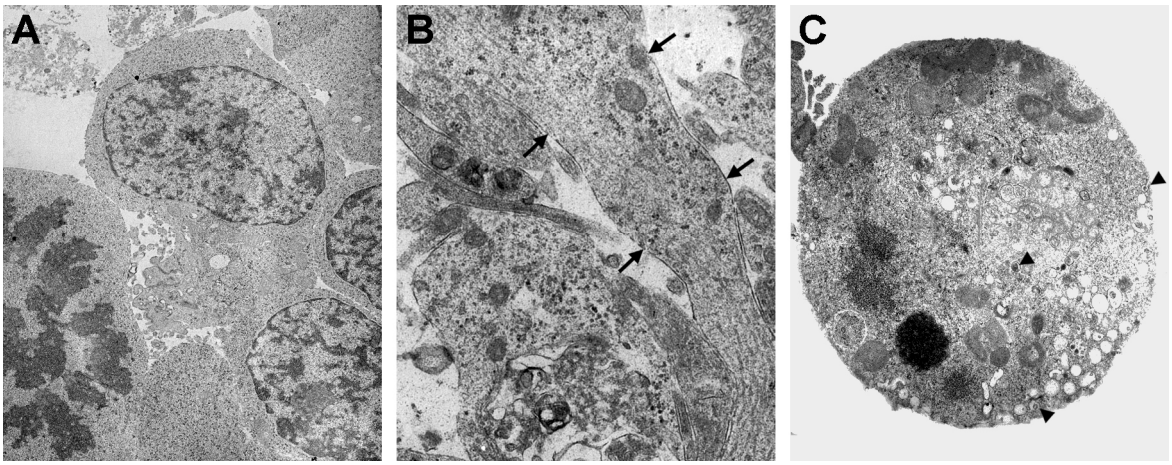

Figure 3 Ultrastructure of ES cells. Ultrastructure of undifferentiated ES cells (**A**), ES cells differentiated according to the reference protocol (**B**) and to our new 4 stage differentiation protocol (**C**). Differentiated ES cells after the reference protocol showed signs of the neuroendocrine development as nerve fibers (*arrows*). ES cells after differentiation with the new protocol showed the signs of an insulin-producing cell as beta cell typical secretory granules (*arrowheads*), a well developed rough endoplasmic reticulum and Golgi apparatus.

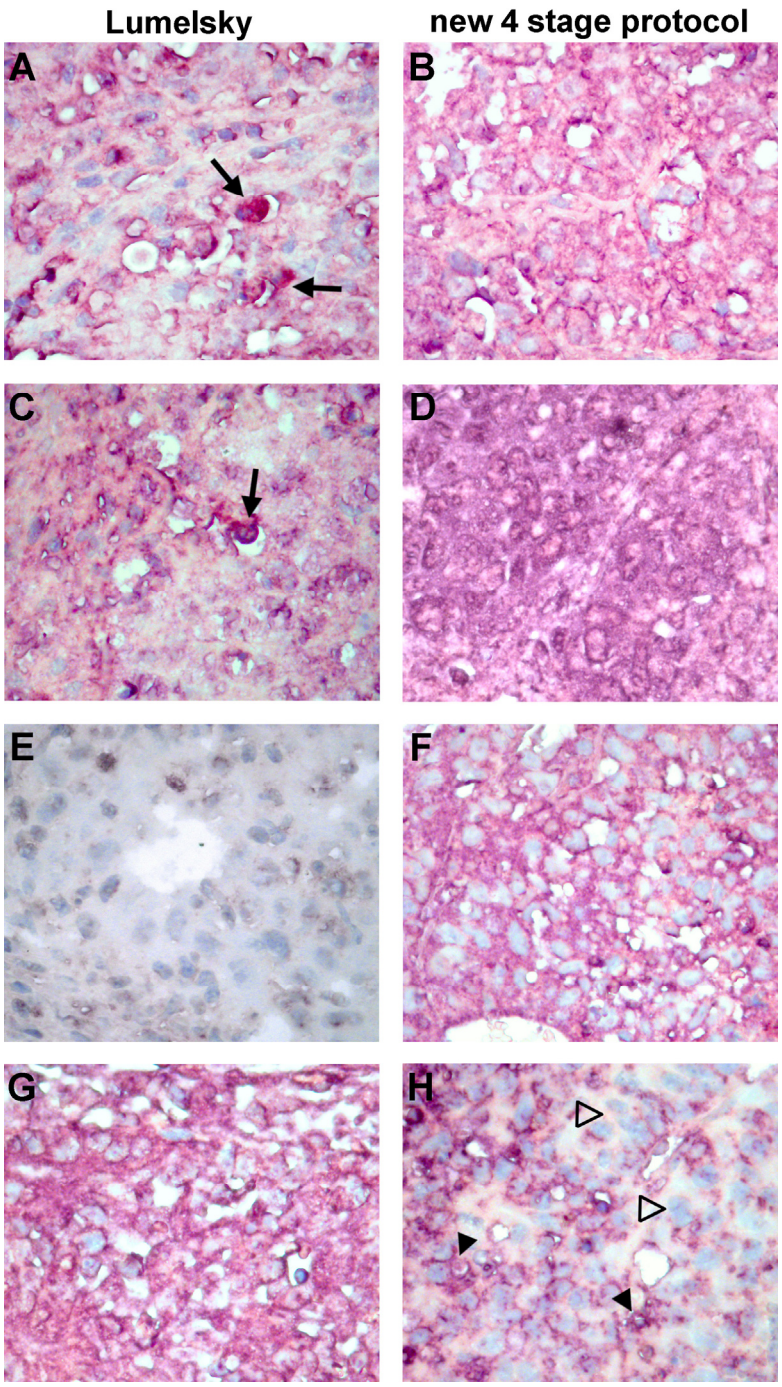

Figure 4 *In situ* PCR of implanted grafts of ES cells after differentiation with the reference protocol or the new 4 stage differentiation protocol. mRNA-transcripts encoding either the endocrine hormones insulin (**A**, **B**) and IAPP (**C**, **D**) or the beta cell transcription factor Pdx1 (**E**, **F**) and the intermediate filament protein nestin (**G**, **H**) were analysed by *in situ* RT-PCR in grafts of ES cell differentiated with either the Lumelsky protocol (**A**, **C**, **E**, **G**) or with the new 4 stage differentiation protocol (**B**, **D**, **F**, **H**). Grafts of ES cells, which were differentiated with the new 4

stage protocol showed fewer signs of nestin mRNA expression (*open arrowheads*), while after differentiation with the Lumelsky protocol ES cells grafts showed abundant nestin transcripts (*arrowheads*). Insulin and IAPP mRNA expression was only found in ES cells after differentiation with the new 4 stage protocol. In addition the Pdx1 mRNA expression was higher in the ES cells differentiated with the new 4 stage protocol compared with cells from the Lumelsky protocol. Apoptotic cells are marked with arrows.

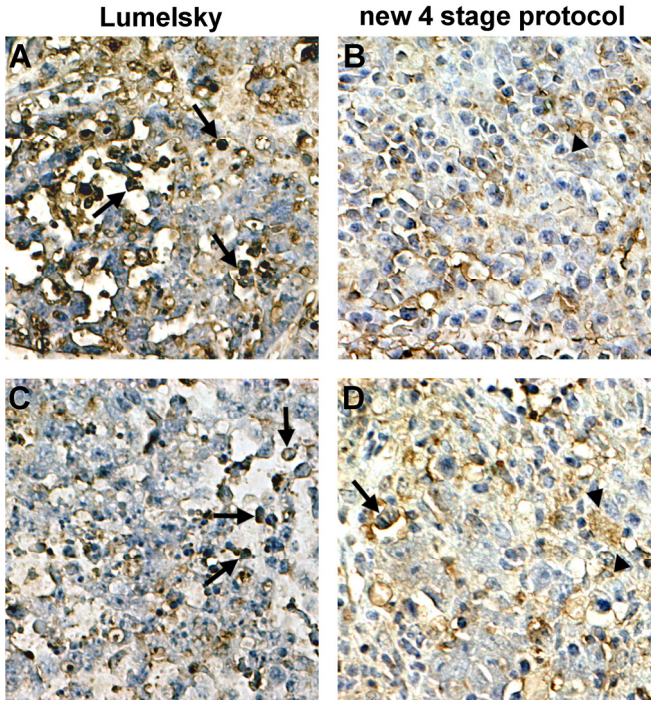

Figure 5 Protein expression of embryonic transcription factors in ES cell grafts after differentiation with the reference protocol or the new 4 stage differentiation protocol and implantation in a diabetic mouse model. Comparison of the *in vivo* protein expression in ES cells of Oct4 (**A**, **B**), and Nanog (**C**, **D**) after implantation under the kidney capsule of STZ diabetic mice. The cells were differentiated according to the reference protocol and (**A**, **C**) or according to the new 4 stage protocol (**B**, **D**) and showed positive staining in apoptotic cells (*arrows*) and occasionally faint staining in viable cells (*arrowheads*).

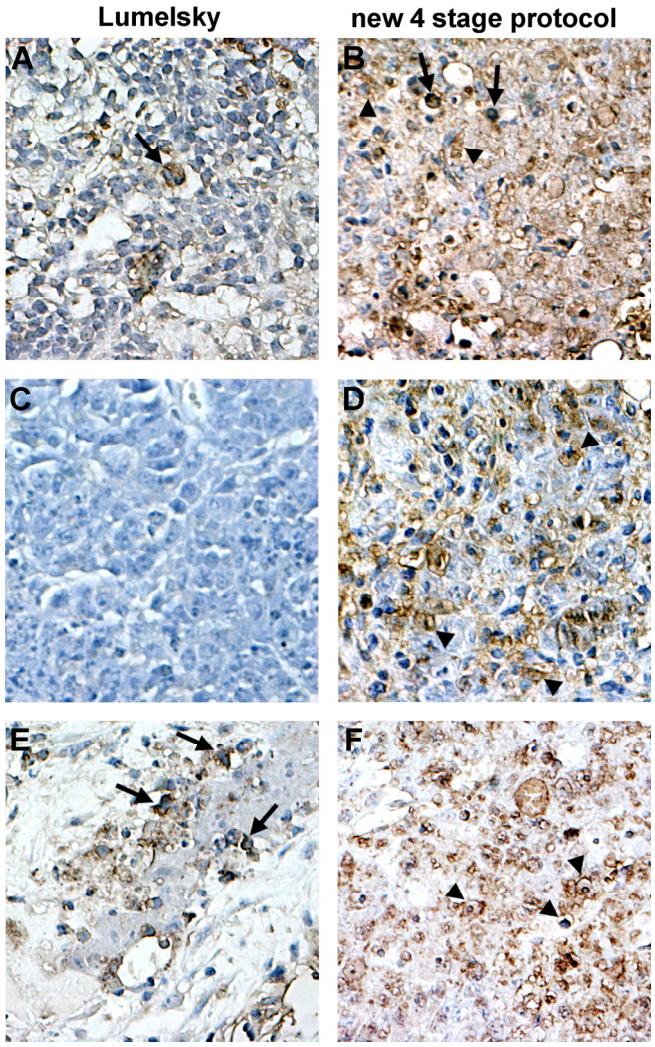

Figure 6 Protein expression of beta cell markers in ES cell grafts after differentiation with the reference protocol or the new 4 stage differentiation protocol and implantation in a diabetic mouse model. Comparison of the *in vivo* protein expression in ES cells of C-peptide (**A**, **B**), glucokinase (**C**, **D**) and GLUT2 (**E**, **F**) after implantation under the kidney capsule of STZ diabetic mice. The cells were differentiated according to the reference protocol (**A**, **C**, **E**) or according to the new 4 stage protocol (**B**, **D**, **F**). The beta cell specific marker C-peptide as well as glucokinase and the GLUT2 glucose transporter showed a moderate to dense immunostaining (*arrowheads*) in intact ES cells differentiated with the new 4 stage protocol. Immunostaining of glucokinase was absent and immunostaining of C-peptide and GLUT2 was restricted to apoptotic cells (*arrows*) in the ES cells developed according to the reference protocol.

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An efficient experimental strategy for mouse embryonic stem cell differentiation and separation of a cytokeratin-19-positive population of insulin-producing cells

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Abstract. *Objectives*: Embryonic stem cells are a potential source for insulin-producing cells, but existing differentiation protocols are of limited efficiency. Here, the aim has been to develop a new one, which drives development of embryonic stem cells towards insulin-producing cells rather than to neuronal cell types, and to combine this with a strategy for their separation from insulin-negative cells. *Materials and methods*: The cytokeratin-19 (CK19) promoter was used to control the expression of enhanced yellow fluorescence protein in mouse embryonic stem cells during their differentiation towards insulin-producing cells, using a new optimized four-stage protocol. Two cell populations, $CK19^+$ and $CK19^-$ cells, were successfully fluorescence sorted and analysed. *Results*: The new method reduced neuronal progeny and suppressed differentiation into glucagon- and somatostatin-producing cells. Concomitantly, β-cell like characteristics of insulin-producing cells were strengthened, as documented by high gene expression of the Glut2 glucose transporter and the transcription factor Pdx1. This novel protocol was combined with a cell-sorting technique. Through the combined procedure, a fraction of glucose-responsive insulin-secreting $CK19^+$ cells was obtained with 40-fold higher insulin gene expression and 50-fold higher insulin content than $CK19^-$ cells. $CK19^+$ cells were immunoreactive for C-peptide and had ultrastructural characteristics of an insulin-secretory cell. *Conclusion*: Differentiated $CK19^+$ cells reflect an endocrine precursor cell type of ductal origin, potentially suitable for insulin replacement therapy in diabetes.

INTRODUCTION

The differentiation protocol by Lumelsky *et al.* (2001), originally established to differentiate mouse embryonic stem cells (ESCs) into neuronal cells (Lee *et al.* 2000), has also been a reference tool for differentiation of mouse ESCs towards insulin-producing cells; however, its efficiency is low (Paek *et al.* 2005). Various other alternative mouse ESC differentiation methods have been developed, but the results of these activities have also been disappointing, due to limited efficiency and reproducibility (Soria *et al.* 2000; Hori *et al.* 2002). The yield of insulin-

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producing cells is usually so low that the amount of insulin they produce cannot be reliably quantified, in particular when tissue culture media contain insulin (Rajagopal *et al*. 2003; Hansson *et al*. 2004). Thus, it has been doubted whether cells obtained during the course of such experiments may be suited for reproducible generation of insulin-producing cells (Sipione *et al*. 2004). With a few exceptions (Soria *et al*. 2000; Leon-Quinto *et al*. 2004; Roche *et al*. 2005; Vaca *et al*. 2006), work on ESC differentiation towards insulin-producing cells has been focused on variation of cell culture conditions (Lumelsky *et al*. 2001; Hori *et al*. 2002; Moritoh *et al*. 2003; Bai *et al*. 2005; Shi *et al*. 2005) or overexpression of transcription factors that are known to be important for pancreatic organogenesis (Blyszczuk *et al*. 2003; Miyazaki *et al*. 2004; Shiroi *et al*. 2005). However, none of these have been suited to separate insulin-producing cells from other cell types, which also develop during differentiation.

In Lumelsky *et al*.'s original method, the idea was put forward that ESCs may differentiate *in vitro* into nestin-positive endocrine precursor cells that would differentiate further towards insulin-producing cells. This approach was based on the assumption that β-cell neogenesis in the pancreas results from differentiation of nestin-positive cells, as such a population is resident in adult pancreas (Hunziker & Stein 2000; Zulewski *et al*. 2001). However, participation of nestin-positive cells in β-cell regeneration is doubtful (Selander & Edlund 2002; Treutelaar *et al*. 2003). In addition to nestin-positive cells, another cell population, namely cytokeratin-19-positive ($CK19^+$) ductal cells, have been considered to be a pool for newly derived β-cells during pancreatic organogenesis (Rutter 1980; Slack 1995; Bouwens *et al*. 1997; Bonner-Weir *et al*. 2000; Bouwens & Rooman 2005). In the mouse, pancreatic primordia develop from the primitive gut tube stage after gastrulation, from a Pdx1-expressing cell population (Edlund 2002). The pancreas at this stage exists as separate dorsal and ventral buds, which grow in size and later fuse to form the definitive pancreatic tissue. In contrast, several studies have doubted the hypothesis of neuroectodermal origin of the β-cells, and that pancreatic endocrine cells may develop from duct-like cells that migrate into the surrounding mesenchyme and differentiate into the four major hormone-expressing cell types (Rutter 1980). Most importantly, these transitional cells would express CK19 and co-express insulin (Bouwens & De Blay 1996; Bouwens *et al*. 1997; Bouwens & Pipeleers 1998). Such ductal cells have shown capacity to proliferate and differentiate *in vitro* into islet cells (Bonner-Weir *et al*. 2000; Gao *et al*. 2003, 2005). Moreover, re-expression of β-cell specific transcription factor Pdx1 has been observed in ductal cells when placed in cell culture, indicating an early endocrine precursor cell type (Gmyr *et al*. 2000). In addition to these findings, participation of CK19 during differentiation towards insulin-producing cells has been observed in mouse (Blyszczuk *et al*. 2004) and recently in human (Jiang *et al*. 2007) embryonic stem cell lines. Not surprisingly, appearance of $CK19^+$ cells in ESCs undergoing *in vitro* differentiation towards insulin-producing cells reflects a mechanism analogous to that described for differentiation of ductal tissue into insulin-producing cells during foetal development of the pancreas (Gao *et al*. 2003; Noguchi *et al*. 2003; Bonner-Weir *et al*. 2004). However, efficient generation of insulin-producing cells from ESCs requires not only a reliable differentiation method, but in addition a sensitive cell separation strategy. To overcome the drawbacks of the reference protocol, we have established a new straightforward and optimized four-stage strategy that minimizes neuronal differentiation potential and suppresses differentiation into glucagon- and somatostatin-producing cells. We have combined this with a cell-sorting technique based on fluorescence-activated cell sorter (FACS) analysis. Specifically, the CK19 promoter was used to control expression of enhanced yellow fluorescence protein (eYFP) in mouse ESCs during their differentiation towards insulin-producing cells. Two distinct cell populations, $CK19^+$ and $CK19^-$ cells, were successfully separated and analysed. This approach yielded an insulin-producing cell type from $CK19^+$ endocrine precursors.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) and DMEM/F-12 tissue culture media, glutamine, non-essential amino acids and basic fibroblast growth factor (bFGF) were obtained from Invitrogen (Karlsruhe, Germany). Foetal calf serum (FCS) embryonic stem cell grade and gentamicin were purchased from PAA (Vienna, Austria) and leukaemia inhibitory factor (LIF) from Chemicon (Temecula, CA, USA). Insulin, transferrin, sodium selenite, putrescine and progesterone were from Sigma (St. Louis, MO, USA). All primer pairs, including random hexamer primers, were synthesized by MWG (Munich, Germany). The RevertAid™ H Minus M-MuLV Reverse Transcriptase was from Fermentas (St. Leon-Rot, Germany). Biotherm™ Taq polymerase as well as the deoxynucleoside triphosphates were from Genecraft (Münster, Germany). SYBR Green I was from Biozym (Hess. Oldendorf, Germany) and plastic ware for real-time polymerase chain reaction (PCR) reactions were from Abgene (Hamburg, Germany). C-peptide antibody was from Linco Research (St. Charles, MS, USA) and insulin antibody from Abcam (Cambridge, UK). Secondary antibodies were provided by Dianova (Hamburg, Germany). Unless otherwise mentioned, chemicals of analytical grade were obtained from Sigma or Merck (Darmstadt, Germany).

Plasmid construction and transfection

The pGL3-CK19 backbone vector was kindly provided by Dr. Shuichi Kaneko (Kanazawa University, Kanazawa, Japan). The luciferase sequence downstream of the 2.9 kb CK19 promoter fragment was removed by DNA digestion with *Hin*dIII and *Xba*I. Subsequently, cDNA of eYFP was purified by DNA digestion from the pEYFP-N1 vector (Clontech, Mountain View, CA, USA) as described above and was subcloned into the linearized pGL3-CK19 vector. In the obtained gene cassette, the human CK19 promoter drives the expression of eYFP followed by a poly(A) signal. ES-D3 cells were co-transfected with pCK19-eYFP and pCDNA3 plasmid featuring a neomycin gene for cell selection. Cell transfections were performed with Effectene (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Resistant clones were selected, using 300 μg/mL G418, and successful integration of the construct was verified by RT-PCR from genomic DNA. Five different YFP clones were generated and on the basis of analysis of fluorescence intensity, the best clone was selected for detailed analyses in the present study. Resulting transgenic embryonic stem cells were designated ES-D3 CK19-eYFP.

Cell lines and culture conditions

The mouse embryonic stem cell line ES-D3 (Doetschman *et al.* 1985) was purchased from the American Tissue Culture Collection (Manassas, VA, USA). These cells were differentiated towards insulin production using a new four-stage differentiation protocol and results were compared to those of a previously published method (Lumelsky *et al.* 2001). In order to maintain the cells in an embryonic state, they were cultured on a feeder layer of mouse embryonic fibroblasts in DMEM containing 25 mM glucose and supplemented with 15% (v/v) FCS, 2 mM L-glutamine, 100 μM non-essential amino acids, 0.1 mM β-mercaptoethanol, 50 μg/mL gentamicin and 1000 U/mL LIF in a humidified atmosphere at 37 °C and 5% CO_2. Medium was changed every day. Subsequently, the cells were transferred for two passages on to gelatin-coated tissue culture dishes to remove them from the feeder layer.

For differentiation with the new protocol, the cells were trypsinized and counted using a haemocytometer. One million cells were transferred to a bacterial culture dish in medium, as

described above, but devoid of LIF. Cells were then grown for up to 5 days in suspension. During this time, embryoid bodies formed that were allowed to settle on to gelatin-coated dishes in serum-free DMEM/F-12 medium supplemented with 25 μg/mL insulin, 50 μg/mL transferrin, 30 nM sodium selenite, 20 nM progesterone, 100 μM putrescine, 2 mM L-glutamine, 100 μM non-essential amino acids and 10 ng/mL bFGF, for 14 days. Thereafter, the cells were cultured for 7 days in DMEM/F-12 medium supplemented with 25 μg/mL insulin, 50 μg/mL transferrin, 30 nM sodium selenite, 20 nM progesterone, 100 μM putrescine, 5% FCS, 2 mM L-glutamine, 1× non-essential amino acids and 10 mM nicotinamide.

FACS analysis and cell sorting

ES-D3 CK19-eYFP cells at different time points along the new differentiation protocol were trypsinized, centrifuged and re-suspended in Krebs–Ringer buffer supplemented with 1 mM EDTA and 20% FCS. They were then filtered through a 40-μM gauze and sorted on a MoFlo cell sorter into $CK19^{+}$ and $CK19^{-}$ cell populations (DakoCytomation, Fort Collins, CO, USA).

Molecular biology

Total RNA was isolated from ESCs using the Chomczynski system (Chomczynski & Sacchi 1987). RNA was quantified photometrically and analysed on a denaturing agarose gel. For cDNA synthesis, random hexamers were used to prime the reaction of RevertAid™ H Minus M-MuLV reverse transcriptase. QuantiTect SYBR Green™ technology (Qiagen, Hilden, Germany), which uses a fluorescent dye that binds only double-stranded DNA, was employed. Reactions were performed using DNA Engine Opticon™ Sequence Detection System (Biozym Diagnostik, Hess. Oldendorf, Germany). Samples were first denatured at 94 °C for 3 min followed by 40 PCR cycles. Each cycle comprised a melting step at 94 °C for 30 s, an annealing step at 62 °C for 30 s, and an extension step at 72 °C for 30 s. Each PCR amplification was performed in triplicate using primers for insulin (detecting both Ins1 and Ins2), glucagon, somatostatin, nestin, Glut2, Pdx1, Nkx6.1, Kir6.2, Sur1, glucokinase, Oct4, neural cell adhesion molecule (NCAM), β-actin, CK19, carbonic anhydrase 2, amylase 2 and albumin (Table 1). Optimal parameters for PCRs were empirically defined. Purity of amplified PCR products was verified by melting curves. Data are expressed as relative gene expressions after normalization to the β-actin housekeeping gene using *Qgene96* and *LineRegPCR* software (Muller *et al*. 2002; Ramakers *et al*. 2003).

Ultrastructural characterization

For electron microscopy, cell pellets of ESCs and MIN6 cells (Miyazaki *et al*. 1990) were fixed in 2% paraformaldehyde plus 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3 and were post-fixed in 1% OsO_4 then finally embedded in Epon resin. Ultrathin sections were contrast-stained with saturated solutions of lead citrate and uranyl acetate and were viewed by electron microscopy (Gurgul *et al*. 2004).

Immunostaining and microscopy

Immunocytochemical co-staining for C-peptide and insulin was performed according to standard procedures. $0.5–1 \times 10^4$ FACS-sorted ESCs and MIN6 cells as positive controls were seeded overnight on glass slides and then fixed in 4% paraformaldehyde for 30 min. Thereafter, cells were blocked for 20 min in phosphate-buffered saline (PBS) plus 0.2% Triton X-100, 1% bovine serum albumin (BSA) and 5% donkey serum. Incubation with anti-C-peptide antibody was performed at room temperature for 60 min, diluted 1 : 100 in PBS with 0.1% BSA followed by incubation with Cy5-conjugated secondary antibody diluted 1 : 500 in PBS + 0.1% BSA for 30 min. Incubation with anti-insulin antibody was also performed at room temperature for

Table 1. Primers used for qRT-PCR. All amplicons were designed exon-spanning and were in a size ranging from 100 to 300 bp

Gene		Primer sequence
Insulin	Fw	5′-CCCACCCAGGCTTTTGTCAAACAGC-3′
	Rv	5′-TCCAGCTGGTAGAGGGAGCAGATG-3′
Glucagon	Fw	5′-CAGGGCACATTCACCAGCGACTAC-3′
	Rv	5′-TCAGAGAAGGAGCCATCAGCGTG-3′
Somatostatin	Fw	5′-ATGCTGTCCTGCCGTCTCCA-3′
	Rv	5′-TGCAGCTCCAGCCTCATCTCG –3′
Nestin	Fw	5′-GAGAGTCGCTTAGAGGTGCA-3′
	Rv	5′-CCACTTCCAGACTAAGGGAC-3′
Glut2	Fw	5′-GAAGACAAGATCACCGGAACCTTGG-3′
	Rv	5′-GGTCATCCAGTGGAACACCCAAAA-3′
Pdx1	Fw	5′-ACCGCGTCCAGCTCCCTTTC-3′
	Rv	5′-CAACATCACTGCCAGCTCCACC-3′
Nkx6.1	Fw	5′-AGAACCGCAGGACCAAGTGGAGAA-3′
	Rv	5′-TCGTCATCCTCCTCATTCTCCGAAG-3′
Kir6.2	Fw	5′-TGCTGTCCCGAAAGGGCATTATC-3′
	Rv	5′-TGCAGTTGCCTTTCTTGGACACG-3′
Sur1	Fw	5′-ACCAAGGTGTCCTCAACAACGGCT-3′
	Rv	5′-TGGAGCCAGGTGCTATGGTGAATG-3′
Glucokinase	Fw	5′-GAGGTCGGCATGATTGTGGGCA-3′
	Rv	5′-GCGCCCCACTCTGTGTTGACACAC-3′
Oct4	Fw	5′-AGGCCCGGAAGAGAAAGCGAACTA-3′
	Rv	5′-TGGGGGCAGAGGAAAGGATACAGC-3′
NCAM	Fw	5′-CGACGAGGCCGAATACGTCTG-3′
	Rv	5′-GCTCCTCTAGTTCCATGGCCGTC-3′
β-Actin	Fw	5′-AGAGGGAAATCGTGCGTGAC-3′
	Rv	5′-CAATAGTGATGACCTGGCCGT-3′
Cytokeratin 19	Fw	5′-GGTGCCACCATTGACAACTC-3′
	Rv	5′-CTGCATCTCCAGGTCAGTCC-3′
Carbonic anhydrase 2	Fw	5′-CCACCACTGGGGATACAGCAAGC-3′
	Rv	5′-GTCCTCCTTTCAGCACTGCATTGTC-3′
Amylase 2	Fw	5′-CTGTGAACACAGATGGCGTCAAATC-3′
	Rv	5′-GCAGGAAGACCAGTCTGTAAAGTGGC-3′
Albumin	Fw	5′-CCTCCTCTTCGTCTCCGGCTCTG-3′
	Rv	5′-GGGATTTGTCACAGTTGGCGGC-3′

Fw, forward (sense) primer; Rv, reverse (antisense) primer; NCAM, neural cell adhesion molecule.

120 min diluted 1 : 100 in PBS with 0.1% BSA, followed by incubation with Texas Red or FITC-conjugated secondary antibody diluted 1 : 500 in PBS + 0.1% BSA for 30 min. Subsequently, the cells were washed and nuclei were counterstained with 300 nM 4′,6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature. Finally, the cells were washed and mounted with Mowiol/DABCO antiphotobleaching mounting media. Stained cells were examined on an Olympus IX81 inverted microscope (Olympus, Hamburg, Germany) and microscopy images were post-processed with *AutoDeblur* and *AutoVisualize* (Autoquant Imaging, New York, USA).

Insulin secretion and insulin content

Cells were seeded at a density of 1×10^5/well in 48-well culture dishes and were grown for 24 h in DMEM/F-12 medium without insulin and supplemented with 5.5 mM glucose; they were then

washed with Krebs–Ringer buffer. Insulin secretion during a 120-min incubation period was measured in Krebs–Ringer buffer containing 0 mM and 30 mM glucose. After incubation, medium was removed and gently centrifuged to remove detached cells. Insulin was measured in the supernatant. For measurement of insulin content, cells were sonicated in Krebs–Ringer buffer. Insulin was determined by radioimmunoassay using rat insulin as standard.

Statistical analyses

Data are expressed as mean values ± SEM. Unless stated otherwise statistical analyses were performed using ANOVA followed by Bonferroni's test for multiple comparisons or *t*-test for paired correlations, using the Prism analysis program (GraphPad, San Diego, CA, USA).

RESULTS

Comparison of the gene expression profile after differentiation of ESCs according to the reference differentiation protocol or to the optimized four-stage differentiation protocol

Quantitative analysis of gene expression in mouse ESCs, which had been differentiated according to the reference protocol by Lumelsky *et al.* (2001), showed that all three major pancreatic endocrine hormones, insulin, glucagon and somatostatin were readily expressed. In contrast, ESCs differentiated according to the new optimized four-stage differentiation protocol expressed insulin almost exclusively, while glucagon expression was not detectable and somatostatin expression was low when compared to the reference protocol (Table 2, Fig. 1).

The structural marker genes *glucokinase*, *Kir6.2* and *Sur1*, which play a crucial role in β-cell stimulus-secretion coupling, were well expressed in cells from both protocols. In addition, expression of CK19 and carbonic anhydrase 2 (CA2), both markers for pancreatic duct cells, were increased significantly by 6-fold and 9-fold, respectively (Table 2). Interestingly, *Sur1*

Table 2. Comparison of the relative gene expression profile of unsorted mouse embryonic stem cells, comprising pancreatic hormones, transcription factors and structural markers, after differentiation into insulin-producing cells, according to the reference protocol by Lumelsky *et al.* (2001), or to the optimized four-stage differentiation protocol

	Reference protocol (%)	Optimized four-stage protocol (%)
Insulin	100 ± 24 (6)	136 ± 27 (13)
Glucagon	100 ± 32 (5)	n.d.
Somatostatin	100 ± 38 (5)	11 ± 4 (11)
Pdx1	100 ± 52 (4)	1184 ± 438 (13)
Glut2	100 ± 24 (4)	338 ± 98 (10)
Glucokinase	100 ± 24 (6)	122 ± 29 (10)
Kir6.2	100 ± 7 (4)	96 ± 24 (12)
Sur1	100 ± 23 (6)	182 ± 47 (10)
Nestin	100 ± 32 (4)	49 ± 12 (10)
NCAM	100 ± 20 (5)	16 ± 4 (6)
Carbonic anhydrase 2	100 ± 29 (4)	561 ± 103 (11)
Cytokeratin-19	100 ± 22 (5)	895 ± 81 (10)

Data shown are gene expression values determined by qPCR on day 26 of differentiation. Depicted are the changes in relative expression in percentage, normalized to the reference protocol by Lumelsky *et al.* (2001). Values shown are means ± SEM of the relative gene expression. n.d., not detectable; NCAM, neural cell adhesion molecule.

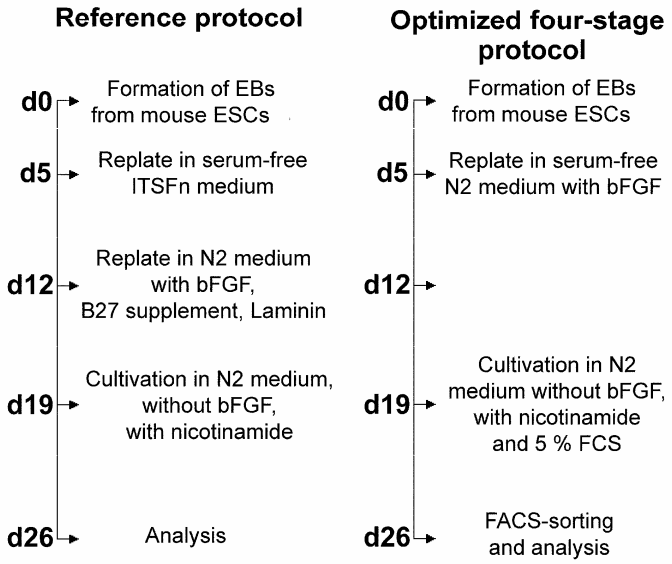

Figure 1. Comparative schematic presentation of the two different protocols used for differentiation of mouse embryonic stem cells (ESCs) into insulin-producing cells.

expression was nearly 2-fold higher in cells differentiated with the new four-stage protocol compared to the reference method (Table 2). The glucose transporter Glut2 and the transcription factor Pdx1 were both weakly expressed. Higher expression levels were observed for Glut2 glucose transporter and β-cell transcription factor Pdx1 after the new optimized four-stage protocol, with a 3-fold increase for Glut2 and 12-fold increase for Pdx1 (Table 2). Nestin and NCAM levels were measured to analyse differentiation towards neuronal progeny. They were both strongly expressed after the reference recipe, while cells from the new optimized four-stage differentiation protocol showed a 2-fold decrease in nestin and a 6-fold decrease in NCAM (Table 2). Gene expression analysis of amylase revealed only traces of mRNA expression in cells from the reference protocol, while in cells from the new optimized four-stage one amylase expression was undetectable (data not shown). Albumin expression, a marker for differentiation towards hepatic progeny, was negligible in both protocols with levels typically at or below the detection limit of real-time PCR (data not shown).

FACS sorting of CK19$^+$ and CK19$^-$ cells differentiated from mouse ESCs, according to the optimized four-stage differentiation protocol

The pCK19-eYFP construct was successfully integrated into ESCs. Cell clones could be selected and maintained in an undifferentiated state with identical morphology when compared to wild-type ESCs. Moreover, growth rate of transfected and non-transfected cells was comparable, indicating that their embryonic properties were not affected by transfection and subsequent selection process. Interestingly, undifferentiated clones displayed eYFP positivity (91.0 ± 0.6%, $n = 4$) that grossly decreased after formation of embryoid bodies to 13.4 ± 1.1% on day 5 ($n = 7$), 11.2 ± 1.2% on day 12 ($n = 10$), 6.8 ± 1.1% on day 19 ($n = 8$) and 1.6 ± 0.3% on day 26 ($n = 16$) of differentiation with the new optimized method (Fig. 2b). Analysis of gene expression of endogenous CK19 significantly revealed 4–7-fold higher CK19 expression in CK19$^+$ FACS-sorted cells when compared to CK19$^-$ cells (Fig. 2c). Carbonic anhydrase 2 was analysed as a

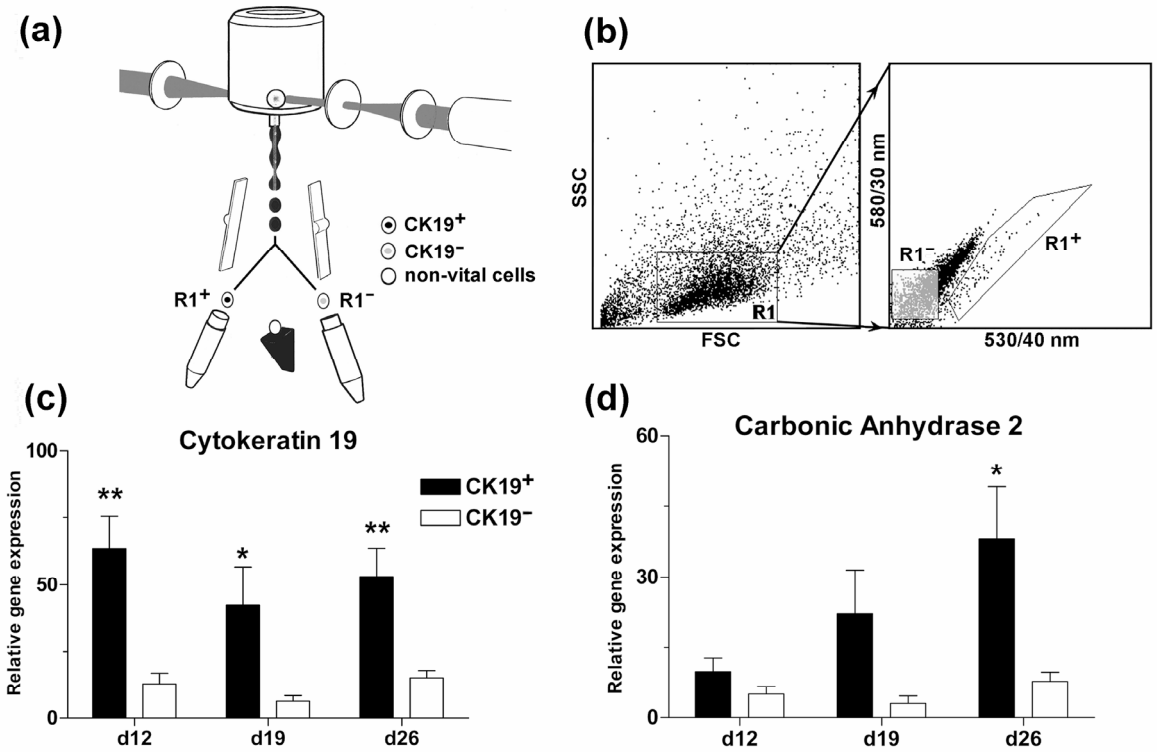

Figure 2. Selection of CK19$^+$ and CK19$^-$ cells through FACS sorting of insulin-producing cells differentiated from mouse embryonic stem cells (ESCs) according to the optimized four-stage differentiation protocol. (a) Schematic presentation of the method used for separation of vital CK19$^+$ and CK19$^-$ cells and non-vital cells by FACS sorting. (b) Clonal ESCs differentiated according to the optimized four-stage differentiation protocol were analysed with respect to size (FSC) and granularity (SSC) on day 26 of differentiation. Vital cells were gated (R1) and analysed for fluorescence intensity. CK19$^+$ (R1$^+$) and CK19$^-$ (R1$^-$) cells were separated. (c) Cytokeratin-19 gene expression in FACS-sorted CK19$^+$ and CK19$^-$ cells. Values shown are means ± SEM of the relative gene expression of 5–13 experiments; $^*P < 0.05$, $^{**}P < 0.01$. (d) Carbonic anhydrase 2 gene expression in FACS-sorted CK19$^+$ and CK19$^-$ cells. Values shown are means ± SEM of the relative gene expression of four to eight experiments; $^*P < 0.05$. SSC, side scatter; FSC, forward scatter.

second marker for duct cell progeny. Analysis of gene expression showed a 2-fold increase in CK19$^+$ cells at day 12 and a significant 9-fold increase in expression in CK19$^+$ cells at day 26, when compared to CK19$^-$ cells (Fig. 2d).

Gene expression profiles of pancreatic hormones and transcription factors in CK19$^+$ and CK19$^-$ cells during differentiation from mouse ESCs

Quantitative analysis of gene expression revealed a low level of insulin in CK19$^+$ and CK19$^-$ cells up to day 19. A significant 80-fold increase in insulin gene expression was observed in CK19$^+$ cells on day 26 as compared to day 12 and day 19 (Fig. 3). Compared to CK19$^-$ cells, insulin gene expression on day 26 was higher by a factor of 38 (Fig. 3), whereas the level of insulin gene expression in CK19$^-$ cells did not change significantly. Glucagon gene expression was not detectable at day 12 and day 19 (Fig. 3), while glucagon gene expression remained barely detectable at day 26 in CK19$^+$ cells, it increased to appreciable levels in CK19$^-$ cells 12 times higher than in CK19$^+$ cells (Fig. 3). Somatostatin gene expression was marginal throughout the whole differentiation protocol at days 12, 19 and 26 in CK19$^+$ cells (Fig. 3). In CK19$^-$ cells, somatostatin gene expression was high at day 12 but had decreased by days 19 and 26 (Fig. 3). Nevertheless, the expression level was seven times higher than in CK19$^+$ cells (Fig. 3). Thus, CK19$^+$

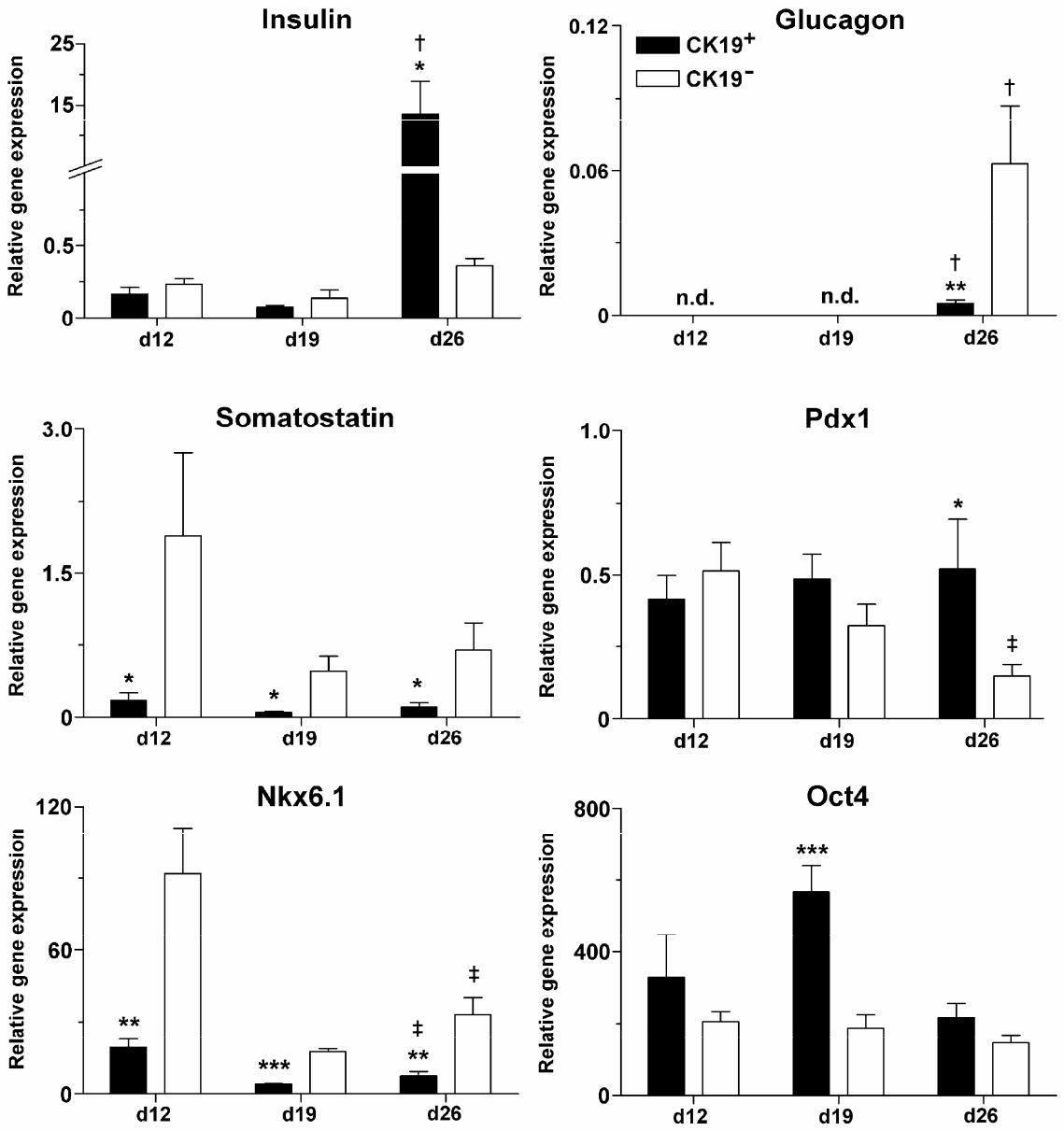

Figure 3. Comparison of the gene expression profile of pancreatic hormones insulin, glucagon and somatostatin, the transcription factors Pdx1 and Nkx6.1 and embryonic marker Oct4, in CK19$^+$ and CK19$^-$ cells, during differentiation of mouse embryonic stem cells at days 12, 19 and 26 of the optimized four-stage differentiation protocol. Values shown are means ± SEM of relative gene expression of 5–14 experiments. n.d., not detectable. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, CK19$^+$ compared to CK19$^-$ cells. †$P < 0.05$, ‡$P < 0.01$, compared to cells on day 12.

cells can be classified as a population of monohormonal insulin-positive cells, while CK19$^-$ cells are polyhormonal cells with similar levels of glucagon and somatostatin and low insulin gene expression.

The transcription factor Pdx1, a transactivator of the insulin gene, was expressed throughout the differentiation from days 12–26 in CK19$^+$ cells, whereas its expression in CK19$^-$ cells decreased significantly from day 12–26 (Fig. 3). On day 26, Pdx1 expression in CK19$^+$ cells was 3.5 times higher than in CK19$^-$ cells. Nkx6.1, a transcription factor found in adult β-cells and during vertebrate central nervous system development, was predominantly expressed in CK19$^-$ cells,

in particular at day 12, while Nkx6.1 expression in CK19$^+$ cells was significantly lower at each time point, between four and five times, as compared to CK19$^-$ cells (Fig. 3). Oct4 was expressed in CK19$^+$ cells and CK19$^-$ cells throughout the differentiation protocol, but it decreased towards the end of the differentiation protocol at day 26 (Fig. 3).

Gene expression profile of β-cell-specific structural markers in CK19$^+$ and CK19$^-$ cells during differentiation of mouse ESCs

A significant increase in Glut2 glucose transporter and *glucokinase* gene expression was observed in CK19$^+$ and CK19$^-$ cells on day 26 as compared to day 12 and day 19 (Fig. 4). The level of *Glut2* expression was significantly higher in CK19$^+$ than in CK19$^-$ cells at day 26 (Fig. 4). Both the *Kir6.2* and *Sur1* genes, which code for ATP-sensitive potassium channel protein and the associated sulphonylurea receptor protein, were expressed throughout the whole differentiation protocol at days 12, 19 and 26 in CK19$^+$ and CK19$^-$ cells (Fig. 4).

Nestin and *NCAM* genes, which code for neuronal marker proteins, were also transcribed throughout the whole differentiation protocol at days 12, 19 and 26 in CK19$^+$ and CK19$^-$ cells (Fig. 4). Both genes were preferentially expressed in CK19$^-$ cells (Fig. 4).

Thus, β-cell-specific expression of genes for glucose-sensing proteins as well as for proteins making the β-cell depolarizing, predominated in CK19$^+$ cells, while neuron-specific gene expression prevailed in CK19$^-$ cells.

Immunocytochemical staining for C-peptide, of differentiated mouse ESCs

CK19$^+$ cells had positive staining for C-peptide and insulin in the cytoplasm with a dot-like appearance, providing evidence for ongoing active preproinsulin biosynthesis and for insulin processing, while CK19$^-$ cells were negative for C-peptide. Nonetheless, staining for insulin revealed that single CK19$^-$ cells exhibited a little immunoreactivity for insulin, albeit at very low levels, which, however, did not co-stain with C-peptide (Fig. 5a). The MIN6 insulin-producing β-cell line was used as positive control and showed distinct co-localization of C-peptide and insulin in a manner similar to CK19$^+$ cells (Fig. 6a).

Ultrastructure of differentiated mouse ESCs

CK19$^+$ cells, in contrast to undifferentiated CK19$^-$ cells, exhibited clear signs of differentiation with respect to subcellular organelles for synthesis, processing, storage and release of insulin. CK19$^-$ cells possessed fewer mitochondria and few dilated cisternae of the endoplasmatic reticulum with a minor number of ribosomes (Fig. 5b). The CK19$^+$ cells showed both well-developed rough endoplasmic reticulum and Golgi apparatus, well suited for insulin synthesis, and insulin-secretory granules, together with a high number of mitochondria providing the basis for sufficient energy supply for the cells' protein synthesis. A distinct proportion of the secretory granules were located in the vicinity of the plasma membrane with some single granules undergoing exocytosis (Fig. 5b). This was comparable to the situation that can be observed in MIN6 insulin-producing β-cells although the number of secretory granules in CK19$^+$ cells was lower than in MIN6 cells.

Insulin content in differentiated mouse ESCs

Embryonic stem cells differentiated according to the four-stage protocol contained detectable amounts of insulin (0.37 ± 0.09 ng insulin/10^6 cells, $n = 6$) in the same range as found in cells grown with the reference protocol (0.25 ± 0.07 ng insulin/10^6 cells, $n = 3$) (Fig. 7a). Analysis of the CK19$^+$ and CK19$^-$ cells revealed increased insulin content in CK19$^+$ cells. Insulin content was 50 times higher in CK19$^+$ cells (113.3 ± 37.7 ng insulin/10^6 cells, $n = 5$) compared to

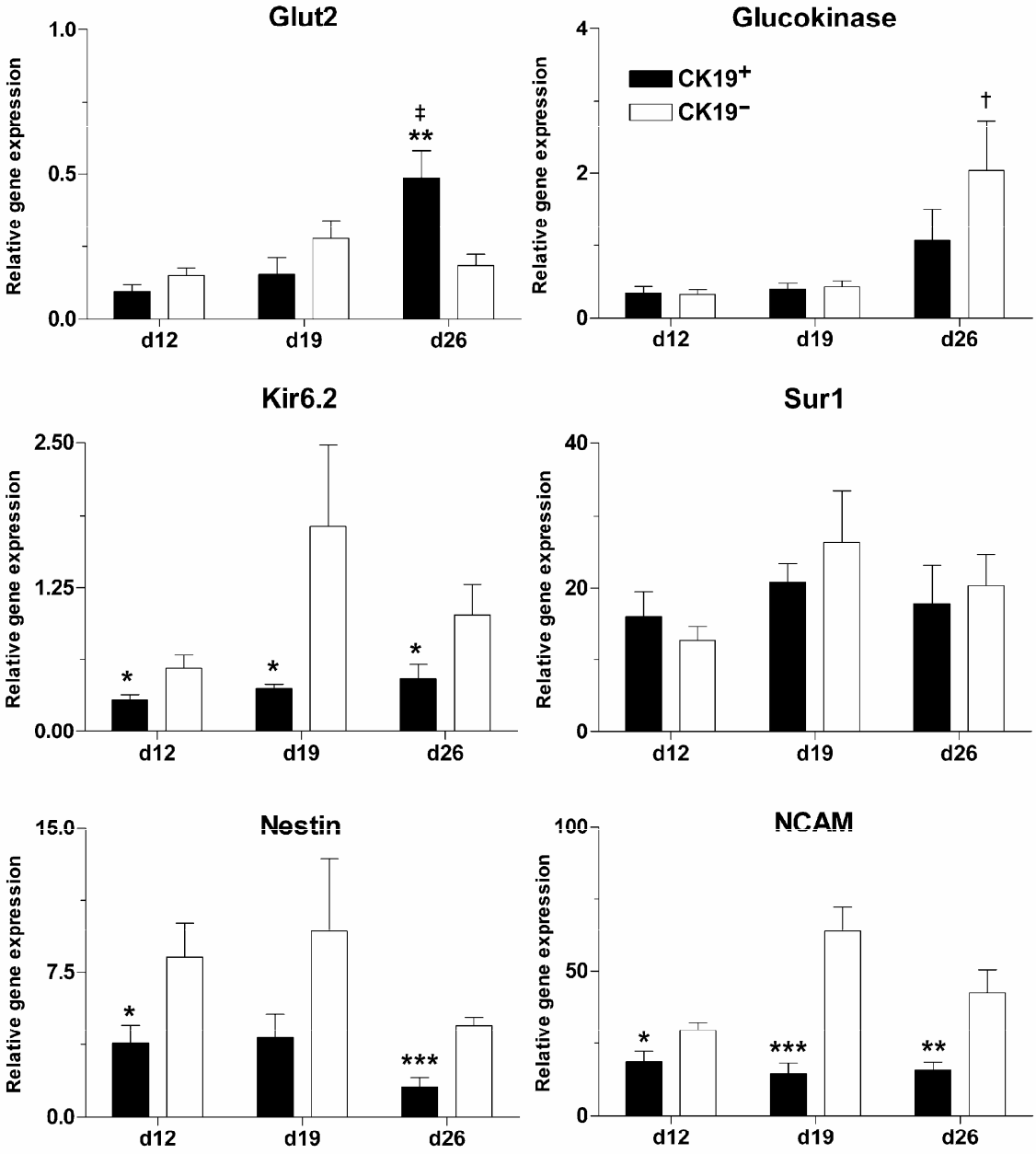

Figure 4. Comparison of gene expression profile of the structural markers Glut2 glucose transporter, glucokinase, Kir6.2, Sur1, nestin and NCAM in CK19$^+$ and CK19$^-$ cells during differentiation of mouse embryonic stem cells at days 12, 19 and 26 of the optimized four-stage differentiation protocol. Values shown are means ± SEM of the relative gene expression of five to eight experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, CK19$^+$ compared to CK19$^-$ cells. †$P < 0.05$, ‡$P < 0.01$, compared to cells on day 12. NCAM, neural cell adhesion molecule.

CK19$^-$ cells (2.1 ± 0.5 ng insulin/10^6 cells, $n = 5$) (Fig. 7a). The removal of damaged and dead cells through FACS-sorting also contributed to increased levels of insulin content in the sorted cells.

Insulin secretion in differentiated CK19$^+$ and CK19$^-$ mouse ESCs

To evaluate whether CK19$^+$ and CK19$^-$ cells release insulin in a glucose-dependent manner, insulin secretion was determined. Both groups showed the same basal insulin release at 0 mM

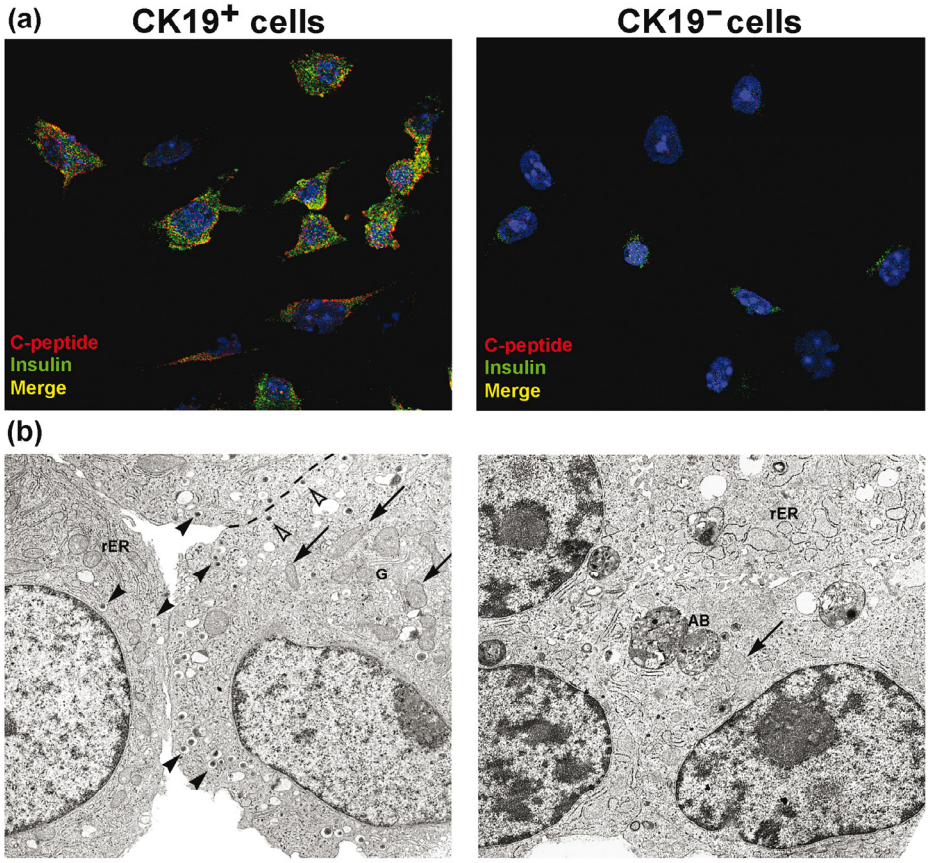

Figure 5. Comparison of C-peptide and insulin staining of CK19$^+$ and CK19$^-$ cells after differentiation of mouse embryonic stem cells (ESCs) at day 26 of the optimized four-stage differentiation protocol. Ultrastructure of CK 19$^+$ and CK19$^-$ ESCs at day 26 of the optimized four-stage differentiation protocol. (a) After FACS sorting CK19$^+$ and CK19$^-$ cells were seeded on glass slides and were stained for C-peptide (red) and insulin (green). Nuclei were counter-stained with DAPI (blue). The merge is shown in yellow. Original magnification ×600. (b) Shown on the left side is an electron micrograph of typical well-differentiated CK19$^+$ cells with characteristics of endocrine insulin-producing cells with well-developed rough endoplasmic reticulum (rER), Golgi apparatus (G) and secretory granules (*arrowheads*) and a high number of mitochondria (*arrows*). Some of the secretory granules are situated near to the plasma membrane (*dashed line*) ready for exocytosis (*open arrowheads*). On the right, shown for comparison, is an electron micrograph of CK19$^-$ cells with a small number of mitochondria (*arrows*), dilated endoplasmic reticulum and a high number of autophagic bodies (AB). Original magnification ×8000.

glucose. Incubation of CK19$^+$ cells with 30 mM glucose resulted in release of 5.0 ± 1.8 ng insulin/10^6 cells (n = 4) in 2 h, whereas CK19$^-$ cells showed no such increase (0.35 ± 0.09 ng insulin/10^6 cells, n = 5) on stimulation, when compared to basal insulin release rate (0.5 ± 0.17 ng insulin/10^6 cells, n = 5) (Fig. 7b).

DISCUSSION

Quantitative analysis of gene expression in mouse ESCs, which were differentiated according to the reference protocol by Lumelsky *et al*. (2001), showed that all three major endocrine

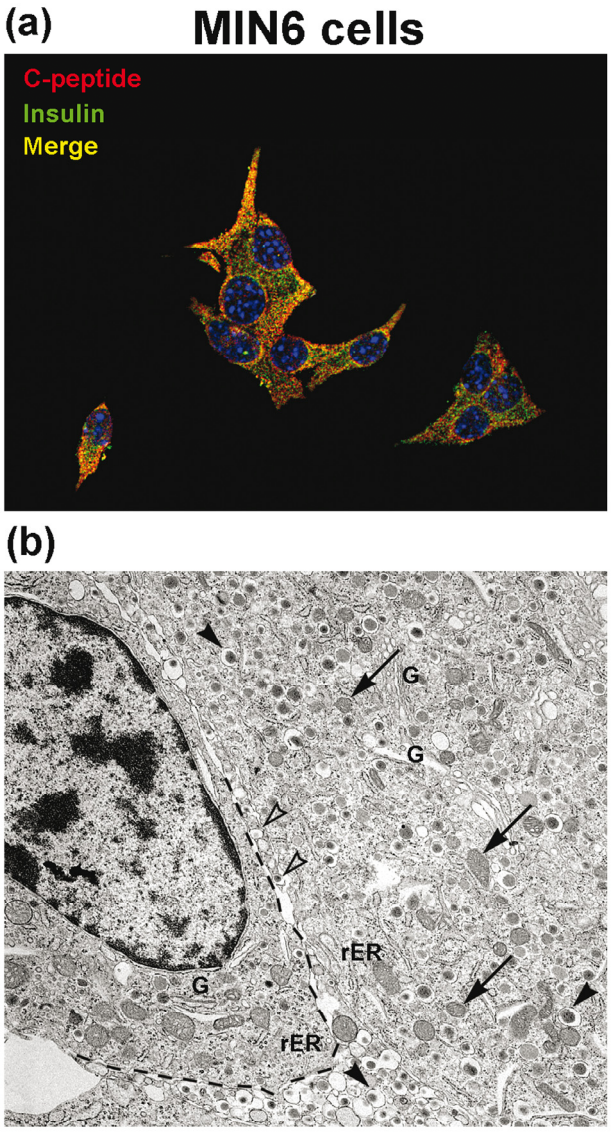

Figure 6. C-peptide and insulin staining and ultrastructure of MIN6 insulin-producing cells. (a) MIN6 cells were seeded on glass slides and were stained for C-peptide (red) and insulin (green); nuclei were counterstained with DAPI (blue). The merge is shown in yellow. Original magnification ×600. (b) The electron micrograph of MIN6 insulin-producing cells shows the typical endocrine morphology with high numbers of mitochondria (arrows), well developed rough endoplasmic reticulum (rER), Golgi apparatus (G) and secretory granules (*arrowheads*) some of them ready for exocytosis (open arrowheads). Original magnification ×8000.

hormones, insulin, glucagon and somatostatin, were readily expressed. Differentiation of ESCs according to the newly developed optimized four-stage differentiation protocol, in contrast to other published differentiation protocols (Lumelsky *et al*. 2001; Blyszczuk *et al*. 2003; Bai *et al*. 2005), yielded monohormonal insulin-producing cells, which virtually exclusively expressed insulin, with no glucagon expression and minor somatostatin expression.

Through removal of nestin selection, we produced cells with a significantly reduced neuronal character, as documented by reduction of expression levels of the neuronal markers nestin and NCAM. At the same time, β-cell-like characteristics of these insulin-producing cells were reinforced

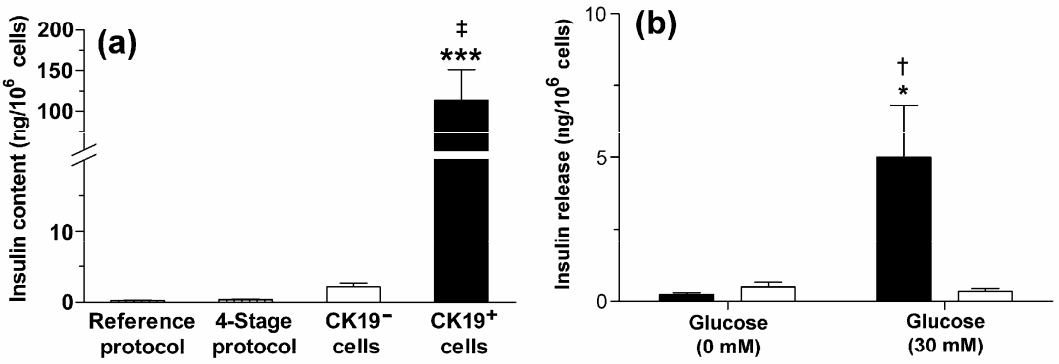

Figure 7. Comparison of insulin content of mouse embryonic stem cells after differentiation into insulin-producing cells, according to the reference differentiation protocol by Lumelsky *et al.* (2001) to the optimized four-stage differentiation protocol, as well as of the latter cells after separation into CK19$^+$ and CK19$^-$ cells. Comparison of insulin release in CK19$^+$ and CK19$^-$ cells after 2 h static incubation with 0 mM and 30 mM glucose. (a) Values shown are means ± SEM of the insulin content (ng/10^6 cells) of three to six experiments. ***$P < 0.001$, CK19$^+$ compared to CK19$^-$ cells. ‡$P < 0.01$, CK19$^+$ compared to cells from the reference protocol. (b) Values shown are means ± SEM of the insulin released (ng/10^6 cells) in 2 h of three to five experiments. *$P < 0.05$, CK19$^+$ compared to CK19$^-$ cells. †$P < 0.05$, compared to CK19$^+$ cells treated with 0 mM glucose. For comparison, insulin content of the MIN6 insulin-producing cells was 817.6 ± 123.5 ng insulin/10^6 cells, $n = 64$.

by high the gene expression level of the Glut2 glucose transporter and the transcription factor Pdx1. High levels of expression were also present for other β-cell characteristic genes, namely of the glucose sensor enzyme glucokinase as well as the potassium channel *Kir6.2* and the sulphonylurea receptor *Sur1*. In parallel, markers for differentiation towards ductal cells, namely CK19 and CA2, were significantly increased. On the other hand, lack of expression of amylase and albumin excluded significant differentiation towards exocrine pancreas and hepatic progeny.

These results support the contention that the removal of the nestin selection step from the differentiation protocol drives ESCs to the direction of insulin-producing cells and reduces neuronal characteristics of the differentiated cells. Thus, selection strategies focused on nestin expression (Lumelsky *et al.* 2001; Hori *et al.* 2002; Blyszczuk *et al.* 2003; Moritoh *et al.* 2003; Miyazaki *et al.* 2004; Bai *et al.* 2005) are not particularly suited to differentiation of ESCs into insulin-producing cells (Rajagopal *et al.* 2003; Hansson *et al.* 2004; Sipione *et al.* 2004). Not surprisingly, therefore, differentiation protocols that yielded cells with increased nestin positivity (Blyszczuk *et al.* 2003) were not significantly superior to the protocol devised by Lumelsky *et al.* (2001).

Therefore, rather than on nestin-positive cells, we focused our attention on CK19$^+$ as a selection marker for insulin-producing cells. CK19$^+$ ductal cells are generally considered to be progenitors for endocrine cells during pancreatic organogenesis (Rutter 1980; Slack 1995; Bouwens & De Blay 1996; Bouwens *et al.* 1997, 1998). *In vitro* analysis of purified CK19$^+$ cells demonstrated the capacity to proliferate and differentiate into islet cells (Bouwens *et al.* 1997, 1998, 2005; Bonner-Weir *et al.* 2000; Gao *et al.* 2003, 2005). In addition, recent work has shown that human embryonic stem cells follow, during their differentiation towards endocrine cells, the same developmental pathway known from *in vivo* development of the pancreas (D'Amour *et al.* 2005, 2006). Thus, it is clear that during *in vitro* differentiation of stem cells the last differentiation step towards β-cell type must occur *via* a CK19$^+$ cell population that expresses insulin. Moreover, the expression of CK19 as a marker of pancreatic ducts is in line with endodermal origin, and

clearly distinguishes such cells from cells of neuroectodermal progeny (Bouwens 2004; Hisatomi *et al*. 2004; Bouwens *et al*. 2005).

Following this assumption, we have on the basis of the new optimized differentiation protocol, set up a separation strategy using FACS sorting of fluorescent $CK19^+$ and non-fluorescent $CK19^-$ cells. We have used the CK19 promoter to control expression of eYFP in mouse ESCs during their differentiation towards insulin-producing cells. Previous studies have shown that the CK19 promoter is particularly potent to target duct cells (Brembeck *et al*. 2001). Two distinct cell populations, $CK19^+$ and $CK19^-$ cells, could be successfully separated and analysed using this FACS sorting procedure. Gene expression analysis of endogenous CK19 revealed that this gene is predominantly expressed in the FACS-sorted $CK19^+$ population. Thus, our selection procedure purifies ductal progenitor cells and separates them successfully from the $CK19^-$, non-ductal cell population. A detailed molecular, biochemical and morphological analysis revealed that $CK19^+$ cells represent a population of differentiated ESCs, which express the insulin prohormone gene, and process, store and release insulin.

We obtained 1.6% $CK19^+$ cells, which, in comparison with the $CK19^-$ cell fraction, had a nearly 40-fold higher level of insulin gene expression and a 50-fold higher content of insulin. This insulin content is around 450 times higher than that obtained using the reference protocol of Lumelsky *et al*. (2001) and still around 300 times higher than the optimized four-stage differentiation protocol, from which we started the CK19 separation procedure. Thus, this separation strategy of FACS sorting is far more efficient then previously published differentiation protocols (Lumelsky *et al*. 2001; Hori *et al*. 2002; Blyszczuk *et al*. 2003, 2004; Miyazaki *et al*. 2004; Bai *et al*. 2005; Shiroi *et al*. 2005).

When compared to the clonal insulin-producing cell lines RINm5F and MIN6, which like differentiated embryonic stem cells also reside in a tissue culture milieu, the level of insulin content in MIN6 insulin-producing cells is about seven times higher, and in RINm5F cells it is about 13 times lower than found in $CK19^+$ cells. Isolated islets of Langerhans, containing β-cells and other endocrine and non-endocrine cells types, lose insulin content and expression within less than 1–2 weeks (Weinberg *et al*. 2007). Thus, it is not surprising that embryonic stem cells differentiated *in vitro* towards insulin-producing cells do not reach insulin content and expression levels comparable to those of mature freshly isolated β-cells.

The $CK19^+$ cells expressed nearly 40 times more insulin but 12 times less glucagon and 7 times less somatostatin than $CK19^-$ cells. Thus, $CK19^+$ cells can be classified as monohormonal insulin-positive cells, while $CK19^-$ cells are polyhormonal cells with similar levels of glucagon and somatostatin but low insulin gene expression. In contrast to the $CK19^-$ ones, $CK19^+$ cells also exhibited a glucose-responsive insulin secretion.

Gene expression of Glut2 glucose transporter and transcription factor Pdx1 were also higher in $CK19^+$ cells. Expression levels of genes, which are known to be present both in β-cells and neuronal cells, namely of the potassium channel Kir6.2 and of the sulphonylurea receptor Sur1, were similar in both fractions, while gene expression levels of the typical neuronal markers such as nestin and NCAM were higher in $CK19^-$ cells. This is in accordance with the observation that NCAM is significantly expressed only in pancreatic α-cells while it is low in β-cells and absent in acinar and duct cells (Cirulli *et al*. 1994). The significantly higher expression of the transcription factor Nkx6.1 in $CK19^-$ cells supports the contention that this is also a gene with primary importance for neuronal development (Liu *et al*. 2003; Przyborski *et al*. 2003). This provides further support for the hypothesis that differentiation *via* neuronal precursors does not generate insulin-producing cells. $CK19^+$ cells exhibit the typical characteristics of pancreatic β-cells. In contrast to the $CK19^-$ cell population, $CK19^+$ cells are also C-peptide and insulin-positive and show, on ultrastructural analysis, typical characteristics of insulin-secretory cells, namely well-developed rough

endoplasmic reticulum, Golgi apparatus and secretory granules. Thus, the small proportion of C-peptide-positive cells that have been reported after ESCs had passed through differentiation protocols towards insulin-producing cells (Blyszczuk *et al*. 2004; Paek *et al*. 2005) can be considered to be the source of the C-peptide-positive cells with typical ultrastructural characteristics of secretory cells, which we have found in the present study in the fraction of the $CK19^+$ cells.

During the course of differentiation from ESCs towards insulin-producing cells, a fraction of the cells undergoes cell death through apoptosis. Thus, at the end of the differentiation procedure, the cell population contains a proportion of dead cells (Rajagopal *et al*. 2003; Hansson *et al*. 2004; Sipione *et al*. 2004; Duval *et al*. 2006). These apoptotic cells are particularly prone to uptake of insulin from the extracellular space (Rajagopal *et al*. 2003). This can lead to overestimation of the efficiency of a differentiation protocol (Hansson *et al*. 2004). However, because differentiation culture media require insulin supplementation for proper proliferation of the cells, it is necessary to separate the dead cells from the total cell population. Therefore, this separation-out of dead cells is part of our FACS sorting procedure (Fig. 2). Thus, the obtained populations of $CK19^+$ and $CK19^-$ cells were not contaminated with apoptotic cells, excluding an overestimation of cellular insulin content due to uptake from the differentiation medium.

In conclusion, our results show that this combined procedure is suitable for obtaining C-peptide-positive, glucose-responsive insulin-secreting $CK19^+$ cells. That the population of insulin-positive cells are, in addition, positive for CK19, provides strong support for the concept of ductal origin of insulin-producing cells (Bonner-Weir *et al*. 2000, 2004; Gao *et al*. 2003, 2005; Bouwens *et al*. 2005). Thus, these cells represent an endocrine precursor cell type with typical ultrastructural characteristics of insulin-secretory cells, potentially suitable for insulin replacement therapy in diabetes.

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3 Diskussion

Laut einer Studie der WHO sind weltweit mehr als 180 Millionen Menschen vom Diabetes mellitus betroffen. Der Anteil an Patienten mit einem Diabetes vom Typ 1 liegt bei 10 % (WHO 2008). Die selektive autoimmun vermittelte Zerstörung der pankreatischen ß-Zellen ist Ursache für den absoluten Insulinmangel beim T1DM. Eine Heilung ist bisher nicht möglich und daher sind die Patienten auf eine lebenslange Substitution von Insulin oder Insulinanaloga angewiesen. Der therapeutischen Strategie durch Transplantation von isolierten Langerhansschen Inseln oder ganzer Pankreata sind durch die geringe Verfügbarkeit von Spenderorganen Grenzen gesetzt. Daher kann die Therapie des T1DM nur bei wenigen Patienten durch Transplantation von humanen Spenderpankreata erfolgen. Außerdem bedingt diese Therapieform eine lebenslange immunsuppressive Behandlung, um eine Abstoßung des transplantierten Gewebes zu verhindern. Aufgrund der Spätkomplikationen, die selbst bei einem behandelten T1DM auftreten können, ist der Typ-1-Diabetiker die primäre Zielgruppe für eine gentherapeutische Behandlung mit Surrogatzellen.

3.1 Ein neues experimentelles Protokoll zur präferentiellen Differenzierung muriner embryonaler Stammzellen in insulinproduzierende Zellen

In dieser Studie wurde zunächst ein neues optimiertes 4-Stadien Protokoll zur Differenzierung muriner embryonaler Stammzellen in insulinproduzierende Surrogatzellen entwickelt und mit einem Referenzprotokoll verglichen, welches durch Lumelsky und Mitarbeiter zuvor veröffentlicht worden war (Lumelsky *et al.* 2001). Außerdem wurde der Einfluss von fötalem Kälberserum (FCS) im Differenzierungsmedium und der mögliche Einsatz von Bioreaktoren (sog. s*pinner cultures*) auf die Differenzierung untersucht. Das Referenzprotokoll wurde reproduziert, aber im Gegensatz zu einer anderen Arbeitsgruppe (Sipione *et al.* 2004) konnten wir eine deutliche Insulingenexpression zusammen mit der Expression anderer pankreatischer Hormone messen. Gleichzeitig wurden jedoch hohe Expressionswerte der neuronalen Marker Nestin und NCAM detektiert, während der endokrine β-Zellmarker Pdx1 noch geringer als in undifferenzierten Stammzellkulturen exprimiert wurde (Moritoh *et al.* 2003).

Bei der Differenzierung mit dem Referenzprotokoll zeigte sich auch eine deutliche Reduzierung des Insulingehaltes der differenzierten Zellen, wenn das Insulin in den letzten Tagen der Differenzierung dem Medium nicht mehr zugesetzt wurde. Die Expression des Insulingens blieb von diesem Phänomen unberührt. Dies kann zumindest in Teilen erklären, warum in verschiedenen Studien stark unterschiedliche Ergebnisse bezüglich des

Insulingehaltes ermittelt wurden, da durch die passive Aufnahme von Insulin aus dem Medium in das Zytoplasma der Zelle ein falscher Eindruck von der Effektivität des Referenzprotokolls vermittelt wurde (Rajagopal *et al.* 2003; Hansson *et al.* 2004; Sipione *et al.* 2004; Paek *et al.* 2005). Das Fehlen der Pdx1-Expression und die Unfähigkeit, ohne Insulin im Medium, trotz einer klar detektierbaren Insulingenexpression, signifikante Mengen an Insulin zu speichern, wies auf eine unregulierte Expression des Insulingens hin, wie es für die Ontogenese des zentralen Nervensystems berichtet wurde (De Pablo *et al.* 1982; Devaskar *et al.* 1993a; Devaskar *et al.* 1993b; Perez-Villamil *et al.* 1994). In der Tat ergaben ultrastrukturelle Analysen, dass diese Zellen phänotypische Merkmale neuronaler Entwicklung aufwiesen. Daher ist es auch wenig erstaunlich, dass diese Zellen nach Implantation in ein diabetisches Mausmodell keine signifikante Erniedrigung der Blutglucosekonzentration bewirken konnten. Das Verteilungsniveau der Genexpression, zusammen mit den ultrastrukturellen Ergebnissen und den Implantationsversuchen, bestätigte, dass die Differenzierung mit dem Referenzprotokoll im Wesentlichen Zellen mit neuronalem Geno- und Phänotyp hervorgebracht hat. Diese Zellen wiesen zwar eine moderate Insulingenexpression auf, ließen aber ansonsten jedes Charakteristikum einer endokrinen, insulinproduzierenden Zelle fehlen.

Das Referenzprotokoll stützte sich auf sie Hypothese, das Nestin-Positivität ein wichtiges und unumgängliches Markerprotein einer möglichen Progenitorzelle des endokrinen Entwicklungsweges sei (Lumelsky *et al.* 2001). Daher wurde die Selektion auf Nestin in das Referenzprotokoll eingearbeitet. Diese Nestinselektion führte bei der Reproduktion des Referenzprotokolls zu einer stark erhöhten Caspase-3 Aktivierung, so dass Zelltod durch Apoptose ein wesentliches Merkmal des Referenzprotokolls zu sein schien. Die Selektion auf Nestin führte in den ES-Zellen zur neuronalen Entwicklung oder zum vorzeitigen Zelltod durch Apoptose und verhinderte damit möglicherweise die Differenzierung in pankreatisches, endokrines Gewebe. Durch Supplementierung mit Serum wurde die neuronale Differenzierung noch zusätzlich verstärkt.

Um diesen Nachteil des Referenzprotokolls zu überwinden, wurde ein neues optimiertes 4-Stadien Protokoll entwickelt. Auf den Nestinselektionsschritt wurde verzichtet und die Medienzusammensetzung verändert, um Bestandteile des Mediums, welche die neuronale Entwicklung fördern, zu entfernen. Außerdem wurde das Medium im finalen Stadium der Differenzierung mit Serum supplementiert, um einen möglichen Mangel an Nährstoffen und den daraus resultierenden Zelltod zu verhindern. Nach der Differenzierung mit dem neuen 4-Stadien Protokoll konnte beobachtet werden, dass die Zellen eine stark erhöhte

Insulingenexpression aufwiesen. Die Expression von Glucagon war nicht mehr detektierbar und die Expression von Somatostatin war im Vergleich zum Referenzprotokoll deutlich reduziert. Die reduzierte Expression von NCAM und Nestin spiegelte die Verlagerung von der neuronalen Differenzierung des Referenzprotokolls zu einer endokrinen Differenzierung in Zellen mit monohormonalem Charakter im neuen Differenzierungsprotokoll wider. Durch die Entfernung der Nestinselektion konnte die Caspase-3 Aktivierung deutlich reduziert werden, was als Hinweis auf eine größere Zellvitalität gewertet werden kann. Außerdem zeigte sich die Zunahme des endokrinen Charakters auch an der erhöhten Expression des Glucosetransporters GLUT2 und anderer β-zellspezifischer Gene, wie Glucokinase, Kir6.2 und Sur1. Markerproteine der duktalen Differenzierung wie CK19 und Carboanhydrase 2 (CA2) waren signifikant erhöht und das Fehlen der Amylase und des Albumins schlossen eine Entwicklung in Richtung des exokrinen Pankreas oder der hepatischen Richtung aus. Es ist weitgehend anerkannt, dass β-Zellen im Verlaufe der Embryonalentwicklung aus Zellen der duktalen Gänge entstehen (Rutter 1980; Slack 1995; Bouwens & De Blay 1996; Bouwens *et al.* 1997; Bouwens & Pipeleers 1998). Daher ist es auch nicht erstaunlich, dass die verstärkte Expression von CK19 und CA2 mit der erhöhten Expression von Insulin und der anderen endokrinen Marker einherging. Hier ergaben sich interessante Parallelen zur Organogenese des Pankreas. Der Transkriptionsfaktor Pdx1 ist ein wichtiger Transaktivator des Insulingens und ist somit für die geregelte Insulingenexpression verantwortlich. Auch die Expression dieses endokrinen Markers konnte im neuen 4-Stadien Protokoll gegenüber dem Referenzprotokoll deutlich erhöht werden.

Es wurde außerdem untersucht, ob die Veränderungen im Genexpressionsmuster auch zu einem veränderten Phänotyp mit dem Erscheinungsbild einer β-Zelle geführt haben könnten. Hier wurden auf der ultrastrukturellen Ebene die entscheidenden Charakteristika einer endokrinen, insulinproduzierenden Zelle in Form von Insulin-Granula beobachtet. In diesem Zusammenhang ist es außerdem wichtig zu erwähnen, dass das Insulin in diesen Zellen synthetisiert wurde und somit unabhängig von einer exogenen Quelle war. Die Ergebnisse der biochemischen und morphologischen Untersuchungen wurden durch die Implantationsversuche unterstützt. Die Implantation von differenzierten Stammzellen aus dem optimierten 4-Stadien Differenzierungsprotokoll in das STZ-diabetische Mausmodell führte zu einer signifikanten Reduktion der Blutglucosekonzentration unter Werte von 200 mg/dl und somit in den Bereich nicht-diabetischer Tiere.

Kürzlich wurde berichtet, dass ein Differenzierungsprotokoll, welches im Wesentlichen auf dem von Lumelsky *et al.* basierte, aber als zusätzliche Neuerung eine 14-tägige Kultivierung

in einem Bioreaktor einführte, zu einem 14fach höheren Insulingehalt führte (Blyszczuk *et al.* 2003). Ein solcher Bioreaktor hält die Zellen im Gegensatz zur Standardgewebekultur und zum hier vorgestellten optimierten 4-Stadien Differenzierungs-protokoll in Suspension. Im Gegensatz zu dieser Veröffentlichung konnte keine Verbesserung der Differenzierung ausgemacht werden. Es wurde auf der Ebene der Genexpression und der Ultrastruktur sogar eine Unterdrückung der Differenzierung beobachtet, so dass diese Zellen keine signifikante Veränderung gegenüber dem undifferenzierten, embryonalen Status erworben haben konnten. Dieser Entwicklungsstop könnte möglicherweise darin begründet sein, dass die Zellen in Suspension nicht in der Lage waren, Zell-Zell Kontakte auszubilden, die aber für eine geregelte Differenzierung unabdingbar sind (Trosko *et al.* 2000). Auf der anderen Seite könnte die Suppression der Differenzierung eine attraktive biotechnologische Methode sein, um humane ES-Zellen undifferenziert in Kultur zu halten.

3.2 Veränderungen der Genexpression und der Morphologie von murinen embryonalen Stammzellen nach Differenzierung in insulinproduzierende Zellen *in vitro* und *in vivo*.

In dieser Studie wurden die Veränderungen der Genexpression und die morphologische Reifung von embryonalen Stammzellen nach Differenzierung *in vitro* und nach Implantation in ein diabetisches Mausmodell *in vivo* untersucht. Die differenzierten ES-Zellen aus der *in vitro* Kultur wurden darüber hinaus immunhistochemisch und mittels Elektronenmikroskopie charakterisiert. Das Implantat wurde auf der Ebene der Genexpression und der Proteinexpression mit den Methoden der *in situ* RT-PCR und Immunhistochemie untersucht. Das Ziel dieser Studie war es, den Effekt einer *in vivo* Umgebung auf die weitere Entwicklung insulinproduzierender Surrogatzellen einzuschätzen, die zuvor mit zwei verschiedenen Protokollen differenziert worden waren. Dazu wurden differenzierte Zellen verwendet, die entweder mit einem Referenzprotokoll (Lumelsky *et al.* 2001) oder mit dem optimierten 4-Stadien Protokoll differenziert wurden. Das Referenzprotokoll wurde ursprünglich für die neuronale Differenzierung entwickelt, aber später für die endokrine Differenzierung modifiziert (Lee *et al.* 2000).

Wenn ES-Zellen unter *in vitro* Bedingungen differenziert wurden, stellten sich in den ersten Tagen schnelle Veränderungen des Phänotyps ein. Der Stammzell-Phänotyp war gekennzeichnet durch eine starke Tendenz der ES-Zellen in Kolonien zu wachsen. Diese Kolonien wiesen eine sehr glatte und runde Morphologie auf, so dass einzelne Zellen in dieser Kolonie nur sehr schwer auszumachen waren. Innerhalb einer einzelnen Zelle war der Zellkern mit den Nucleoli größer als das Zytoplasma. Unter den

Differenzierungsbedingungen des Referenzprotokolls wurde dieser Phänotyp sehr schnell aufgegeben und die Zellen bildeten vermehrt axonale Verzweigungen aus. Diese Tendenz war bereits in vorhergehenden Arbeiten beschrieben worden (Sipione *et al.* 2004). Im Gegensatz dazu konnte bei den Zellen des optimierten 4-Stadien Protokolls nach Differenzierung eine solche Entwicklung nicht beobachtet werden. Hier zeigten sich grundsätzlich zwei Zellpopulationen in zwei verschiedenen, dreidimensionalen Ebenen der Kulturschale. Der Boden der Platte wurde durch einen einschichtigen Zellrasen bedeckt, während auf diesem *Monolayer* Sprossungen von Zellclustern auswuchsen. Einige Arbeiten im Bereich der adulten Stammzellen weisen darauf hin, dass solche Cluster möglicherweise den Bereich umfassen, in dem endokrine Progenitorzellen heranreifen (Gao *et al.* 2005).

Die Suppression der neuronalen Differenzierung im neuen 4-Stadien Protokoll wurde anhand der verminderten Immunoreaktivität für Nestin gezeigt, da Nestin positive Zellen den größten Anteil der Zellpopulation beim Referenzprotokoll ausmachten. Bemerkenswerterweise ging die Reduzierung der Nestin-Positivität mit einer Erhöhung der Zellen einher, die positiv für Insulin und C-Peptid waren. Dieser Effekt konnte auch von einer anderen Arbeitsgruppe gezeigt werden (Blyszczuk *et al.* 2004), was jedoch interessanterweise im Gegensatz zu einer zuvor veröffentlichten Arbeit derselben Gruppe und einer anderen Arbeitsgruppe steht (Hori *et al.* 2002; Blyszczuk *et al.* 2003). Die immunhistochemischen Resultate in Bezug auf die Nestinexpression wurden auch durch elektronenmikroskopische Untersuchungen gestützt. In diesen konnte der verbesserte Differenzierungsstatus anhand β-zelltypischer Strukturen wie z.B. ausgeprägtes rauhes Endoplasmatisches Reticulum, Golgi Apparat und Insulin-Granula in Zellen des neuen Protokolls gezeigt werden. Außerdem zeigten diese Zellen einen vitalen Phänotyp ohne Anzeichen von Zelltod durch Nekrose oder Apoptose. Eine Übereinstimmung dieser Resultate ergibt sich auch zu Veröffentlichungen anderer Arbeitsgruppen, in denen die Entfernung der Nestinselektion ebenfalls als die Voraussetzung für die endokrine Entwicklung angesehen wurde (Shi *et al.* 2005; D'Amour *et al.* 2006; Jiang *et al.* 2007).

Trotzdem ist für ES-Zellen der Maus bisher nicht beschrieben worden, inwieweit die *in vivo* Situation den Reifungsprozess der Stammzellen fördern kann. Hier sind speziell der weitere Verlust der Pluripotenz und die Zunahme an Funktionalität der implantierten Zellen interessant. Stammzellen, die mit dem optimierten 4-Stadien Differenzierungsprotokoll behandelt wurden, konnten die metabolische Situation STZ-diabetischer Mäuse signifikant verbessern. Auch andere Studien konnten die Funktionalität von differenzierten ES-Zellen belegen (Soria *et al.* 2000; Hori *et al.* 2002; Blyszczuk *et al.* 2004; Leon-Quinto *et al.* 2004; Fujikawa *et al.* 2005; Shi *et al.* 2005). Allerdings sind in diesen Arbeiten keine vertiefenden

Analysen über die Veränderungen der Blutglucosekonzentration der diabetischen Mäuse hinaus durchgeführt worden. Die vorliegende Studie dokumentiert damit zum ersten Mal die Zunahme wichtiger β-Zellfaktoren, ebenso wie den weiteren Verlust an Embryonalität der differenzierten Stammzellen. Nach der Implantation von ES-Zellen, die mit beiden Protokollen differenziert wurden, war eine distinkte Reduktion der Oct4- und Nanog-positiven Zellen auszumachen. Zellen, die noch positiv für einen der beiden embryonalen Marker waren, wiesen allerdings erhebliche Anzeichen von Apoptose auf. Daher ist anzunehmen, dass diese Zellen möglicherweise durch Apoptose aus dem Implantat entfernt werden. Bei ES-Zellen, die vor der Implantation entsprechend dem Referenzprotokoll differenziert worden waren, blieb eine weitere Reifung in insulinproduzierende Zellen weitestgehend aus, was als Fehlschlagen der endokrinen Differenzierung gewertet werden kann. Im Gegensatz dazu war bei den Zellen, die mit dem neuen 4-Stadien Protokoll differenziert wurden, eine weitere Verstärkung der Pdx-1 Expression im Implantat zu beobachten. Pdx1 ist einer der wichtigsten Aktivatoren der Insulingenexpression und ist auch während der Ontogenese des Pankreas für die Entwicklung des Organs verantwortlich (Jonsson *et al.* 1995; Ahlgren *et al.* 1996; Ahlgren *et al.* 1998). Die Detektion von Pdx-1 ist bislang aber nur in kultivierten Zellen *in vitro* gelungen (D'Amour *et al.* 2006; Shim *et al.* 2007). Durch die Detektion von Pdx1 erschien auch die detektierte Expression von Insulin plausibler, da die Expression beider Gene untrennbar miteinander verknüpft ist. Die beobachtete Genexpression von Insulin wurde auch durch die beobachtete Aktivität des IAPP-Gens bestätigt. IAPP wird normalerweise mit Insulin zusammen in den sekretorischen Granula gespeichert. Die Detektion von IAPP unterstützt daher die These, dass das Insulin in den Zellen nur durch *de novo* Synthese entstanden sein konnte und nicht durch passive Aufnahme aus dem Kultivierungsmedium.

Der Glucosetransporter GLUT2 und das Glucose-phosphorylierende Enzym Glucokinase sind verantwortlich für eine regulierte Aufnahme und den Stoffwechseln von Glucose in der β-Zelle und spielen eine entscheidende Rolle bei der Initiierung der physiologischen und Glucose-induzierten Insulinsekretion. Die Analyse dieser Proteine zeigte nur eine geringe Proteinexpression in Implantaten von Zellen, die mit Referenzprotokoll differenziert worden waren. Nur sehr wenige Zellen ließen sich schwach für diese Marker anfärben, während die Anfärbung der Zellen des neuen Protokolls ausgeprägt war und mehr Zellen diese Marker exprimierten. Morphologisch wurden diese beiden Marker in den Zellkompartimenten detektiert, in denen sie sich typischerweise befinden. Auch dies zeigt deutlich die

unterstützende Rolle der *in vivo* Situation, die sich allerdings nur dann positiv auswirken kann, wenn die ES-Zellen zuvor mit einem adäquaten Protokoll differenziert wurden.

3.3 Eine effiziente neue Strategie zur Differenzierung und Aufreinigung einer CK19$^+$ insulinproduzierenden Zellpopulation

Die quantitative Analyse der Genexpression von ES-Zellen, die mit dem Lumelsky-Protokoll differenziert wurden (Lumelsky *et al.* 2001), zeigte die Expression der drei wichtigen Hormone Insulin, Glucagon und Somatostatin. Das neu entwickelte 4-Stadien Differenzierungsprotokoll konnte im Gegensatz zu bisherigen Publikationen (Lumelsky *et al.* 2001; Blyszczuk *et al.* 2003; Bai *et al.* 2005) ES-Zellen in annährend monohormale Zellen differenzieren, die eine deutliche Insulin- aber eine fast nicht mehr nachweisbare Glucagon- und Somatostatinexpression aufwiesen. Vor allem durch die Entfernung des Nestinselektionsschritts des ursprünglichen Differenzierungsprotokolls konnte der neuronale Charakter der differenzierten Zellen signifikant reduziert werden. Diese Reduzierung konnte anhand der neuronalen Markergene Nestin und NCAM deutlich gezeigt werden. Parallel zu dieser Veränderung der Differenzierung wurde der endokrine, β-zellähnliche Charakter der differenzierten ES-Zellen verstärkt. Die Expression typischer β-zellspezifischer Marker, wie GLUT2 und Pdx1 konnte erheblich erhöht werden.

Das hohe Expressionsniveau konnte auch für Gene des Glucose-sensorischen und sekretorischen Apparats der β-Zelle, wie für die Glucokinase, den ATP-abhängigen Kaliumkanal Kir6.2 sowie den assoziierten Sulfonylharnstoffrezeptor Sur1 festgestellt werden. Gleichzeitig konnte eine signifikante Erhöhung der Expression der duktalen Marker Cytokeratin 19 und CA2 nachgewiesen werden.

Um die Differenzierung in Richtung des exokrinen Pankreas oder des hepatischen Zelltyps zu untersuchen, wurde die Expression von Amylase und Albumin bestimmt. Selbst mit der hochsensitiven *real-time* RT-PCR war keine mRNA Expression zu detektieren, was auf ein vollkommenes Fehlen von Zellen mit diesen Charakteristika in den differenzierten Zellkulturen hinweist. Die Annahme, dass die Nestin-Selektion förderlich für die Differenzierung in insulinproduzierende Zellen sei, wird mit der vorliegenden Arbeit grundsätzlich widerlegt. Daraus folgt die Schlussfolgerung, dass bereits veröffentlichte Differenzierungs- oder Selektionsstrategien auf den Marker Nestin (Lumelsky *et al.* 2001; Hori *et al.* 2002; Blyszczuk *et al.* 2003; Moritoh *et al.* 2003; Miyazaki *et al.* 2004; Bai *et al.* 2005) nicht besonders geeignet sind, um insulinproduzierende Zellen *in vitro* zu erzeugen. Eine ganze Reihe von weiteren Arbeiten konnte ebenfalls die Sinnhaftigkeit dieser bisher

verfolgten Strategie entkräften (Rajagopal *et al.* 2003; Hansson *et al.* 2004; Sipione *et al.* 2004). Es überrascht daher nicht, dass minimale Modifikationen des urprünglichen neuronalen Differenzierungsprotokolls von Lumelsky *et al.* (Lumelsky *et al.* 2001) trotz genetischer Modifikation keine wesentliche Verbesserung erzielen konnten (Blyszczuk *et al.* 2003). Daher war es ein Ziel dieser Arbeit, den Fokus von der Theorie der Nestin-Selektion auf einen anderen Marker für die Selektion zu richten. $CK19^+$ Zellen werden allgemein als Vorläuferzellen für β-Zellen während der Embryogenese betrachtet (Rutter 1980; Slack 1995; Bouwens & De Blay 1996; Bouwens *et al.* 1997; Bouwens & Pipeleers 1998). Die Analyse aufgereinigter duktaler $CK19^+$ Zellen aus humanen Spenderpankreata hat eindrucksvoll das Differenzierungspotential in Richtung β-Zelle belegt (Bonner-Weir *et al.* 2000; Gao *et al.* 2003; Bonner-Weir *et al.* 2004; Gao *et al.* 2005; Yatoh *et al.* 2007). Arbeiten an humanen ES-Zellen konnten zeigen, dass Stammzellen während der *in vitro* Differenzierung den gleichen entwicklungsbiologischen Linien folgen, wie bei der Organogenese des Pankreas (D'Amour *et al.* 2005; D'Amour *et al.* 2006). Hieraus ergab sich auch die Hypothese der vorliegenden Arbeit, dass eine Differenzierung von ES-Zellen in insulinproduzierende β-Zellen über einen intermediären Zelltyp mit duktalem Charakter erfolgen muss. Der Marker CK19 ist ein charakteristisches Protein duktaler Zellen und ist darüber hinaus endodermaler Herkunft (Bouwens 2004; Hisatomi *et al.* 2004; Bouwens & Rooman 2005).

Auf der Basis des optimierten 4-Stadien Protokolls wurde eine Sortierungsstrategie für eYFP+ und eYFP- Zellen in einem Hochgeschwindigkeitszellsortierer realisiert. Ein Fragment des CK19 Promoters wurde in ein Reporterkonstrukt subkloniert, um die Expression des Fluoreszenzproteins eYFP zu kontrollieren und während der Differenzierung von ES-Zellen die Aktivierung des CK19 Promoters sichtbar zu machen. Vorhergehende Studien haben das Potential des CK19 Promoters für die Darstellung duktaler Zellen gezeigt (Brembeck *et al.* 2001). Durch die Zellsortierung konnten zwei Zellpopulationen, namentlich $CK19^+$ und $CK19^-$ Zellen, gewonnen werden.

Die Charakterisierung erfolgte auf der Ebene der mRNA Expression. $CK19^+$ Zellen exprimierten verstärkt endogenes CK19 und endogene CA2, so dass das eYFP unter der Kontrolle des CK19 Promoters offenbar Zellen mit duktalem Charakter darstellt, während die nicht-duktale Fraktion negativ für CK19 war. Beide Zellpopulationen wurden einer detaillierten biochemischen und morphologischen Analyse unterzogen, wobei gezeigt werden konnte, dass $CK19^+$ Zellen das Insulingen exprimierten, das Prohormon Proinsulin prozessierten und in der Lage waren, Insulin auf den Stimulus Glucose hin freizusetzen.

Die Fraktion der $CK19^+$ Zellen betrug am Tag 26 der Differenzierung etwa 1,6 % der gesamten vitalen Population. Diese Zellen exprimierten etwa 40mal mehr Insulin und hatten einen 50mal höheren Insulingehalt im Vergleich zu $CK19^-$ Zellen. Im Vergleich zu den Ergebnissen des Lumelsky-Protokolls war dieser Insulingehalt 450mal höher und im Vergleich zu unsortierten Zellen aus dem optimierten 4-Stadien Protokoll 300mal höher. Diese Ergebnisse zeigten eindeutig, dass Sortierungsverfahren mittels eines FACS-Sorters wesentlich effizienter sind als bisher publizierte Differenzierungsprotokolle für ES-Zellen der Maus (Lumelsky *et al.* 2001; Hori *et al.* 2002; Blyszczuk *et al.* 2003; Blyszczuk *et al.* 2004; Miyazaki *et al.* 2004; Bai *et al.* 2005; Shiroi *et al.* 2005). Vergleicht man diese Ergebnisse mit immortalen und insulinproduzierenden Tumorzelllinien, wie der RINm5F und der MIN6 Zellline, so ist festzustellen, dass der Insulingehalt der MIN6 Zelllinie etwa 7mal höher ist und der RINm5F Zelllinie etwa 13mal niedriger. Damit ist der Insulingehalt dem Gehalt etablierter insulinproduzierender Gewebekulturzellen vergleichbar. Isolierte Pankreasinseln enthalten neben den endokrinen Zelltypen auch nicht-endokrine Zellen. Wenngleich der Insulingehalt in diesem heterogenen Gewebe höher ist, verlieren diese Pankreasinseln durch die *in vitro* Kultivierung innerhalb von 1-2 Wochen ihren typischen, endokrinen Phänotyp, so dass bereits nach kurzer Zeit nur noch wenig Insulin, weder auf Protein- noch auf mRNA-Ebene, nachgewiesen werden kann (Weinberg *et al.* 2007). Schließt man diesen Aspekt in die Beurteilung der Ergebnisse mit ein, so ist zu schlussfolgern, dass die Differenzierung und Kultivierung von ES-Zellen *in vitro* höchstwahrscheinlich nie den Grad der Ausdifferenzierung einer reifen β-Zelle unmittelbar nach der Isolierung aus dem Pankreas erreichen wird.

Die $CK19^+$ Zellen exprimierten im Vergleich zu $CK19^-$ Zellen etwa 40mal mehr Insulin, aber 12mal weniger Glucagon und 7mal weniger Somatostatin. Insgesamt kann daher konstatiert werden, dass $CK19^+$ Zellen einen monohormalen Charakter aufwiesen, während $CK19^-$ Zellen zumindest zwei Hormone gleichzeitig auf ähnlichem Niveau exprimierten, aber eine deutliche Insulingenexpression vermissen ließen. Auch die Expression von GLUT2 und Pdx1 war in $CK19^-$ Zellen geringer. Das Expressionsniveau von Genen des Sekretionsapparats, wie Kir6.2 und Sur1, war in beiden Fraktionen ähnlich stark ausgeprägt, während die Expression der neuronalen Marker Nestin und NCAM präferentiell in $CK19^-$ Zellen gemessen werden konnte. Diese Ergebnisse decken sich mit früheren Studien, in denen für das Oberflächenprotein NCAM eine erwähnenswerte Expression nur in α-Zellen, nicht aber in β-Zellen, duktalen oder azinären Zellen beobachtet werden konnte (Cirulli *et al.* 1994).

Die signifikant höhere Expression des Transkriptionsfaktors Nkx6.1 in $CK19^-$ Zellen unterstützt die Annahme, dass auch dieses Gen bei der Differenzierung embryonaler Stammzellen an der neuronalen Entwicklung beteiligt ist (Liu *et al.* 2003; Przyborski *et al.* 2003). Auch dies unterstützt die Hypothese, dass die Differenzierung über neuronale Progenitorzellen keine insulinproduzierenden Zellen hervorbringen kann.

$CK19^+$ Zellen zeigten das typische Erscheinungsbild einer pankreatischen β-Zelle. Im Gegensatz zu $CK19^-$ Zellen belegten ultrastrukturelle Untersuchungen mittels Elektronenmikroskopie bei $CK19^+$ Zellen einen ausgeprägten Differenzierungsstatus, was an der Ausbildung des rauen Endoplasmatischen Reticulums, des Golgi Apparats und sekretorischer Granula im Zytoplasma festzumachen war. Der geringe Anteil C-Peptid-positiver Zellen, der auch von anderen Arbeitsgruppen berichtet wurde, könnte aufgrund der vorgelegten Daten der $CK19^+$ Zellfraktion zuzuschreiben sein (Blyszczuk *et al.* 2004; Paek *et al.* 2005). Während der Differenzierung in insulinproduzierende Zellen geht ein Teil der Zellen durch apoptotischen Zelltod unter (Rajagopal *et al.* 2003; Hansson *et al.* 2004; Sipione *et al.* 2004; Duval *et al.* 2006). Verschiedene Arbeitsgruppen konnten eine passive Aufnahme von Insulin aus dem Medium in diese apopotischen Zellen nachweisen (Rajagopal *et al.* 2003). Dieser Prozess führte bei der Beurteilung der Effizienz einer Differenzierung zu einer Überschätzung des Insulingehalts (Hansson *et al.* 2004). Da das Differenzierungsmedium aber in jedem Fall mit Insulin zur Anregung der Proliferation supplementiert werden muss, ist eine Separation der toten bzw. apoptotischen Zellen von der vitalen Zellpopulation unbedingt erforderlich. Eine solche Separation toter Zellen fand im Sortierungsverfahren statt, da nur die Lebendpopulation auf Fluoreszenz hin sortiert wurde. In den beiden sortierten Zellfraktionen, $CK19^+$ und $CK19^-$ Zellen, sind solche kontaminierenden Zellen mit passiv aufgenommenem Insulin nicht enthalten, so dass der gemessene Insulingehalt tatsächlich als *de novo* synthetisiertes Insulin betrachtet werden kann.

Daher repräsentieren $CK19^+$ Zellen einen endokrinen Progenitorzelltyp, der in der Lage ist das Prohormon Proinsulin zu exprimieren, in Insulin und C-Peptid zu prozessieren und auf den Stimulus Glucose hin Insulin freizusetzen. Mit diesen Merkmalen einer insulinsezernierenden Zelle, könnten $CK19^+$ Zellen als Surrogatzellen in der Zellersatztherapie des Diabetes mellitus genutzt werden.

4 Zusammenfassung

In Deutschland leiden ca. 6 Millionen Menschen unter einem Diabetes mellitus. Davon sind etwa 10 % insulinpflichtige Typ-1-Diabetiker. Typ-1-Diabetiker sind auf eine Therapie durch eine Insulinsubstitution angewiesen, welche die Ausbildung von Spätkomplikationen jedoch nicht verhindern kann. Die Transplantation von humanen Spenderpankreata oder isolierten Pankreasinseln ist durch die limitierte Anzahl geeigneter Spenderorgane nur in wenigen Fällen möglich. Daher ist ein artifizielles Organsystem aus Surrogatzellen mit den Charakteristika endokriner β-Zellen von großer medizinischer Bedeutung. Die Implantation von insulinproduzierenden Surrogatzellen, die aus embryonalen Stammzellen (ES-Zellen) generiert werden, ist eine vielversprechende Strategie zur Therapie des insulinpflichtigen Diabetes mellitus. Das Ziel dieser Dissertation war die Entwicklung eines Differenzierungsprotokolls, das verlässlich insulinproduzierende Zellen aus ES-Zellen generiert. Dieses Protokoll sollte mit einem Sortierungsverfahren kombiniert werden, um unerwünschte Zellen abzutrennen und insulinproduzierende Zellen aufzureinigen. Dazu wurde zunächst ein zuvor veröffentlichtes Differenzierungsprotokoll von Lumelsky *et al.* (2001) reproduziert und als Referenz verwendet. Die Differenzierung mit dem Referenzprotokoll erzeugte Zellen mit einem neuronalen Geno- und Phänotyp. Diese Zellen exprimierten die inselzellspezifischen Hormone Insulin, Somatostatin und Glucagon, ließen aber ansonsten typische Charakteristika einer endokrinen Zelle fehlen. Darüber hinaus zeigte sich, dass die differenzierten Zellen Insulin aus dem Kultivierungsmedium passiv aufgenommen hatten, was zu einer deutlichen Überschätzung des Insulingehalts führen kann. Ein neu entwickeltes 4-Stadien Differenzierungsprotokoll konnte im Gegensatz zum Referenzprotokoll ES-Zellen in annähernd monohormonale Zellen differenzieren. Durch die Entfernung des Nestinselektionsschritts des Referenzprotokolls konnte der neuronale Charakter der differenzierten Zellen signifikant reduziert werden. Die Reduzierung der Nestin-Positivität ging mit einer Erhöhung der Zellen einher, die positiv für Insulin und C-Peptid waren, was ein Nachweis für *de novo* Synthese von Insulin ist und damit ausschließt, dass das Insulin durch passive Aufnahme aus dem Differenzierungsmedium in die Zellen gelangt ist. Parallel zu dieser Verbesserung konnte der endokrine, β-zellähnliche Charakter der differenzierten ES-Zellen auch durch eine erhöhte Expression des GLUT2 Glucose Transporters, Glucokinase, Pdx1 und Sur1 belegt werden. Gleichzeitig konnte eine signifikante Erhöhung der Expression der duktalen Marker Cytokeratin 19 und Carboanhydrase 2 nachgewiesen werden.

Die Implantation von differenzierten Stammzellen aus dem Referenzprotokoll und dem optimierten 4-Stadien Differenzierungsprotokoll in das durch Streptozotocin-Behandlung induzierte diabetische Mausmodell führte nur bei Zellen des neuen Differenzierungsprotokolls zu einer signifikanten Reduktion der Blutglucosekonzentration in den Bereich nicht-diabetischer Tiere. Bei ES-Zellen, die vor der Implantation mit dem Referenzprotokoll differenziert wurden, blieb eine weitere Reifung in insulinproduzierende Zellen in der *in vivo* Umgebung aus, während für Zellen des neuen 4-Stadien Differenzierungsprotokolls eine weitere Verstärkung des β-zellähnlichen Charakters gezeigt werden konnte.

Ein weiteres Ziel dieser Dissertation war die Etablierung eines Sortierungsverfahrens für $CK19^+$ Zellen. Duktale Zellen, die positiv für den Marker CK19 sind, spielen eine entscheidende Rolle bei der Organogenese des Pankreas. Hieraus ergab sich für die vorliegende Arbeit die Hypothese, dass eine Differenzierung von ES-Zellen in insulinproduzierende β-Zellen über einen intermediären Zelltyp mit duktalem Charakter erfolgt. Es konnte gezeigt werden, dass aufgereinigte $CK19^+$ Zellen das Insulingen exprimierten, das Prohormon Proinsulin in Insulin und C-Peptid prozessierten und in der Lage waren, Insulin auf den Stimulus Glucose hin freizusetzen. $CK19^+$ Zellen exprimierten 40mal mehr Insulin und hatten einen 50mal höheren Insulingehalt im Vergleich zu $CK19^-$ Zellen. Darüber hinaus zeigten $CK19^+$ Zellen den typischen Phänotyp einer pankreatischen β-Zelle mit einem ausgeprägten Differenzierungsstatus und Insulin-Granula im Zytoplasma. Die Tatsache, dass diese Zellen positiv für Insulin und CK19 waren, unterstützt die These, dass der duktale Zelltyp für die Organogenese von β-Zellen während der Embryonalentwicklung verantwortlich ist. Daher repräsentieren diese Zellen einen endokrinen Progenitorzelltyp, mit phänotypischen und genetischen Merkmalen einer insulinsezernierenden Zelle, die als mögliche Surrogatzellen in der Zellersatztherapie des Diabetes mellitus genutzt werden können.

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6 Publikationen

Teilergebnisse der vorliegenden Dissertation sind bereits veröffentlicht.

Kurzbeiträge:

Naujok O., Francini F., Tiedge M., Jörns A. & Lenzen S. (2004). Expression Betazell-spezifischer Gene und Morphologie von murinen embryonalen Stammzellen während der Differenzierung zu insulinproduzierenden Zellen. *Diabetes und Stoffwechsel* **13**: Suppl. 1, V-88, 40, Deutscher Diabetes-Kongress, Hannover, Mai 2004.

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Naujok O, Francini F, Jörns A, Tiedge M, & Lenzen S: **(2005).** Unterschiede in der Differenzierung von murinen und embryonalen Stammzellen in insulin-produzierenden Zellen. *Diabetes und Stoffwechsel* **14**: Suppl. 1, 124, P-268, Deutscher Diabetes- Kongress, Berlin, Mai 2005.

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Naujok, O., Francini, F., Jörns, A. & Lenzen, S. (2008). An efficient experimental strategy for mouse ES cell differentiation and separation of a cytokeratin 19 positive population of insulin-producing cells. *Cell Prolif* 2008; **41**: 607-624.

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7 Curriculum Vitae

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Schulausbildung

1981 – 1985	Grundschule Bonner Straße in Hannover
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1987 – 1994	Elsa-Brändström-Gymnasium in Hannover
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Zivildienst

01/95-06/95	Wirtschaftsdienst im Clementinenkrankenhaus in Hannover
07/95-01/96	Individuelle Schwerstbehindertenbetreuung

Studium

04/96-09/96	Ein Semester Studium der Fächer Philosophie und Geschichte an der Universität Hannover
10/96-05/01	Studium der Biologie an der Universität Hannover
	Beginn der Diplomarbeit: „Charakterisierung apikaler Transportvesikel in polaren Endothelzellen“, erstellt am Institut für physiologische Chemie der Tierärztlichen Hochschule Hannover
05/02	Abschluss des Studiums als Diplom-Biologe

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Berufliche Tätigkeit

Seit 11/02	Wissenschaftlicher Angestellter an der Medizinischen Hochschule Hannover, Institut für Klinische Biochemie

Promotion

07/04	Beginn der Dissertation mit dem Thema : „Differenzierung muriner embryonaler Stammzellen in insulinproduzierende Zellen“ am Institut für Klinische Biochemie der Medizinischen Hochschule Hannover

Die vorliegende Dissertation wurde im Juli 2004 unter Anleitung von Herrn Professor Dr. S. Lenzen am Institut für Klinische Biochemie der Medizinischen Hochschule Hannover begonnen und im Mai 2008 abgeschlossen.

8 Erklärung

Hierdurch erkläre ich, dass die Dissertation

Differenzierung muriner embryonaler Stammzellen in insulinproduzierende Zellen

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 17.06.2008

Ortwin Naujok

9 Danksagung

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