CREB-vermittelte Mechanismen der Adaptation und endokrinen Regulation embryonaler Zellen

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Inhalt

1. Einleitung

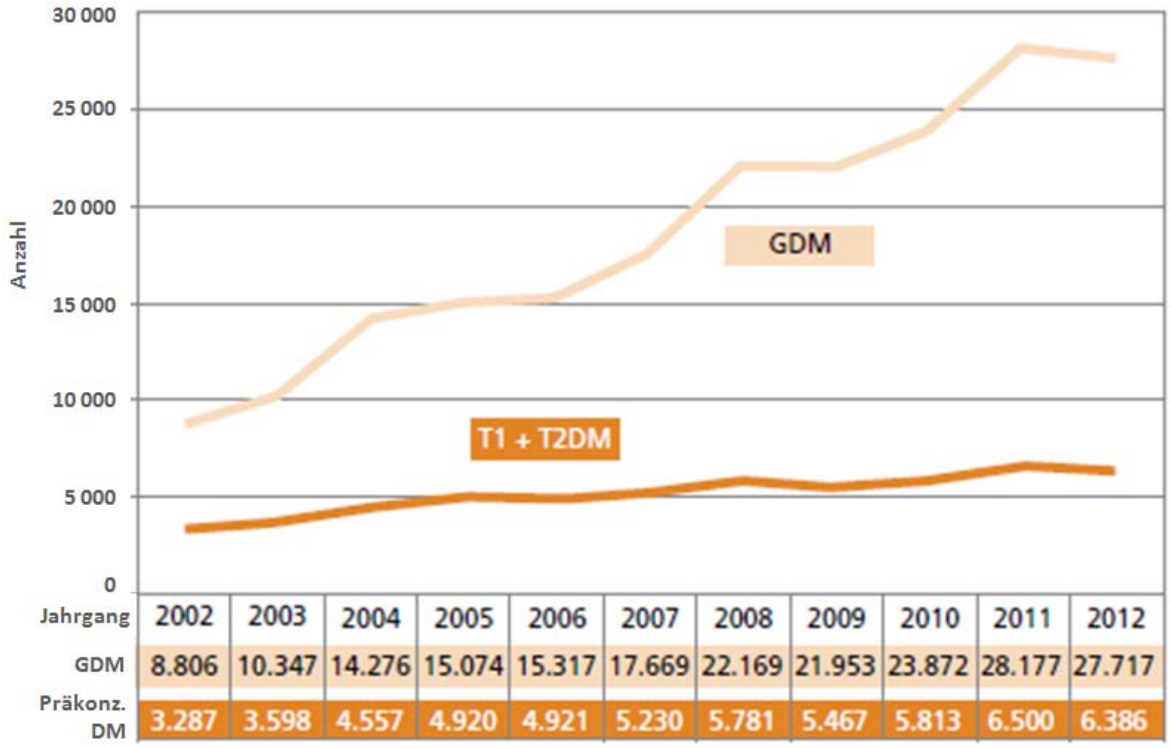
Um eine möglichst optimale Versorgung und Entwicklung des Embryos zu gewährleisten, finden im Verlauf einer gesunden Schwangerschaft im mütterlichen Organismus eine Reihe von endokrinen und metabolischen Anpassungsprozessen statt. Während der Präimplantationsphase, zu einem Zeitpunkt, an dem der Embryo noch nicht in den Uterus implantiert ist, sichern lokale Regulationsmechanismen die Bereitstellung eines optimalen intrauterinen Milieus. Die mütterliche Versorgung mit Nährstoffen und Wachstumsfaktoren hat grundlegende Auswirkungen auf den embryonalen Metabolismus und damit langfristige Konsequenzen für die Gesundheit des Kindes (metabolische Programmierung). Das Auftreten bestimmter Erkrankungen im Erwachsenenalter ist mit den Entwicklungsbedingungen *in utero* verbunden. So ist das Risiko metabolischer Erkrankungen, wie Übergewicht und Diabetes mellitus, bei Nachkommen diabetischer Mütter deutlich erhöht (Übersichtsartikel Plagemann 2011). Dabei besteht ein direkter Zusammenhang zwischen der Art und der Stärke der intrauterinen Veränderung und den Konsequenzen für den Embryo. Je „gravierender" der mütterliche Stoffwechsel gestört ist, desto häufiger treten Veränderungen des embryonalen Metabolismus und eine erhöhte Krankheitsdisposition der Nachkommen auf (Newnham et al. 2002). Als Ursache für diesen Zusammenhang ist die Anpassung des embryonalen Stoffwechsels an die intrauterinen Entwicklungsbedingungen wahrscheinlich.

1.1. Diabetes mellitus

Seit der erstmaligen Erfassung 1960 wird jährlich eine kontinuierliche Zunahme von Diabetikern beobachtet (Deutscher Diabetes-Gesundheitsbericht 2013). 2011 waren weltweit 366 Millionen Menschen an einem Diabetes mellitus erkrankt. Die Zahl wird bis 2030 auf 552 Millionen steigen. Zudem tritt die Erstmanifestation in einem immer früheren Lebensalter auf (Diabetes Atlas Update, International Diabetes Federation 2012). Diese Entwicklung spiegelt sich auch in den steigenden Zahlen schwangerer Frauen mit einem Diabetes mellitus wieder. Diabetes mellitus ist, neben mütterlichem Übergewicht, die häufigste metabolische Schwangerschaftskomplikation. Dabei kann ein Diabetes mellitus entweder durch den absoluten Mangel an Insulin (Typ 1 Diabetes mellitus, T1DM), als Folge eines Sensitivitätsverlustes gegenüber Insulin (Typ 2 Diabetes mellitus, T2DM) oder als Glukoseinterolanz während der Schwangerschaft (Gestationsdiabetes, GDM) hervorgerufen werden. Dies führt in allen drei Fällen zu einer Erhöhung des Blutglukosewertes der Patientinnen.

1.1.1. Konsequenzen für den mütterlichen Metabolismus und Auswirkung auf den entwickelnden Embryo

Die Erhebungen der Perinatalstatistik 2012 zeigen, dass in Deutschland bei ca. 1 Prozent aller Schwangeren bereits vor der Konzeption ein Diabetes (T1DM/T2DM) bekannt war. Bei mehr als 4 Prozent der Schwangerschaften tritt ein GDM auf. Diese Aussagen entsprechen einer Verdopplung bei einem präkonzeptionellen Diabetes und einer Verdreifachung bei einem GDM innerhalb der letzten 10 Jahre. Zudem wird vermutet, dass in Deutschland rund 50% der Frauen mit einem GDM aufgrund fehlender Diagnostik übersehen werden (Deutscher Diabetes-Gesundheitsbericht 2014) (Abb. 1).



Jahrgang	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012
GDM	8.806	10.347	14.276	15.074	15.317	17.669	22.169	21.953	23.872	28.177	27.717
Präkonz. DM	3.287	3.598	4.557	4.920	4.921	5.230	5.781	5.467	5.813	6.500	6.386

Abb. 1 **Absolute Häufigkeiten des präkonzeptionellen Diabetes (T1DM und T2DM) und des Gestationsdiabetes (GDM) in Deutschland von 2002 bis 2011**

(aus Deutscher Diabetes-Gesundheitsbericht 2014)

Neben dem klassischen Leitsymptom - der Hyperglykämie - konnten bei Diabetikerinnen unter anderem Veränderungen von Wachstumshormonen (insulinartigen Wachstumsfaktoren, IGFs), bei Adipozytokinen (Adiponektin) und im Aminosäure- und Lipidmetabolismus nachgewiesen werden (Felig 1977, Imagawa et al. 2002, Bloomgarden 2002, Teppala and Shankar 2010).

Ein mütterlicher Diabetes mellitus verändert das intrauterine Milieu in der Schwangerschaft. Die Mechanismen, die durch die gestörte Versorgung mit Nährstoffen, Wachstumsfaktoren und Hormonen den Fötus beeinflussen und die damit verbundenen Folgen sind noch nicht ausreichend aufgeklärt. Bekannt ist, dass die Konsequenzen viel stärker zum Tragen kommen, je gravierender der

mütterliche Metabolismus betroffen ist, zum anderen, dass der Zeitpunkt der Störung in der Embryogenese entscheidend für das Überleben des Embryos ist. Ein präkonzeptioneller Diabetes mellitus während des 1. Trimesters führt häufig zu Wachstumsverzögerung, Fehlgeburten und angeborenen Fehlbildungen (Übersichtsartikel Walkinshaw 2001, Platt et al. 2002 Walkinshaw 2005). Tritt ein Diabetes mellitus im 2. und/oder 3. Trimester auf, sind die Kinder häufig makrosom (*large for gastational age,* LGA), können aber auch wachstumsverzögert (*small for gastational age,* SGA) sein und/oder weisen metabolische Adaptationsstörungen aufgrund einer Hyperinsulinämie auf (Übersichtsartikel Walkinshaw 2001, Evers et al. 2004, Buchanan and Xiang 2005). Epidemiologische Studien belegen zudem langfristige Konsequenzen für die Nachkommen, wie eine eingeschränkte motorische und intellektuelle Entwicklung und metabolische Veränderungen, die zu einem erhöhten Risiko für Adipositas und Diabetes mellitus führen (Churchill et al. 1969, Stehbens et al. 1977, Pettitt et al. 1983, Silverman et al. 1991, Sells et al. 1994, Dörner and Plagemann 1994, Dabelea et al. 1999, Dabelea and Pettitt 2001, Clausen et al. 2009, Crume et al. 2011, Übersichtsartikel Plagemann 2011).

Sowohl die steigenden Zahlen schwangerer Frauen mit einem Diabetes mellitus als auch die dramatischen Auswirkungen auf den Verlauf der Schwangerschaft und Nachkommen begründen die Dringlichkeit, mit der weitere Erkenntnisse über den Einfluss einer Glukosestoffwechselstörung auf die Schwangerschaft gewonnen werden müssen. Aus ethischen Gründen können experimentelle Untersuchungen der embryonal-maternalen Interaktionen und potentielle Adaptationsprozesse während der Präimplantationsphase nicht an Diabetikerinnen untersucht werden. Um die wissenschaftlichen Grundlagen zu klären, werden deshalb *in vivo*-Ansätze mit diabetischen Tiermodellen und *in vitro*-Versuche mit Embryonen durchgeführt (Vercheval et al. 1990, De Hertogh et al. 1991, De Hertogh et al. 1992, Pampfer et al. 1997, Moley 2001 Ramin et al. 2010).

1.1.2. Experimentell induzierter Diabetes mellitus Typ 1 (T1DM) beim Kaninchen

Versuchstiere mit einem Diabetes mellitus weisen deutlich geringere Embryonenzahlen, einen höheren Anteil an fehlgebildeten Embryonen und erhöhte Apoptoseraten embryonaler Zellen auf (Vercheval et al. 1990, De Hertogh et al. 1992, Ramin et al. 2010). In der vorliegenden Arbeit sollten die Zusammenhänge zwischen einer diabetischen Mutter und dem Metabolismus des entwickelnden Embryos an Blastozysten von Kaninchen mit einem experimentell induzierten Diabetes mellitus untersucht werden.

Der T1DM wurde bei weiblichen, fertilen Kaninchen durch eine einmalige Gabe der diabetogenen Chemikalie Alloxan induziert. Alloxan, welches selektiv durch den Glukosetransporter Typ 2 (GLUT2, Gen: SLC2A2) in die pankreatische Beta-Zelle aufgenommen wird, führt intrazellulär zu einer massiven Anhäufung von reaktiven Sauerstoffspezies (ROS). Da die pankreatische Beta-Zelle kaum mit protektiven Enzymen ausgestattet ist, kommt es zu einer Anhäufung von ROS mit nachfolgendem

provozierten Zelltod, einer Nekrose. Innerhalb von 48 Stunden nach der chemischen Behandlung etabliert sich ein insulinabhängiger Diabetes mellitus mit einer Hyperglykämie (15-30mmol/l) und einer Hypoinsulinämie (Ramin et al. 2010). Um eine Ketoazidose der Mutter zu vermeiden, wurde der Blutzucker der Versuchstiere täglich kontrolliert und durch eine subkutane Insulininjektion auf einen Blutzuckerwert von 14-25mmol/l eingestellt. Sechs Tage nach der Verpaarung (Tag 6 *p.c.*) der diabetischen Kaninchen wurden die Blastozysten gewonnen. Das diabetische Kaninchen wurden als Reproduktions- und embryologisches Versuchstiermodell bereits vor längerem etabliert und Versuchsbeschreibung und Ergebnisse dazu publiziert (Ramin et al. 2010, Thieme et al. 2012a, Schindler et al. 2013, Schindler et al. 2014) (Abb. 2).

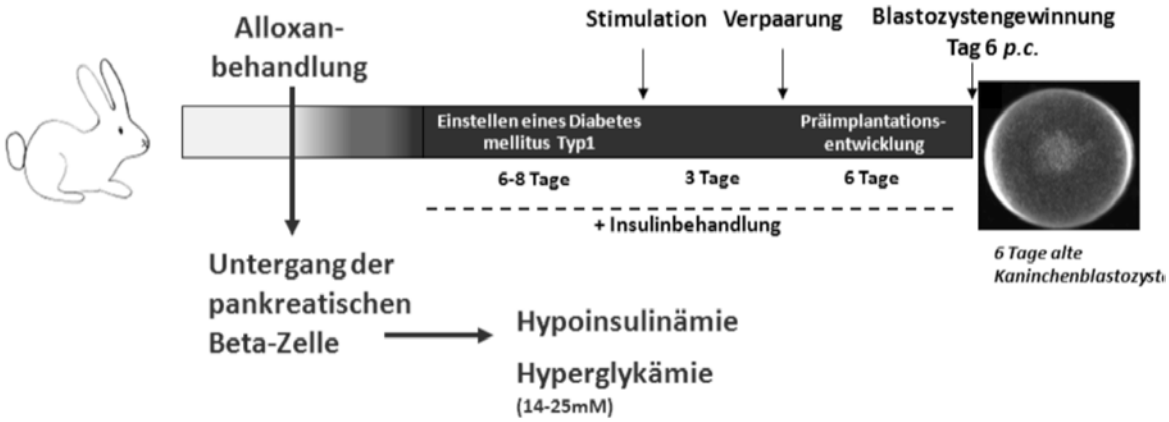


Abb. 2 **Schematische Darstellung des diabetischen Kaninchenmodels**

Der Diabetes mellitus Typ 1 wurde beim Kaninchen durch die einmalige *intravenöse* (*i.v.*) Gabe der diabetogenen Chemikalie Alloxan in die *Vena auricularis lateralis* induziert. Täglich wurde der Blutglukosespiegel zweimal kontrolliert und durch die Gabe von Insulin auf ≥14 mmol/l bis ≤25mmol/l eingestellt. Zur Steigerung der Embyronenzahl erfolgte die Stimulation mit *Pregnant Mare Serum Gonadotropin* (PMSG) und 3 Tage später die Verpaarung mit zwei fertilen Böcken unter zusätzlicher Gabe von humanen Choriongonadotropin (hCG). 6 Tage nach der Paarung wurden die Spenderkaninchen getötet und die Blastozysten mit Spülmedium (Basales Synthetisches Medium (BSM II) mit 0,1% BSA) aus dem Uterus ausgespült. (*p.c.* – *post coitum*)

1.2. Die Präimplantationsentwicklung des Kaninchens

Die Präimplantationsphase umfasst die Entwicklung des Embryos von der Konzeption bis zur Einnistung (Nidation). Das Kaninchen ist ein bekanntes embryologisches Modell für die Frühentwicklung von Säugetierembryonen (Fischer et al. 2012). Eine Besonderheit des Kaninchens besteht darin, dass die Ovulation durch die Verpaarung ausgelöst wird (induzierte Ovulation).

Durch die Kenntnis des Verpaarungszeitpunktes (*post coitum, p.c.*) ist eine exakte Bestimmung des Alters der Embryonen möglich. Die Präimplantationsentwicklung bis zur Blastozyste wurde im Kaninchen intensiv untersucht und ist im Übersichtsartikel Fischer *et al.* detailliert beschrieben

(Fischer et al. 2012). Zirka 14 Stunden *p.c.* ist das 2-Zellstadium sichtbar. Am Tag 2,5 *p.c.* entsteht die Morula, die aus 32 annähernd gleichgroßen Blastomeren besteht. Bis zum Tag 3,5 *p.c.* verdichtet sich die Morula weiter (sogenannte kompaktierte Morula) und gelangt vom Eileiter in den Uterus, wo sie sich zur frühen Blastozyste entwickelt. Durch erste Zelldifferenzierungsprozesse entsteht der Embryoblast (EB; *inner cell mass*, *ICM*), aus dem im weiteren Verlauf der Embyrogenese der eigentliche Embryo entsteht, und der Trophoblast (TB), der sich zusammen mit dem mütterlichen endometrialen Gewebe zur Plazenta entwickelt.

Ab dem 6. Entwicklungstag (d6 *p.c.*) differenzieren sich die Zellen des Embryoblasten weiter. Es entsteht die dreiblättrige Keimscheibe (Entoderm, Mesoderm, Ektoderm) mit einer charakteristischen, entwicklungsabhängigen Morphologie (Gastrulation) (siehe Abb. 3). Auf Grundlage der sich differenzierenden Keimscheibe können bis zur Implantation vier verschiedene Entwicklungsstadien (Stadien 0-4) des Embryos lichtmikroskopisch beurteilt und eingeteilt werden (Viehbahn 1995). Im Stadium 0 ist die Keimscheibe (Embryoblast) nach außen locker abgegrenzt und besteht aus morphologisch gleichen Zellen. Eine axiale Differenzierung ist erst ab Stadium 1 erkennbar. Am anterioren Ende der Keimscheibe bildet sich eine halbmondförmige Verdichtung der Zellen, der vordere Randbogen (VRB). Im Stadium 2 bildet sich am posterioren Ende die sichelförmige Gastrulationsextension (PGE). In diesem Stadium ist auch der erste mesodermale Marker, Brachyury, nachweisbar (Thieme et al. 2012b). Die Bildung des Primitivstreifens von posterior nach anterior erfolgt im Stadium 3. Das Stadium 4 ist durch die Vollendung des Primitivstreifens und der Anlage des Primitivknotens (Hensen-Knoten) am posterioren Ende charakterisiert. Die Präimplantationsentwicklung endet mit der Implantation am Tag 6,8 p.c. (6 Tage und 18 Stunden).

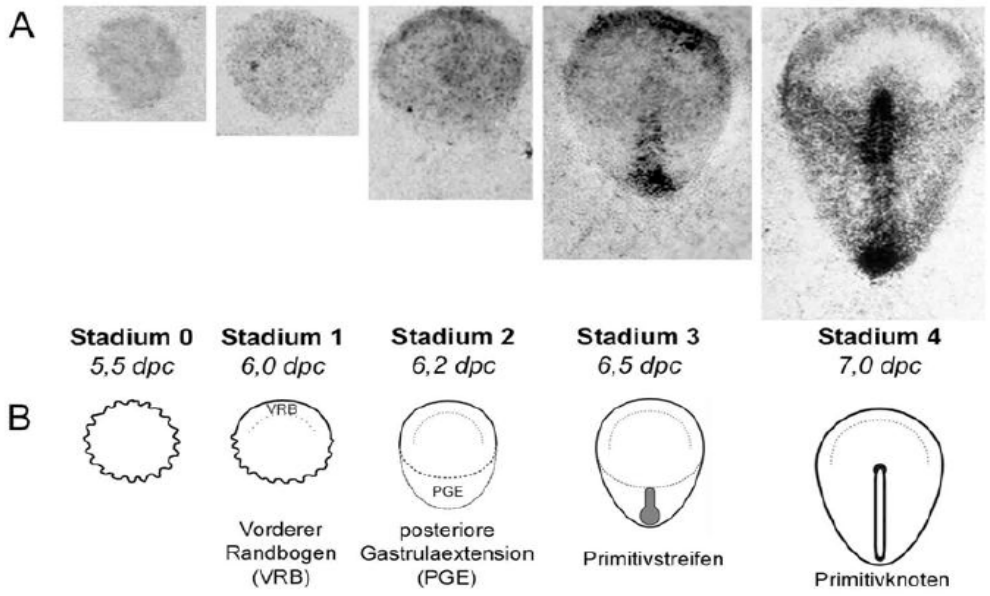


Abb. 3 **Morphologische Einteilung der frühen Keimscheibendifferenzierung des Kaninchens**

A) Dorsale Aufsichten Osmium (OsO_4)-fixierter Keimscheiben (Embryoblast) der Gastrulationsstadien 0 bis 4 und **B)** entsprechende schematische Darstellung. Stadium 0 ist durch eine locker abgrenzte Keimscheibe, Stadium 1 ist durch den Vorderen Randbogen (VRB), Stadium 2 durch die posteriore Gastrulaextension (PGE), Stadium 3 durch den Primitivstreifen und Stadium 4 durch den Primitivknoten gekennzeichnet. Unter den Stadien ist das durchschnittliche Entwicklungsalter in Tagen nach der Verpaarung angegeben (*days post coitum, dpc*). (aus Viehbahn, Mayer und Miething 1995)

Die Verwendung des Kaninchens als Versuchsmodell bietet zahlreiche Vorteile. Zum einen besitzt das Kaninchen einen höheren phylogenetischen Verwandtschaftsgrad zum Menschen als zum Nager (Graur et al. 1996, Springer and Murphy 2007). Außerdem ist es als vergleichendes Modell zur menschlichen Embryogenese gut geeignet, da es zwischen beiden Spezies Gemeinsamkeiten in der Morphologie der Keimscheibe während der Gastrulation und der Art der Plazentation gibt (Übersichtsartikel Fischer et al. 2012).

Zudem bietet die Verwendung von Kaninchenembryonen neben der exakten Bestimmung des Embryonalalters weitere experimentelle Vorteile. Durch die vergleichsweise späte Implantation (Tag 6 und 18 Stunden p.c.) (Denker, 1977) und die Größe der Blastozysten (ca. 5mm am Tag 6,5 p.c.), sind die Embryonen leicht zu gewinnen. Die hohe Zellzahl ermöglicht die Analyse individueller Blastozysten und der isolierten Zelllinien Embryoblast (EB) und Trophoblast (TB) sowie der Blastozystenhöhlenflüssigkeit.

1.3. Der embryonale Metabolismus

Seit Beginn der in vitro-Embryokultur wird deutlich, dass der Metabolismus einen weitaus größeren Einfluss auf die Entwicklung und Vitalität des heranwachsenden Embryons ausübt, als vorher angenommen. Im Jahre 2002 wurde von H. Leese die „*Quiet Embryo Hypothesis*“ aufgestellt (Leese 2002). Metabolisch „inaktive“ Embryonen, so Leese, seien vitaler, da Genom, Transkriptom und Proteom nicht überflüssig beansprucht werden. Somit werden von diesen Embryonen weniger Nährstoffe und Sauerstoff verbraucht. Hingegen weisen Embryonen mit einer schlechten Qualität einen hohen Nährstoff- und Sauerstoffumsatz auf. Diese metabolisch „hyperaktiven“ Embryonen entstehen vor allem durch exogene Stressoren, wie mütterliche Erkrankungen und Stress oder suboptimale *in vitro*-Kulturbedingungen (Leese et al. 2007, Leese et al. 2008). Belege, dass der embryonale Metabolismus *in vitro* sich deutlich vom *in vivo*-Nährstoffumsatz unterscheidet, gibt es vielfältige (Fischer 1987, Übersichtsartikel Barnett and Bavister 1996, Leese et al. 2007, Leese et al. 2008). Die langfristigen Konsequenzen für die Nachkommen werden momentan intensiv diskutiert. Beim Menschen wird beobachtet, dass Kinder, die durch IVF oder ICSI (assistierten Reproduktionsmedizin (ART)) gezeugt wurden, ein höheres Frühgeburtsrisiko, ein geringeres Geburtsgewicht, einen höheren Blutdruck, höhere Gewichtszunahme sowie höhere Nüchternglukose- und Triglyceridwerte aufweisen (Helmerhorst et al. 2004, Jackson et al. 2004, Ceelen et al. 2007, Ceelen et al. 2008, Ceelen et al. 2009, McDonald et al. 2009, Dumoulin et al. 2010, van Montfoort et al. 2012). Diese Beobachtungen lassen vermuten, dass die Anpassung des embryonalen Metabolismus an die *in vitro*-Kulturbedingungen langfristige Effekte haben kann. Die Vermittler dieses Adaptationsprozesses sind bisher jedoch unbekannt.

Während der frühen Embryonalentwicklung wird der Embryo durch die Mutter mit Nährstoffen (Glukose, Aminosäuren und Lipide) und Wachstumsfaktoren versorgt. Grundlegende Erkenntnisse zum Metabolismus in Präimplantationsembryonen konnten bereits in den 1960er und frühen 1970er gewonnen werden (Fridhandler 1961, Suguwara and Umezu 1961, Mills and Brinster 1967, Biggers and Stern 1973) und bildeten die Grundlage für die Entwicklung von Kulturmedien in der assistierten Reproduktionsmedizin (ART). Der Präimplantationsembryo ist von der Versorgung mit Nährstoffen von außen abhängig. Bis zur Kompaktierung der Morulae werden hauptsächlich Laktat und Pyruvat als Substrate verbraucht (Fridhandler 1961, Brinster 1965, Brinster 1967, Brinster 1968). Mit der Blastozystenentwicklung wird der embryonale Stoffwechsel auf Glukose umgestellt (Mensch (Wales et al. 1987, Dan-Goor et al. 1997), Rind (Thompson et al. 1996, Khurana and Niemann 2000), Schwein (Flood and Wiebold 1988), Kaninchen (Pike 1981), Maus (Leese and Barton 1984, Gardner and Leese 1988), Ratte (Dufrasnes et al. 1993)). Diese Umstellung ist an die Aktivierung des Schrittmacherenzyms der Glykolyse, die Phosphofruktokinase, gebunden (Barbehenn et al. 1974, Barbehenn et al. 1978) und ist durch einen erhöhten Bedarf an Metaboliten für verschiedene

endogene Biosynthesen bedingt. Der Wechsel von einer Pyruvat-/Laktatverwertung zu Glukose wird durch die Umstellung des aeroben auf einen anaeroben Stoffwechsel begleitet und passt den Embryo an die sauerstoffarme Umgebung vor und während der Implantation an (Fischer and Bavister 1993, Johnson et al. 2003).

Während die Bedeutung von Glukose, Pyruvat und Laktat für die Präimplantationsphase seit mehr als 60 Jahren erforscht wird (Hammond 1949, Whitten 1956, Whitten 1957), entdecken erst neuere Arbeiten die Bedeutung anderer Metabolite. Vor allem Lipide rücken verstärkt in den Fokus (Sturmey et al. 2006, Sturmey et al. 2009a, Sturmey et al. 2009b, Dunning et al. 2010, Jungheim et al. 2011, Van Hoeck et al. 2011). Präimplantationsembryonen werden über das uterine Sekret mit Lipiden versorgt und sind in der Lage, diese aufzunehmen und zu metabolisieren. Der Embryo selbst ist in der Lage, Fettsäuren und komplexere Lipide zu synthetisieren und zu speichern (Übersichtsartikel (Tsujii et al. 2001)). Eine erhöhte intrazelluläre Speicherung von Lipiden scheint jedoch negative Auswirkungen auf die Präimplantationsentwicklung zu haben. Ein hoher Gehalt an Lipidvesikeln ist mit einer geringeren Entwicklungskompentenz, schlechteren Embryoqualität und Kryokonservierbarkeit beim Rind und Schwein assoziiert (Dobrinsky et al. 1999, Nagano et al. 2006, Jeong et al. 2009). In einem kürzlich erschienenen Übersichtsartikel von Krisher und Prather wird postuliert, dass vitale Embryonen Glukose nur zu einem geringen Anteil für die Energiegewinnung verwenden und die Deckung des Energiebedarfs über Fettsäuren abgesichert wird. Die aufgenommene Glukose hingegen wird als Grundgerüst für die endogene Biosynthese von Makromolekülen, wie DNA und RNA, verwendet (Krisher and Prather 2012). Dies würde die Bedeutung von Fettsäuren für die Präimplantationsentwicklung unterstreichen. Diese Hypothese wird durch einen Befund aus den 90iger Jahren in Kaninchenembryonen unterstützt, bei dem gezeigt wurde, dass Glukose hauptsächlich für den Aufbau von Makromolekülen, wie Lipiden, Glukogen, Proteinen und Nukleinsäuren anstatt für die Energiegewinnung verwendet wird (Robinson and Benos 1991).

Inwieweit Kohlenhydrate, Lipide oder Proteine zur Engergiebereitstellung verwendet werden unterliegt einer strengen Kontrolle, an der unter anderem Hormone, Wachstumsfaktoren und Adipokine beteiligt sind. Das Hormon Insulin, das zum Insulin-IGF-Rezeptor-System (IIRS) gehört, ist Teil der Stoffwechselhomöostase.

1.4. Das Insulin-IGF-Rezeptor-System (IIRS)

Als Vermittler der embryo-maternalen Kommunikation dienen unter anderem Hormone und Wachstumsfaktoren. Diese werden zum einen von der Mutter bereitgestellt, können aber auch vom Embryo selbst produziert werden. Auf diese Weise beeinflussen sich der mütterliche Organismus und der heranwachsende Embryo gegenseitig und ermöglichen dadurch eine optimale Anpassung der Entwicklung des Embryos und sind damit essentiell für die Aufrechterhaltung der Schwangerschaft. Teil dieses komplexen Netzwerkes ist das IIRS.

Zu den Komponenten des IIRS gehören die Liganden Insulin, die Insulinähnlichen Wachstumsfaktoren (*Insulin-like growth factors,* IGF) 1 und 2, sowie deren Rezeptoren Insulin-Rezeptor (IR), IGF1-Rezeptor (IGF1R) und IGF2-Rezeptor (IGF2R). Aufgrund der strukturellen Homologie in der Peptidstruktur binden die Liganden nicht nur ihren jeweils zugeordneten Rezeptor, sondern auch an die anderen Rezeptoren des IIRS, jedoch meist mit einer geringeren Affinität (Rotwein 1991, Van den Brande 1999, Chao and D'Amore 2008) (siehe Abb. 4). Durch die unterschiedlichen Liganden-Rezeptor-Bindungseffizienzen, aber auch durch die Fähigkeit, unterschiedliche intrazelluläre Signalmoleküle zu aktivieren, können Insulin und die IGFs in physiologischen Konzentrationen diverse Zellfunktionen steuern (Chao and D'Amore 2008). Der IR und der IGF1R weisen hohe strukturelle Ähnlichkeiten auf (Ullrich et al. 1986). Beide Rezeptoren bestehen aus jeweils zwei alpha- und zwei beta-Untereinheiten. Die alpha-Untereinheit enthält die Bindungsdomäne für die Liganden und die beta-Untereinheit, die in der Membran verankert ist, die Tyrosinkinasedomäne, die nach Ligandenbindung aktiviert wird und zur Autophosphorylierung der Insulin-Rezeptor-Substrate (IRS) führt. Der IR existiert in zwei verschiedenen Isoformen, IR-A und IR-B. Beide Isoformen besitzen eine unterschiedliche Bindungsaffinität zu den Liganden (Abb. 4). So kann der IR-A IGF2 mit der gleichen hohen Affinität wie Insulin binden (Frasca et al. 1999, Sciacca et al. 2003, Lawrence et al. 2007). Der IGF2R weist dagegen keine Tetramere-Struktur auf, sondern ähnelt im Aufbau den Mannose-6-Phosphat-Rezeptoren. Diese einkettigen integralen Membranproteine besitzen einen langen extrazellulären und einen kurzen intrazellulären Anteil (Ghosh 2003), der den lysosomalen Abbau der gebunden Liganden einleitet (Oka et al. 1985, Kiess et al. 1987, Nolan and Lawlor 1999).

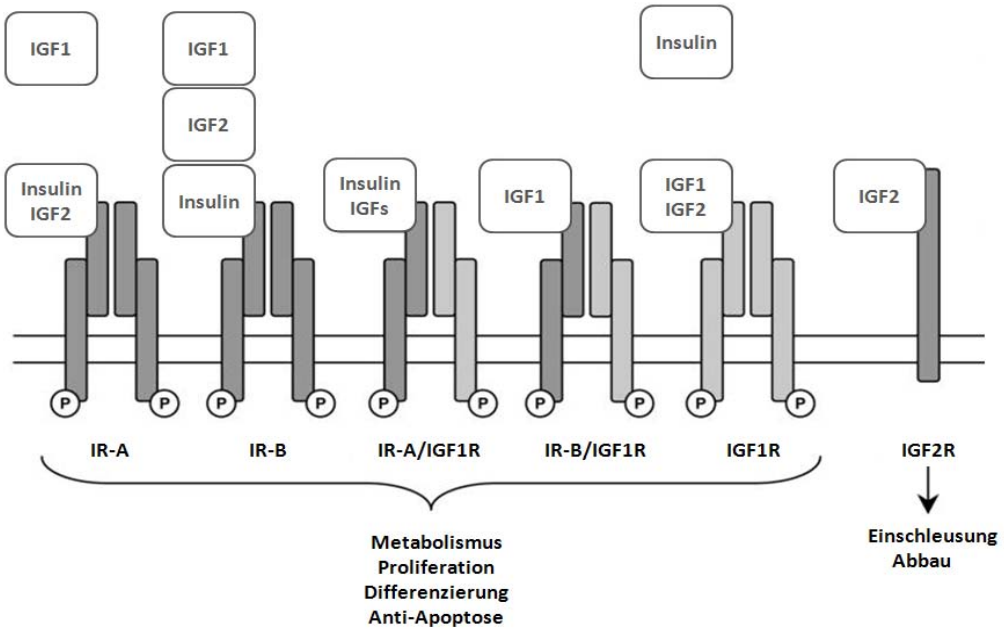


Abb. 4 **Schematische Darstellung der Liganden und Rezeptoren des Insulin-IGF-Rezeptor-Systems (IIRS)**

Die Liganden wurden unter Berücksichtigung ihrer Affinität den Rezeptoren zugeordnet. Der Insulinrezeptor (IR) existiert in 2 verschiedenen Isoformen: IR-A und IR-B. Der IR und *Insulin-like growth factor 1 receptor* (IGF1R) können Hybridrezeptoren (IR/IGF1R) bilden. Diese Rezeptoren entstehen durch das Zusammenlagern einer α-β-Domäne des IR und einer zweiten des IGF1R. Nach Ligandenbindung werden über den IR und IGF1R der zelluläre Metabolismus, Differenzierung und proliferative und antiapoptotische Prozesse reguliert. Der IGF2R bindet nur IGF2, das anschließend internalisiert und abgebaut wird.
(modifiziert nach Chao 2008)

Bedeutung des IIRS für die Präimplantationsentwicklung

Das IIRS spielt während der Präimplantationsentwicklung als Mediator im embryo-maternalen Dialog eine zentrale Rolle. Der zelluläre Metabolismus und zellproliferative Prozesse werden während dieser sehr frühen Phase der embryonalen Entwicklung über das IIRS koordiniert. Der Embryo selbst kann IGF1 und IGF2 als auto- und parakrin wirkende Faktoren produzieren, nicht jedoch Insulin (Maus (Doherty et al. 1994, Pantaleon and Kaye 1996), Rind (Watson et al. 1992), Schaf (Watson et al. 1994). Die weitere Versorgung wird aus dem mütterlichen Blutkreislauf über das Uterussekret bewerkstelligt (endokrin). Zusätzlich können auch die mütterlichen reproduktiven Organe, Uterus und Eileiter, über die lokale Produktion der IGFs die Präimplantationsentwicklung beeinflussen (parakrin) (Maus (Harvey and Kaye, 1990), (Zakaria, 2007), Rind (Watson et al., 1992), (Perks et al., 1995) (Wathes et al., 1995) und Mensch (Giudice, 1993). Eine Regulation erfolgt somit über lokale und zentrale Mechanismen. Insulin hingegen kann nicht selbst vom Präimplantationsembryo produziert werden (Kaye, 1997), (Lighten et al., 1997). Somit muss eine Versorgung über das uterine Sekret der Mutter erfolgen, um eine normale Entwicklung zu gewährleisten. Die Tyrosinkinase-

Rezeptoren des IIRS werden sowohl vom Präimplantationsembryo als auch im Uterus und Eileiter der Mutter exprimiert (Mensch (Lighten et al. 1998), Maus (Rappolee et al. 1992, Heyner et al. 1989), Kaninchen (Navarrete Santos et al. 2008, Ramin et al. 2010, Thieme et al. 2012a). In Kaninchenblastozysten weisen die Rezeptoren ein spezifisches Verteilungsmuster auf. Während der Embryoblast vor allem den IGF1R und IR-A exprimiert, werden vom Trophoblasten beide IR-Isoformen synthetisiert (Navarrete Santos et al. 2008). Somit werden über die IGFs proliferative und mitogene Signale im Embryoblasten forciert. Im Trophoblasten hingegen können die IGFs in physiologischen Konzentrationsbereichen aufgrund der geringen IGF1R-Dichte kaum wirken. Insulin steuert sowohl im Embryoblasten und auch im Trophoblasten proliferative und metabolische Prozesse.

Verschiedene transgene Tiermodelle beweisen die essentielle Bedeutung der IGFs in der Embryogenese und Schwangerschaft. Der Mangel an IGF1-Protein führt im Mausmodell zu einer fetalen Wachstumsretardierung und einer hohen perinatalen Letalität (Liu et al. 1993, Baker et al. 1993, Powell-Braxton et al. 1993). Zudem weisen IGF1-defiziente Mäuse verschiedene Störungen in der Organogenese auf und sind infertil (Baker et al. 1993, Camacho-Hübner and Savage 2001). IGF2-null-Mutanten sind ebenfalls wachstumsretardiert, entwickeln sich nach der Geburt jedoch normal (DeChiara et al. 1990, DeChiara et al. 1991). Im Vergleich zu IGF1 ist IGF2 somit nicht essentiell für die embryonale und fetale Entwicklung. Die Bedeutung von IGF1 für die humane Präimplantationsentwicklung bleibt umstritten, da im Vergleich zum Kaninchen und Maus eine IGF1-Produktion im humanen Embryo bis zum Blastozystenstadium nicht nachgewiesen werden konnte (Lighten et al. 1997).

Die Gabe von Insulin und IGFs fördert die mesodermale Zellproliferation und die kardiogene Differenzierung (Thieme et al. 2012b, Engels et al. 2014), essentielle Prozesse der frühen Embryogenese. Zudem reguliert das IIRs die Aufnahme des zentralen Metaboliten Glukose (Übersichtsartikel (Barnett and Bavister 1996)). In Mausblastozysten wird die Glukoseaufnahme durch Insulin und IGF1 forciert (Pantaleon and Kaye 1996, Riley et al. 2005). In Kaninchenblastozysten führen weder Insulin noch IGF1 zu einer Erhöhung der Glukoseaufnahme (d4 *p.c.*) bzw. zu einer Translokation des Glukosetransporter Typ 4 (d6 *p.c.*) (GLUT4, Gen: SLC2A4) und somit zu einer insulinabhängigen Steigerung der Glukoseaufnahme (Navarrete Santos et al. 2004). Neben Insulin ist auch das Hormon Adiponektin in der Lage, die Glukosehömeostase zu regulieren (Fischer et al. 2010). Somit ist Adiponektin ein weiterer potentieller Kandidat für eine direkte und indirekte Wirkung in der embryo-maternalen Interaktion.

1.5. Adiponektin-Rezeptor-System (ARS)

Adiponektin, das auch als AdipoQ und Acrp30 bekannt ist, wird seit der ersten Beschreibung Mitte der 1990er Jahren intensiv untersucht (Scherer et al. 1995, Hu et al. 1996, Maeda et al. 1996, Nakano et al. 1996). Es gehört zu der Gruppe der Adipozytokine (Adipokine), die hauptsächlich von Adipozyten produziert werden. Im Vergleich zu den anderen Adipozytokinen ist die Serumkonzentration von Adiponektin mit dem Körpergewicht invers korreliert (Hu et al. 1996, Arita et al. 1999) und in einer 1000-fach höheren Konzentration als die anderen Adipozytokine im Blut zu finden (Stefan et al. 2003). Der Hauptteil des im Serum vorkommenden Adiponektins stammt aus dem Fettgewebe. Auch in der Leber (Kaser et al. 2005), in Muskelzellen (Staiger et al. 2003, Delaigle et al. 2004), Osteoblasten (Berner et al. 2004) und im Präimplantationsembryo (Schmidt et al. 2008, Cikos et al. 2010) konnte Adiponektin mRNA und Protein nachgewiesen werden. Für die Produktion und Sekretion von Adiponektin aus dem viszeralen Fettgewebe nimmt Insulin eine zentrale Stellung ein (Bogan and Lodish 1999, Bacha et al. 2004).

Adiponektin ist wie Insulin in der Lage, den Zellmetabolismus zu regulieren. Es verfügt über ein eigenes Rezeptor- und Signalsystem. Adiponektin entfaltet seine metabolische Wirkung über zwei Rezeptoren: AdipoR1 und AdipoR2 (Yamauchi et al. 2003). Während der AdipoR1 ubiquitär vorkommt, findet man den AdipoR2 vor allem in der Leber (Tsuchida et al. 2004). Des Weiteren bindet Adiponektin den T-Cadherin-Rezeptor (Hug et al. 2004). Die Aktivierung ist für die metabolische Regulation durch Adiponektin nicht relevant, kann aber die Angiogenese beeinflussen(Hug et al. 2004, Whitehead et al. 2006, Parker-Duffen et al. 2013, Parker-Duffen and Walsh 2014).

Beide AdipoRs sind aus sieben Transmembrandomänen aufgebaut und aktivieren nach Ligandenbindung über das *Adaptor Protein Containg Pleckstrin Homology Domain* 1 (APPL1) und AMP-aktivierte Proteinkinase (AMPK) eine Vielzahl an Schlüsselenzymen unterschiedlicher Stoffwechselprozesse. Auf diesem Wege können die Glukoseaufnahme, Glukoneogenese, die Verstoffwechselung von Fettsäuren, die Triglycerid- und Cholesterinsynthese gesteuert werden (Fruebis et al. 2001, Mao et al. 2006, Guerre-Millo 2008, Zhao et al. 2009, Übersichtsartikel Yamauchi et al. 2014). Über die Aktivierung des Peroxisomen-Proliferator-aktivierter Rezeptors alpha (PPARα) sowie einer erhöhten Transkription von Carnitin-Palmitoyltransferase 1 B (CPT1B) und der damit verbundenen gesteigerten Verstoffwechselung von Fettsäuren, kommt Adiponektin eine seiner metabolischen Funktionen nach: der Regulation der Insulinsensitivität (Yamauchi et al. 2003, Combs et al. 2004). In Abbildung 5 sind die Signalwege und Funktion von Adiponektin zusammengefasst.

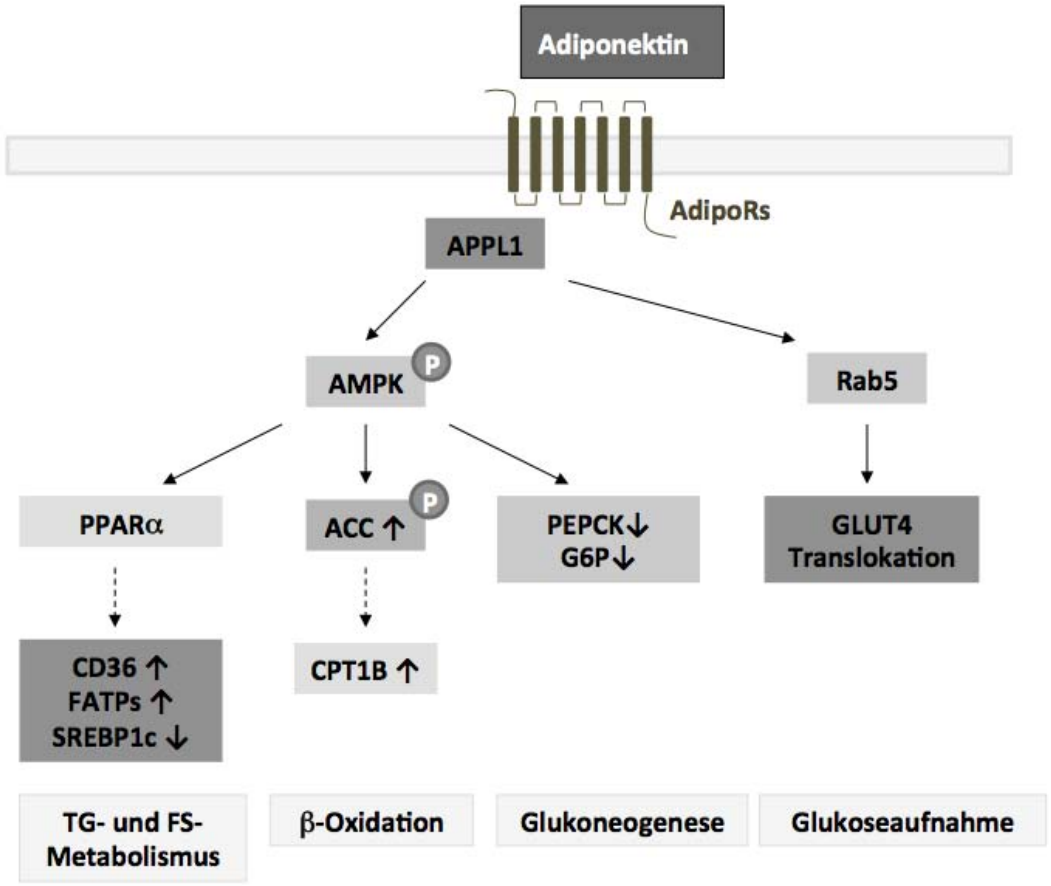


Abb. 5 **Schematische Darstellung des metabolischen Adiponektin-Rezeptorsignalweges**

Nach Bindung von Adiponektin an dessen Rezeptor (AdipoRs; Adiponektin Rezeptor 1 (AdipoR1) und Adiponektin Rezeptor 2 (AdipoR2)) interagiert dieser mit dem A*daptor Protein Containg Pleckstrin Homology Domain* (APPL1). Nach Phosphorylierung der AMP-aktivierter Proteinkinase (AMPK) kann zum einen die Glukoneogenese über die Regulation der Phosphoenolpyruvatcarboxykinase (PEPCK) und der Glukose-6-Phosphatase (G6P) gesteuert werden. Zum anderen wird der Lipidmetabolismus reguliert über die Phosphorylierung der Acetyl-CoA Carboxylase (ACC) und anschließenden transkriptionellen Regulation der Carnitin-Palmitoyltransferase 1 (CPT1B) sowie der Aktivierung des Transkriptionsfaktors Peroxisom-Proliferator-aktivierter Rezeptor α (PPARα). PPARα kontrolliert die Promotoraktivität vieler Gene des Fettsäuremetabolismus. Dazu zählen *Cluster of Differentiation* 36 (CD36), *sterol regulatory element-binding protein* 1 (SREBP1) und *fatty acid transporter proteins* (FATPs). Die intrazelluläre Glukoseaufnahme wird AMPK-unabhängig über *RAS-related protein 5 (Rab5)* und die Translokation des Glukosetransporters 4 (GLUT4) gesteuert.

Bedeutung des Adiponektin-Rezeptor-Systems (ARS) für die Präimplantationsentwicklung

Im Menschen ist über die Funktion von Adiponektin während der frühen Phase der Schwangerschaft bisher wenig bekannt. In verschiedenen Säugetierspezies sind Adiponektin und seine Rezeptoren AdipoR1 und AdipoR2 in Präimplantationsembryonen und reproduktiven Organen nachgewiesen worden (Maus (Schmidt et al. 2008, Cikos et al. 2010, Kim et al. 2011), Schwein (Chappaz et al. 2008), Rind (Maillard et al. 2010), Kaninchen (Schmidt et al. 2008, Fischer et al. 2010), Übersichtsartikel Palin et al. 2012). Die Rezeptoren werden hauptsächlich im Embryoblasten und zu einem geringen

Anteil im Trophoblasten der Blastozysten exprimiert (Schmidt et al. 2008, Kim et al. 2011, Fischer et al. 2010). Während die Rezeptoren schon ab dem Oozytenstadium nachgewiesen werden konnten, erfolgt die Synthese von Adiponektin selbst in Maus- und Kaninchenembryonen erst ab dem Blastozystenstadium (Schmidt et al. 2008, Cikos et al. 2010). Die Komponenten des Adiponektin-Signalweges, wie AMPK, PPARα und *fatty acid transport protein* (FATP) 1 und ein funktioneller Adiponektin-Signalweg sind im Embryo vorhanden (Fischer et al. 2010, Kim et al. 2011). Die *in vitro*-Stimulation mit physiologischen Adiponektinkonzentrationen steigert die Phosphorylierung der AMPK und Akt, was durch die Translokation von GLUT4 in die Membran zu einer erhöhten Glukoseaufnahme in die Zelle und einer Hemmung der PEPCK führt (Fischer et al. 2010). Adiponektin ist in der Lage, in Präimplantationsembryonen klassische metabolische Funktionen des Insulins zu übernehmen. Mütterliche Erkrankungen, die das embryonale Adiponektin-Rezeptor-System beeinflussen, können über die Adiponektinregulation einen gravierenden Einfluss auf den Metabolismus und die weitere Entwicklung des Embryos haben. Mütterliche Adipositas oder Erkrankungen wie Diabetes mellitus Typ 1 und 2 sowie das polyzystisches Ovarsydrom (PCOS) sind sowohl mit einem veränderten Adiponektinspiegel als auch mit Subfertilität und einer erhöhten embryonalen Letalität assoziiert (Imagawa et al. 2002, Groth 2010). Im Mausmodell konnten Ma und Koautoren keinen Zusammenhang zwischen dem Mangel an funktionellem Adiponektin und der Fertilität zeigen (Ma et al. 2002).

1.6. Die CREB/ATF-Familie

Extrazelluläre Signale können über Signalkaskaden ausgehend von der Rezeption bis zum Transkriptionsfaktor die Expression von Genen regulieren und kontrollieren. Der Transkriptionsfaktor *cyclic AMP (cAMP) responsive element binding protein* (CREB) fungiert als zentraler Vermittler zellulärer Prozesse. Hormone, Wachstumsfaktoren, Neurotransmitter, aber auch Metabolite wie Glukose führen zu einer Aktivierung von CREB und somit zur transkriptionellen Regulation von Zielgenen. Bislang sind mehr als 300 Stimuli bekannt, die über verschiedene Kinasen CREB am Serin-133 phosphorylieren und somit aktivieren (Gonzalez and Montminy 1989, Mayr and Montminy 2001). Diese transiente CREB-Phosphorylierung erreicht nach ca. 10-15min ein Maximum und sinkt dann kontinuierlich wieder ab (Wu et al. 2001, Jin and O'Neill 2010). Das phosphorylierte CREB bindet als Dimer an seine spezifische DNA-Erkennungssequenz, das *cAMP responsive elment* (CRE), in regulatorischen Regionen des Zielgenes. Dabei erfolgt die Bindung von CREB an CRE-Sequenzen entweder als Homodimer oder als Heterodimer zusammen mit *activating transcription factor 1* (ATF1) oder *CREB modulatory protein* (CREM) (Hai et al. 1989, Habener et al. 1990, Foulkes et al. 1991, Meyer and Habener 1992). Die Bindung als Heterodimer führt immer zu einer geringeren transkriptionellen Aktivität (Loriaux et al. 1994). Sowohl ATF1 als auch CREM gehören, wie CREB,

innerhalb der Leucinzipperproteine zur hochkonservierten CREB/ATF-Unterfamilie und besitzen sehr hohe strukturelle Ähnlichkeiten zueinander (Hai et al. 1989, Ruppert et al. 1992, Meyer and Habener 1993, Delmas et al. 1993) (siehe Abb. 6).

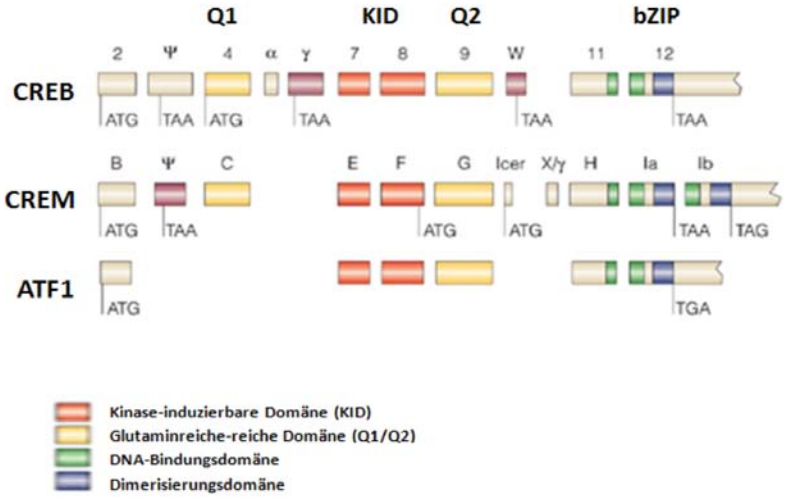


Abb. 6 **Schematische Darstellung der Genstruktur der CREB/ATF-Familie**

Die humanen *cyclic AMP (cAMP) resposive element binding protein* (CREB), *activating transcription factor 1* (ATF1) oder *CREB modulatory protein* (CREM) besitzen hohe strukturelle Ähnlichkeiten in ihrem genomischen Aufbau. Übereinstimmende Bereiche in der Glutaminreichen (Q1/2)-, Kinase-induzierbare (KID)- und Leucin-Zipper (bZIP)-Domäne sind unterschiedlich farbig dargestellt.
(modifiziert nach Mayr and Monteminy 2001)

Die Phosphorylierung von CREB ist nicht ausreichend, um die Transkription der Zielgene zu modulieren (Zhang et al. 2005). Zusätzlich muss eine Translokation in den Zellkern und eine weitere Interaktion mit den Koaktivatoren CREB-*binding protein* (CBP) und p300 erfolgen (Chrivia et al. 1993, Mayr and Montminy 2001). Umfassende Analysen zeigen, dass mehr als 1300 murine und 1600 menschliche Gene eine CRE-Sequenz aufweisen (Conkright et al. 2003, Zhang et al. 2005). Der große Umfang und die funktionelle Vielfalt an potentiellen CREB-Zielgenen zeigt die zentrale Stellung von CREB in der Regulation zellulärer Prozesse, wie zum Beispiel Zellproliferation und Zelldifferenzierung. Der Großteil der CRE-Sequenz-enthaltenden Gene kontrolliert jedoch den zellulären Metabolismus. Dazu gehören zum Beispiel das *fatty acid binding protein* (FABP) 4, die Fettsäure-Synthase (FAS), Phosphoenolpyruvat-Carboxykinase (PEPCK), Hexokinase 2, *Uncoupling protein* (UCP) 1 und 2 (Dooley et al. 1999, Reusch et al. 2000, Zhang et al. 2005, Übersichtsartikel: Altarejos and Montminy 2011, Datenbank: http://natural.salk.deu/CREB). CREB erhöht die Verfügbarkeit von Glukose und Triglyceriden und weiterer Metabolite. Weiterhin reguliert CREB aber auch die Expression von Transkriptionsfaktoren wie Peroxisom-Proliferator-aktivierte Rezeptoren gamma (PPARγ), *activating*

transcription factor 3 (ATF3) und c-Fos, sowie Wachstumsfaktoren und Adipokine, wie Insulin, Leptin und Adiponektin. CREB kann sowohl zu einer Forcierung als auch zu einer Inhibierung der Zielgentranskription führen. Die Transkriptionshemmung wird unter anderem über eine Dimerisierung mit anderen Familienmitgliedern, wie der Repressor-Isoform von CREM (CREM-ICER), ATF3 oder ATF4 realisiert (Hai and Hartman 2001, Johannessen et al. 2004).

Neben CREB und ATF1 gehören auch ATF2 bis ATF6 zur CREB/ATF-Familie. Im Gegensatz zu CREB oder ATF1 besitzen die weiteren ATFs eine andere strukturelle Morphologie (Hai and Hartman 2001). Ihre funktionellen Eigenschaften sind im Vergleich zu CREB noch weitgehend unbekannt (Persengiev and Green 2003). ATF2, ATF3 und ATF6 scheinen eine Rolle in der zellulären Stressantwort zu übernehmen (Hai et al. 1999, Hai and Hartman 2001, Bhoumik et al. 2002). ATF4 ist vor allem als Inhibitor der CREB-abhängigen Transkription bekannt (Hai and Hartman 2001). Zudem reguliert ATF4 den Glukosemetabolismus in Mäusen durch die Hemmung der Insulinsekretion und Regulation der Insulinsensitivität in Leber-, Muskel- und Fettgewebe (Yoshizawa et al. 2009, Sundaram et al. 2003). ATF5, auch bezeichnet als ATF-x oder ATF7, wird besonders hoch in der adulten Leber exprimiert (Shimizu et al. 2009).

Bedeutung der CREB/ATF-Familie während der Embryonalentwicklung

In Mausembryonen konnte die Expression von CREB und ATF1 mittels RT-PCR und Immunhistochemie nachgewiesen werden (Bleckmann et al. 2002, Jin and O'Neill 2007, Jin and O'Neill 2014), wobei es Unterschiede in der Verteilung gibt. Während ATF1 in der gesamten Blastozyste exprimiert wird, kann ein funktionelles CREB-Protein nur in der *Inner Cell Mass* (ICM; Embryoblasten) detektiert werden (Bleckmann et al. 2002). Eine Expression von CREM konnte während der murinen Präimplantationsentwicklung nicht nachgewiesen werden (Bleckmann et al. 2002). Beide Transkriptionsfaktoren, CREB und ATF1, regulieren während der Embryonalentwicklung vor allem proliferative Prozesse (Bleckmann et al. 2002, Jin and O'Neill 2007, Jin and O'Neill 2014). Sowohl CREB als auch ATF1 können das Fehlen des anderen während der frühen Embryonalentwicklung kompensieren (Hummler et al. 1994, Bleckmann et al. 2002). Ein Doppel-knock out von CREB und ATF1 führt zur embryonalen Letalität noch vor der Implantation (Bleckmann et al. 2002). Das unterstreicht die zentrale Bedeutung von CREB und ATF1 für die Präimplantationsentwicklung. ATF1 besitzt jedoch eine geringere transkriptionelle Aktivität als CREB, da ihm die Glutaminreiche Domäne und somit eine Transaktivierungsdomäne fehlt (Rehfuss et al. 1991) (Abb. 6). Das zeigt sich darin, dass der Phänotyp der $CREB^{-/-}$-Maus ausgeprägter ist, als bei der $ATF1^{-/-}$-Maus. Mäuse, die kein funktionelles CREB-Protein produzieren können, weisen ein deutlich vermindertes Geburtsgewicht auf und versterben kurz nach der Geburt an einer Atelektase (Bleckmann et al. 2002). ATF1-null-Mutanten sind dagegen phänotypisch unauffällig (Bleckmann et

al. 2002). In *Xenopus leavis* wurde mit Hilfe der Injektionen von dominant-negativem CREB die Bedeutung von CREB für die Gastrulation und Bildung des Neuralrohres bewiesen (Lutz et al. 1999, Sundaram et al. 2003).

Die Bedeutung der anderen CREB/ATF-Familienmitglieder in der frühen Embryogenese ist noch weitgehend unbekannt. Einen möglichen Einfluss der ATFs auf die pränatale Entwicklung lässt sich bisher nur anhand von *knockout*-Tiermodellen ableiten. Durch ATF2-transgene Mäusen ist belegt, dass ATF2 eine wesentliche Rolle für die Organogenese und postnatale Überlebensfähigkeit spielt. ATF2-defiziente Mäuse sterben kurz nach der Geburt und weisen komplexe Entwicklungsstörungen auf, die vor allem das Nervensystem, den Atemtrakt und die chondrale Ossifikation betreffen (Reimold et al. 1996, Maekawa et al. 1999, Breitwieser et al. 2007). ATF3 ist nicht essentiell für die embryonale und fetale Entwicklung, da $ATF3^{-/-}$-Mäuse keinen auffälligen Phänotyp aufweisen (Hartman et al. 2004). ATF4-null-Mutanten sind dünn und wachstumsretardiert (Wang et al. 2009). ATF4 reguliert den embryonalen und fetalen Lipidmetabolismus, indem es die Lipolyse steigert und Lipogenese reduziert (Seo et al. 2009, Wang et al. 2010). ATF5 wird nach der Implantation vom Embryo exprimiert und übernimmt eine Rolle bei der osteogenen Differenzierung mesenchymaler Stammzellen (MSC) (Leong et al. 2012) und Neurogenese (Angelastro et al. 2003, Angelastro et al. 2005, Mason et al. 2005).

Zusammenfassend lässt sich einschätzen, dass ATFs nicht essentiell für die Präimplantationsphase sind, aber eine regulatorische Rolle nicht ausgeschlossen werden kann und daher weitere Untersuchungen erfordert.

2. Zielstellung der Arbeit

Ein mütterlicher Diabetes mellitus verändert das intrauterine Milieu und damit die Entwicklungsbedingungen des Embryos. Diese Veränderungen führen im Rahmen einer Adaptation des Embryos zu einer Umstellung im embryonalen Metabolismus.

Aus vorangegangen Studien war die zentrale Bedeutung des *Insulin-IGF-Rezeptor-System (IIRS)* für die Präimplantationsphase im Kaninchen bekannt (Navarrete Santos 2004, 2008). Deshalb war das erste Ziel der vorliegenden Arbeit, den Einfluss eines maternalen Diabetes mellitus auf das IIRS weiter zu charakterisieren. Für die Untersuchungen wurden als Versuchstiermodell das bereits etablierte „diabetische Kaninchen" verwendet (Ramin 2010) und zunächst folgende Fragestellungen beantwortet:

- Wie wirkt sich ein Insulinmangel auf die Expression der Liganden und Rezeptoren des IIRS im uterinen Umgebungsmilieu der Blastozyste aus?
- Welche Auswirkungen sind infolge des mütterlichen Insulinmangels auf das embryonale IIRS messbar?

Die experimentellen Untersuchungen wurden im Tiermodell des diabetischen Kaninchens während der Präimplantationsphase durchgeführt. Dafür wurden Blastozysten und Uteri am Tag 6 der Gravidität gesunder und diabetischer Kaninchen hinsichtlich der Expression des IIRS verglichen. Um die Wirkung von Insulin und Glukose zu unterscheiden, wurden 6 Tage alte Blastozysten *in vitro* mit Insulin und Glukose stimuliert. Die Ergebnisse sind in zwei Publikationen „*Maternal Diabetes impairs gastrulation and insulin and IGF-1 receptor expression in rabbit blastocysts*" und „*Insulin growth factor adjustment in preimplantation rabbit blastocysts and uterine tissue in response to maternal type 1 diabetes*" erschienen (Ramin et al. 2010 , Thieme et al. 2012a).

Als zentraler Vermittler möglicher Insulin- und IGF-abhängiger Wirkungen auf den embryonalen Stoffwechsel wurde der Transkriptionsfaktor CREB mit folgenden Fragestellungen untersucht:

- Welche Familienmitglieder der CREB/ATF-Transkriptionsfaktoren werden im Präimplantationsembryo des Kaninchens exprimiert?
- Kann die Aktivität von CREB über exogene Wachstumsfaktoren (Insulin, IGFs) gesteuert werden?
- Über welche CREB-vermittelten Mechanismen verfügt der Embryo, um den Mangel an Insulin zu kompensieren?

Auch hier wurde eine umfassende Analyse der Präimplantationsembryonen gesunder und diabetischer Kaninchen durchgeführt. Zusätzlich wurden Tag 6 Blastozysten gesunder Kaninchen *in vitro* mit Insulin oder IGFs stimuliert. Auf Grund der unterschiedlichen Rezeptorverteilung des IIRS im Embryo- und Trophoblasten erfolgte eine Zelllinien-spezifische Analyse. Da das CREB-Zielgen Adiponektin eine zentrale Rolle im zellulären Stoffwechsel des Kaninchenembryos einnimmt, wurde dessen Transkription unter den gewählten Bedingungen analysiert.

Die Ergebnisse sind in der Publikation *„cAMP-responsive element binding protein: a vital link in embryonic hormonal adaptation*" veröffentlicht (Schindler et al. 2013). .

Ein weiteres Ziel war es, mögliche Konsequenzen eines mütterlichen Diabetes mellitus auf den embryonalen Fettstoffwechsel zu untersuchen. Dabei sollten folgende Fragen geklärt werden:

- Ist bereits in dem frühen Stadium der Schwangerschaft eine veränderte intrazelluläre Speicherung von Lipiden in Blastozysten diabetischer Kaninchen nachweisbar?
- Welche zentralen Gene des Lipidmetabolismus werden durch einen mütterlichen Diabetes reguliert?
- Welche Wirkmechanismen sind ursächlich für die Regulation lipogener Schlüsselmoleküle?

Die Ergebnisse zum Lipidmetabolismus sind in der Publikation *„Maternal diabetes leads to unphysiological high lipid accumulation in rabbit preimplantation embryos*" (Schindler et al. 2014), sowie im Ergebnis- und Diskussionsteil dieser Arbeit dargestellt (Kapitel 4.4).

Potentiell langfristige Auswirkungen eines mütterlichen Diabetes mellitus auf die metabolische Programmierung in der Embryonalentwicklung wurden mit Hilfe der *in vitro* Stammzelldifferenzierung an einer pluripotenten embryonalen Stammzelllinie der Maus, CGR8-ESC, nachgestellt. Die Zellen sind in der Lage, zu reifen Adipozyten zu differenzieren. Dabei lag der Fokus auf einer möglichen regulatorischen Funktion von CREB. Folgende Fragen wurden bearbeitet:

- Welche Rolle spielt eine CREB-Aktivierung während der frühen Embryonalentwicklung für die Adipogenese und was sind potentielle Zielgene von CREB in Stammzellen?
- Zu welchem Zeitpunkt und wie beeinflusst Adiponektin die CREB-Aktivierung und was sind die möglichen Konsequenzen für die Determinierung und Differenzierung von Adipozyten?

Die Ergebnisse sind bisher noch nicht publiziert und werden im Ergebnis- und Diskussionsteil dieser Arbeit ausführlicher dargestellt und diskutiert (Kapitel 4.5.).

3. Originalarbeiten

3.1. **Ramin N*, Thieme R*, Fischer S, Schindler M, Schmidt T, Fischer B, Navarrete Santos A. Maternal diabetes impairs gastrulation and insulin and IGF-I receptor expression in rabbit blastocysts. Endocrinology. 2010;151:4158–4167.**

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Maternal Diabetes Impairs Gastrulation and Insulin and IGF-I Receptor Expression in Rabbit Blastocysts

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Women with type 1 diabetes are subfertile. Diabetes negatively affects pregnancy by causing early miscarriage and poor prenatal outcomes. In this study we examine consequences of maternal type 1 diabetes on early embryo development, metabolic gene expression, and the pattern of insulin receptor (IR) and IGF-I receptor (IGF-IR) distribution in rabbit blastocysts. In female rabbits, type 1 diabetes was induced by alloxan treatment. Six-day-old blastocysts were recovered and assessed for receptor distribution and metabolic gene expression. *In vitro* culture of blastocysts was performed in medium containing 1 mM, 10 mM, or 25 mM glucose, simulating normo- and hyperglycemic developmental condition *in vitro*. The fertility rate of the diabetic rabbits clearly mirrored subfertility with a drop in blastocyst numbers by 40% (13.3 blastocysts in diabetic *vs.* 21.9 in control females). In blastocysts onset and progression of gastrulation was delayed and expression of IR and IGF-IR and their metabolic target genes (hexokinase, phosphoenolpyruvate carboxykinase), both *in vivo* and *in vitro*, was down-regulated. The amount of apoptotic cells in the embryonic disc was increased, correlating closely with the reduced transcription of the *bcl-x(L)* gene. Blastocyst development is clearly impaired by type 1 diabetes during early pregnancy. Insulin-stimulated metabolic genes and IR and IGF-IR are down-regulated, resulting in reduced insulin and IGF sensitivity and a delay in development. Dysregulation of the IGF system and embryonic glucose metabolism are potential reasons for diabetogenous subfertility and embryopathies and start as soon as during the first days of life. **(*Endocrinology* 151: 4158–4167, 2010)**

Women with type 1 diabetes have a significantly higher risk of pregnancy loss and embryopathies compared with healthy women, indicating that a tight control of glucose and/or insulin is essential for proper embryo development. One of the most sensitive and vulnerable periods in ontogenesis is the formation and development of the embryoblast and trophoblast during blastocyst formation and differentiation. The mammalian preimplantation embryo is supplied with nutrients and growth factors such as glucose, amino acids, insulin, and IGFs by oviductal and uterine secretions. Glucose is an essential energy source of the blastocyst (1). Earlier cleavage stages favor pyruvate or lactate as the main energy substrate. The switch from pyruvate/lactate to glucose is considered to account for the increased energy requirements due to blastocyst expansion and Na^+/K^+-ATPases that facilitate blastocyst cavitation and expansion (2). The embryo is able to tolerate variations in glucose concentration, but both, a diabetic condition and deprivation from glucose, are known to be detrimental for the developing embryo. Cell numbers are decreased and can result in abnormal morphogenesis (3–6) and developmental arrest (7). Glucose itself is able to modulate the transcription of many genes, encoding enzymes typical for glycolytic and lipogenic pathways (8–10) and gluconeogenesis (11, 12).

Glucose homeostasis in mammals is tightly controlled by a balanced interaction of peripheral tissues and their sensitivity to insulin. The glycemic control of insulin is mediated by the insulin receptor (IR). The IR is highly

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Abbreviations: HK, Hexokinase; IGF-IR, IGF-I receptor; IR, insulin receptor; p.c., post coitum; PEPCK, phosphoenolpyruvate carboxykinase; PVA, polyvinyl alcohol.

homologue to the IGF-I receptor (IGF-IR). In IR-deficient cells insulin can mediate its metabolic effects via the IGF-IR (13). In physiological concentrations insulin acts only by the IR. The IR is detectable in embryos in all preimplantation stages in most species including the rabbit (14–16). Hyperglycemia or type 2 diabetes induce an altered IR expression pattern in adult tissues leading to various degrees of insulin insensitivity (17, 18).

Type 1 diabetes can be experimentally induced by alloxan or streptozotocin, an established type 1 diabetic model in laboratory animals (19). Alloxan causes a pancreatic β cell-specific necrosis resulting in type 1 diabetes with insulinopenia and hyperglycemia (20, 21). In mice both agents were used to investigate the influence of maternal type 1 diabetes on embryo development (22–24). For rabbits the alloxan-induced diabetes is routinely used in biomedical and pharmaceutical experimental approaches [see Winiarska *et al.* (25) for references]. At the third day after alloxan treatment the blood glucose concentration in the treated rabbits is in a hyperglycemic range (>14 mmol/liter).

The rabbit embryo implants late at d 6 after mating [at 6 d 18 h, (26)]. During d 6 post coitum (p.c.; *i.e.* during the preimplantation period), the embryonic disc of the rabbit blastocysts develops from a nondifferentiated two cell layer gastrulation stage (epiblast and hypoblast) to three germ layers, allowing a subtle analysis of diabetogenous effects on gastrulation in this species. Using the advantages of the rabbit model such as induced ovulation, high embryo numbers, and large-sized blastocysts (with 2–5 mm in diameter at d 6 p.c.) and well defined gastrulation stages before implantation, we investigated the effects of type 1 diabetes on the maternal fertility rate and uterine glucose and serum insulin concentrations on the one hand and on the embryonic metabolic and insulin/IGF receptor gene expression, the early gastrulation, and apoptosis in the preimplantation blastocyst on the other hand. The *in vivo* findings were complemented by *in vitro* cultures of blastocysts in high glucose media with or without insulin supplementation.

Materials and Methods

Alloxan treatment

Female rabbits were adjusted to a feeding regime with a 3-h food interval in the morning and in the evening. Ten days before mating rabbits were fasted overnight, anesthetized with Dormitor im (0.25 mg/kg body weight, Pfizer, Karlsruhe, Germany) and analgized with Ketanest im (15 mg/kg body weight, Pfizer, Karlsruhe, Germany). Alloxan (120 mg/kg body weight, Sigma-Aldrich, Taufkirchen, Germany) was injected in the marginal ear vein with a Multyfly needle followed by a 10 ml saline injection (0.9%). Fifteen minutes after alloxan injection 50 ml glucose solution [27.5% (wt/vol)] was injected sc for a subcutaneous deposit of glucose. Furthermore, the drinking water was supplemented with 5% (wt/vol) glucose. These precautions prevented critical hypoglycemic conditions as the first phase response of the alloxan treatment after 6–8 h (see glucose profile in Fig. 1C). Hyperglycemia of the rabbit with a permanent blood glucose concentration of >14 mmol/liter was established 36 to 48 h after alloxan injection (Fig. 1C).

Rabbits were hold in diabetic conditions for 9 to 11 d before they were mated. Ketoacidosis was prevented by regular insulin supplementation (Insuman Rapid 40 IU/ml, Aventis, Frankfurt a.M., Germany) after feeding. The blood glucose level was monitored with MediSense Precision Xceed Diabetes Management System (Abbott, Wiesbaden, Germany) two times a day and kept in the range of 14–25 mmol/liter (Fig. 1C). All animal experiments were in accordance with the principles of laboratory animal care and had been approved by the local ethical commission of the Landesverwaltungsamt Dessau (reference number: 42502-2-812).

Embryo recovery and *in vitro* culture

Embryos were collected from alloxan and control rabbits stimulated with 150 IU pregnant mare serum gonadotropin sc (PMSG, Intervet, Tönisvorst, Germany) 3 d prior to mating. After mating 75 IU human choriogonadotropin iv (hCG, Scher-

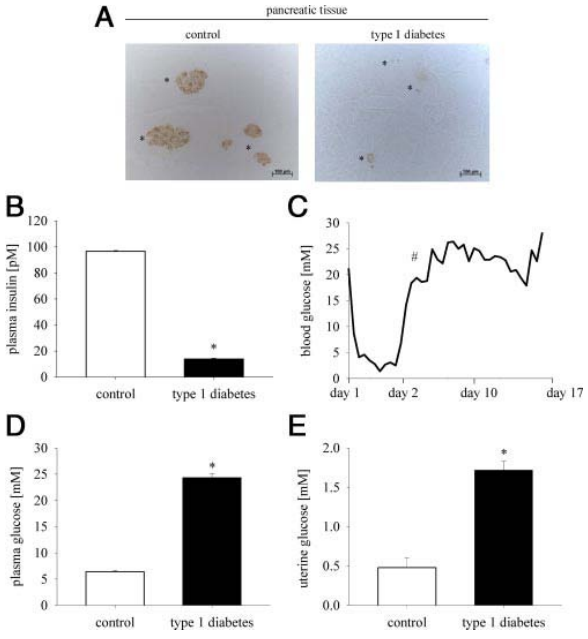

FIG. 1. Insulin and glucose concentrations in type 1 diabetes and normoglycemic rabbits. A, Alloxan-treated type 1 diabetic females had no or only remnants of insulin-positive cells in pancreatic tissue. The β cells in the Langerhans Islands (*) were visualized by immunohistochemical detection (*brown color*) with an anti-insulin antibody. The plasma insulin concentrations were significantly decreased (B). A blood glucose profile of an individual female is shown in C, starting with the day of alloxan treatment. # indicates the starting point of insulin supplementation. A constant hyperglycemic level was achieved 3 d after alloxan treatment. Due to reduced insulin amounts, the glucose concentration was significantly increased in plasma (D) and uterine fluids (E) of type 1 diabetic rabbits (mean ± SEM; n = 3; n ≥ 5; *, $P < 0.05$). *Bar*, 100 μm.

ing AG, Berlin, Germany) was injected. Mating, embryo recovery (27), and embryo culture (28) were performed as described before. At d 3, 4, and 6 p.c., embryos were flushed from the oviducts or uteri and washed two times with PBS and processed for subsequent analyses or *in vitro* experiments. At d 3 p.c. embryos are in the morula stage and develop to early blastocysts at d 4 p.c. The d 3 and d 4 embryos were analyzed by RT-PCR pooled in groups of 10 embryos. Individual d 6 blastocysts were analyzed directly after recovery or used for *in vitro* culture. The gastrulation stage was assessed by morphological examination of the embryonic disc (embryoblast) according to Viebahn *et al.* (29). For *in vitro* culture, embryos were pooled and randomly divided among experimental groups. To study the effect of varying glucose concentrations and insulin, d 6 blastocysts were cultured in groups of four to 10 embryos in basal synthetic medium II at 37 C in a saturated atmosphere of 5% O_2, 5% CO_2, and 90% N_2 in a water-jacketed incubator (BB6060, Heraeus, Hanau, Germany). The standard glucose concentration in culture media for rabbit embryos is 10 mM (15, 30, 31). In current *in vitro* study, we mimicked normal *in vivo* uterine glucose concentration (1 mM) and hyperglycemic developmental conditions (25 mM) *in vitro*. After preculture for 2 h in serum- and insulin-free basal synthetic medium II containing 1 mM, 10 mM, or 25 mM glucose, 17 nM insulin (Sigma-Aldrich, Taufkirchen, Germany) was added to the culture medium for further 1 to 4 h. Controls were cultured in defined glucose concentrations without insulin but otherwise handled identically. After culture, embryos were washed twice in ice-cold PBS and transferred into ice-cold 0.05% polyvinyl alcohol (PVA)/PBS buffer. To investigate gene expression of receptors and key enzymes, blastocyst coverings (neozona, mucin coat) were mechanically removed and the single blastocysts were stored at −80 C. For Western blot analysis, ten embryos were randomly pooled in each group. Samples were homogenized in 100 μl RIPA buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholat, 0.1% SDS) with protease and phosphatase inhibitor cocktail (Roche, Mannheim, Germany) and stored at −80 C.

Insulin-ELISA

The US Insulin ELISA (DRG Instruments GmbH, Marburg, Germany) was used for measurement of the insulin levels according to the manufacturer's protocol using EDTA-plasma.

Measurement of glucose concentration

The glucose concentrations in the plasma and uterine fluid of alloxan-treated and control rabbits were assessed with an enzymatic assay based on a turnover of glucose by exogenous added hexokinase (HK). The product of NADPH was measured spectrophotometrically at a wavelength of 334 nm. 2.8IU HK (Roche, Mannheim, Germany) was added to semimicro cuvettes (Brand, Wertheim, Germany) containing 2.5 mM Mg^{2+}-ATP (Sigma-Aldrich, Taufkirchen, Germany) and 0.5 mM NADP (Applichem, Darmstadt, Germany) in 100 mM triethanol-buffer with a final volume of 1 ml. Before supplementation of HK, 3.5IU glucose-6-phosphat dehydrogenase (Roche, Mannheim, Germany) was added to remove endogenous glucose-6-phosphate. The extinction is proportional to the developed NADPH concentration reflecting the glucose concentration. The uterine fluid was absorbed directly from the endometrial surface by using glucose-free paper strips without any additional flushing, getting approximately 5 μl of uterine fluid per uterus. Before measurement the fluid was washed out of the paper with a defined volume of deionized water (dilution 1:3).

RNA extraction, RT reaction, and PCR detection of insulin

RNA extraction was performed as previously described (15) by Dynabeads mRNA DIRECT Kit (Dynal, Oslo, Norway) according to the manufacturer's instructions. The final volume for cDNA reaction was adjusted to 100 μl.

Total RNA extraction from pancreatic tissues and RT-reaction were performed as published before. PCR amplification was carried out with 3 μl cDNA from tissues and blastocysts in a 50-μl volume containing 200 μM each dNTP and 2.5IU *Taq* polymerase, using the primer combination insulinfw 5′-TTTATACACCCAAGTCCCGCCG-3′/ insulinrev 5′-GAGCAGATGCTGGTGCAACACT-3′ (acc. U03610). Resulting PCR products of 147 bp were separated by electrophoresis on 2.0% agarose gel and stained with ethidium bromide.

Cloning and sequencing of partial cDNA for rabbit HK

A new partial HK (118bp, Acc.No. FJ848380) cDNA was amplified from rabbit kidney tissue using human primers. The sequence was identical with human HK-2 to 92% and 98% at the mRNA and the protein level, respectively, using alignment BLASTN and BLASTX modus.

Real time RT-PCR for IR, IGF-IR, phosphoenolpyruvate carboxykinase (PEPCK), HK, Bcl-x(L), and GAPDH

Samples were analyzed by real-time RT-PCR as described previously (15). The appropriate primer sets: rabGAPDHfw 5′-GCCGCTTCTTCTCGTGCAG-3′/ rabGAPDHrev 5′-ATGGATCATTGATGGCGACAACAT-3′ (acc. L23961), IRfw 5′-CCTGAAGGAGGTGGAGGAG-3′/IRrev 5′-GAGAATCCTGGGACTGTGG-3′ (acc. AY339877), IGF-IRfw 5′-CCCAAGCTCACGGTCATCACTG-3′/IGF-IRrev 5′-ATGGGCTTCTCCTCCAAGGTCC-3′ (acc. EF616472), rabPEPCKfw 5′-TGCGGCCTCCAAAGATGATG-3′/rabPEPCKrev 5′-CCCTGGAAACCTGGTGACAAGG-3′ (acc. EF616471), rabHKfw 5′-ACAGCAACCACATCCAGGTCAAAC-3′/HKrev5′-TTCTCCTCAAGTGGACGAAAGGCT-3′ (acc. FJ848380), and rabbcl-x(L)fw 5′-GGTATTGGTGAGTCGGATCG-3′/rabbcl-x(L)rev 5′-TGTTGCCGTAGAGTTCCACA-3′ (acc. AY005131) for rabbit GAPDH, IR, IGF-IR, PEPCK, HK, and bcl-x(L), respectively. Each assay included duplicates of each cDNA sample and a no template control for each primer set. Relative mRNA expression for IR, IGF-IR, PEPCK, HK, bcl-x(L), and GAPDH was calculated using the ΔΔCT method. The expression of GAPDH RNA was used to normalize samples for the amount of cDNA used per reaction. To confirm the amplification, the resulting real-time RT-PCR products were analyzed by dissociation curves, visualized in an agarose gel (GAPDH 144bp, IR 150bp, IGF-IR 347bp, PEPCK 143bp, HK 118bp, bcl-x(L) 113bp) and sequenced. In each real-time RT-PCR, a calibration curve was included that was generated from serial dilutions (10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 copies) of primer-specific DNA probes generated from cDNA plasmid clones. Analysis of the individual data therefore yielded values relative to these standards. For each group the number of analyzed

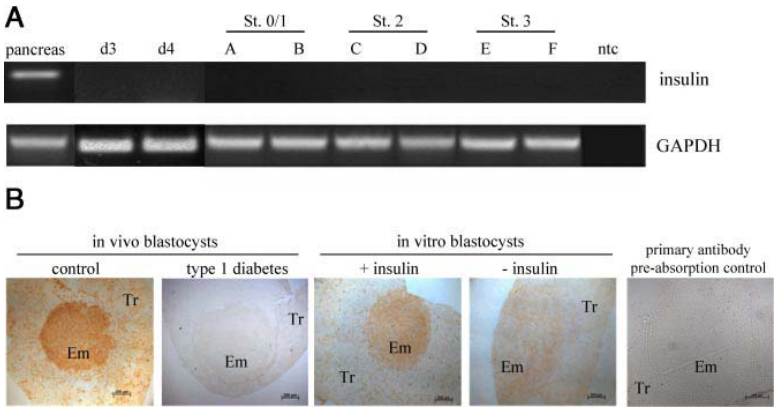

FIG. 2. Insulin detection in rabbit embryos. A, Rabbit embryos did not express insulin mRNA neither at the morula (d 3), the early blastocyst (d 4), nor at the expanded blastocyst stage (d 6) during gastrulation [stage (St.) 0/1, 2, or 3]. RT-PCR analysis was performed at cDNA pools of 10 embryos for d 3, d 4, and individual blastocyst for d 6. Representative results of two individual blastocysts per gastrulation stage (A–F) are shown (n = 2, n = 3). Pancreas was used as positive control for RT-PCR amplification. ntc, No template control. B, In *in vivo* rabbit blastocysts (control) insulin protein is localized in the cytoplasm of embryoblast (Em) and trophoblast cells (Tr). The insulin was provided to the embryo by uterine secretions. An *in vitro* culture of blastocysts without insulin for 10 h strongly diminished the protein amount, proving the degradation of insulin in embryonic cells. Blastocysts from type 1 diabetic females had no detectable insulin protein. No staining was detectable in blastocysts when the antiinsulin antibody was preabsorbed with insulin, demonstrating the specificity of the antibody reaction (n = 3, primary antibody preabsorption control). *Bar*, 200 μm.

blastocysts is given in the diagram bar. Data are expressed as relative values (%) to control blastocysts.

Protein preparation and immunoblotting

Protein isolation and Western blot analyses were performed with 20 μg protein from whole blastocysts. The IR and IGF-IR protein expression was investigated with a monoclonal mouse IR antibody (1:2000, Calbiochem, Darmstadt, Germany) and rabbit IGF-IR antibody (1:1000, Santa Cruz, Heidelberg, Germany). After blotting, the nylon membrane was stained with Ponceau. Apparent molecular weights were determined by comparison with PageRuler prestained molecular weight marker (Fermentas, St. Leon-Rot, Germany). Afterwards the immunoreactive signals were visualized by enhanced chemiluminescence detection (Millipore, Schwalbach, Germany) and quantified by ChemiDoc-It Imaging System (LTF Labortechnik, Wasserburg, Germany). The amounts of receptor protein were evaluated by stripping the membranes and reblotting with a mouse monoclonal β-actin antibody (1:40000, Sigma-Aldrich, Taufkirchen, Germany). Protein amounts were calculated as ratio of band intensities (IR or IGF-IR *vs.* β-actin) in the same blot to correct for differences in protein loading. The relative expression of IR and IGF-IR in embryos of normoglycemic or diabetic animals was calculated as ratio of expression signals of diabetic to normoglycemic controls.

Immunohistochemical localization of insulin

The localization of insulin was investigated in blastocysts and pancreatic tissue of type 1 diabetic and healthy female rabbits. The embryonic disc with the surrounding trophoblast was dissected from whole blastocysts. *In vivo* derived or *in vitro* cultured blastocysts were washed twice in ice-cold PBS and fixed in 4% paraformaldehyde for at least 1 h. The fixed blastocysts were washed again in ice-cold PBS and transferred to 0.05% PVA/PBS. The embryonic disc was mechanically dissected with surgical forceps and scissors (Fine Science Tools GmbH, Heidelberg, Germany) and either used immediately for immunohistochemistry or stored in 100% methanol at −20 C until use. Stored embryonic discs were rehydrated through a series of graded alcohols. The pancreatic tissue was fixed in 4% paraformaldehyde for 1 h, embedded in paraffin, and fixed on a slide in working sections of 5 μm. Nonspecific antibody binding was blocked with 10% normal goat serum in PBS at room temperature for 1 h. The specimens were incubated with the monoclonal anti-insulin IgG1 mouse-antibody (1:200, I2018, Sigma-Aldrich, Taufkirchen, Germany) in 1% BSA/PBS overnight at 4 C. Samples were washed with 0.05% PVA/PBS and incubated with the secondary antiserum goat-antimouse-IgG (Dako EnVision, Hamburg, Germany). Embryonic discs and tissue sections were examined by light microscopy (Axioplan, Carl Zeiss, Jena, Germany). The specificity of immunostaining of the primary antibody was proven by blocking the antibody binding with insulin, using insulin as blocking peptide (bovine 40 mg/ml, Sigma-Aldrich, Taufkirchen, Gemany). The antibody was preincubated with insulin (1:200 in 1% BSA/PBST) for 2 h at 37 C and further 24 h at 4 C. Subsequent application of the neutralized antibody to the IHC reaction did not show any positive staining (Fig. 2B, primary antibody preabsorption control), demonstrating the specificity of the antibody to insulin. The specificity of immunostaining of the secondary antibody reaction was proven by the absence of signals in sections processed after omission of the primary antibody.

TUNEL

Blastocysts exposed to a diabetic or normoglycemic uterine environment were fixed in 4% paraformaldehyde and their embryonic discs were obtained as described before. The discs were permeabilized with 0.1% Tween 20 and incubated in fluorescein-labeled dUTP and terminal transferase in the dark for 1 h at 37 C to label fragmented 3′ DNA (TUNEL, Cell Death In Situ Kit, Roche, Mannheim, Germany). A rabbit kidney cell line (RK13) treated with or without DNase was used to establish and test the TUNEL assay in rabbit cells (data not shown). The TUNEL assays were performed with 9 and 15 blastocysts in control and diabetic blastocysts, respectively. Counterstaining of all nuclear DNA was achieved by incubating the embryos with Hoechst staining (1 mg/ml, Sigma-Aldrich, Taufkirchen, Germany) for 20 min. Embryos were visualized using fluorescence light microscopy (Axioplan, Carl Zeiss, Jena, Germany). The cell number of the embryoblast cells was counted with ImageJ software (Fa. Wayne Rasband, National Institutes of Health, Bethesda, MD) using the Cell Counter plug-in for digital images. An average cell number was counted. The number of apoptotic cells was related to the total number of counted embryoblast cells.

Statistics

Levels of significance between groups were calculated using Student's *t* test after proving normal distribution (SigmaPlot v. 11.0). Data are expressed as mean ± SEM. The difference in the mean values of the two groups is greater than would be expected by chance. There is a statistically significant difference between the input groups. The levels of significance were $P < 0.05$, $P < 0.01$, and $P < 0.001$.

Results

Insulin and glucose concentration in uterine fluids and in blastocysts

Alloxan caused a destruction of pancreatic β cells (Fig. 1A) and a massively reduced insulin secretion (Fig. 1B).

In the serum of females with type 1 diabetes (d 6 p.c.) the plasma insulin concentration was decreased by 85% to 15 pM (Fig. 1B), and blood glucose concentration rose to ≥14 mmol/liter (Fig. 1, C and D). At the third day after alloxan treatment the blood glucose concentration was held steady to 18–25 mmol/liter, defining strong hyperglycemia. All females were profiled twice daily for their blood glucose levels (example in Fig. 1C). Blood glucose levels ≥25 mmol/liter were prevented by external insulin supply for each individual female. Hyperglycemia was reflected by 3.5-fold elevated uterine glucose levels (Fig. 1E).

The rabbit blastocyst itself does not express insulin. The cDNA samples from morulae at d 3, early blastocysts at d 4, and expanded blastocysts at d 6 from gastrulation stage 0/1, 2, and 3 were tested negatively for insulin RNA by RT-PCR (Fig. 2A). The protein, however, was present in the cytoplasm of *in vivo* grown blastocysts when analyzed directly after recovery embryoblast and trophoblast cells (Fig. 2B). In blastocysts of type 1 diabetic rabbits neither the embryoblast nor the trophoblast showed a positive staining for insulin (Fig. 2B). *In vitro* culture of blastocysts in serum-free media for up to 10 h diminished the amount of insulin protein substantially (Fig. 2B).

Developmental competence of blastocysts from type 1 diabetic rabbits

In type 1 diabetic animals the number of corpora lutea (Fig. 3A) and flushed blastocysts (Fig. 3B) were significantly lower than in non-alloxan-treated controls. Blastocysts from type 1 diabetic rabbits were developmentally retarded. At d 6.0 p.c. a lower number had developed to stage 2, and no embryo had reached gastrulation stage 3 (Fig. 3C).

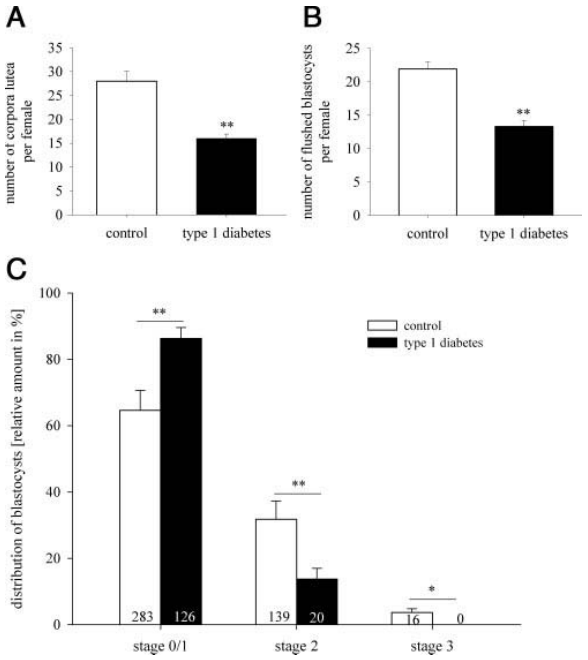

FIG. 3. Fertility of type 1 diabetic rabbits. The number of (A) corpora lutea and (B) blastocysts at d 6 p.c. (n = 5) of type 1 diabetes (n = 11) and normoglycemic animals (control, n = 20) were significantly reduced (mean ± SEM; **, $P < 0.001$). C, The developmental competence was assessed by morphological classification of the embryonic disc. A significantly higher rate of blastocysts from type 1 diabetes animals was arrested in stage 0/1. Only a low number developed to stage 2, and no blastocysts in stage 3 were detectable at d 6.0 p.c. (*, $P < 0.01$; **, $P < 0.001$). The results of three individual experiments (mean ± SEM, n = number in the bar) are shown.

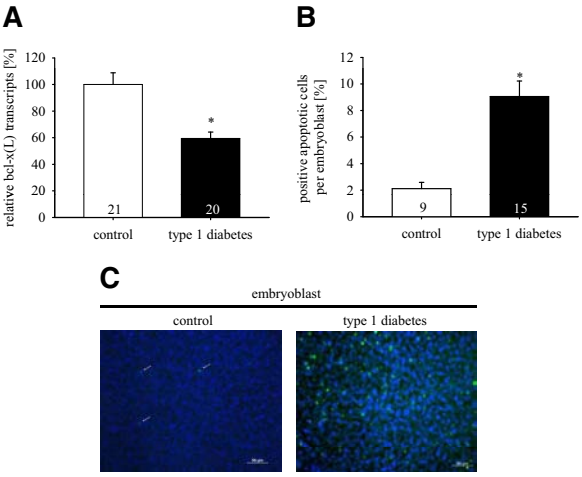

FIG. 4. Apoptosis in blastocysts from type 1 diabetic females. A, RNA expression of bcl-x(L) in d 6 blastocysts of normoglycemic (control) and type 1 diabetes rabbits was significantly decreased by type 1 diabetes developmental conditions (*, $P < 0.001$). The result of three individual experiments are summarized in the diagram (mean ± SEM, n = number in the bar). B, Standardized counting of embryoblast resulted in an increase in TUNEL-positive nuclei in embryos grown under type 1 diabetic conditions. The average number of apoptotic cells per embryoblast is given in percent of totally counted embryoblast cells (mean ± SEM, n = 2, n = number in the bar; *, $P < 0.001$). C, Embryos were counterstained with the nuclear dye Hoechst (*blue*). The panel shows representative embryos grown in a normoglycemic (control) or type 1 diabetic uterine milieu. *Bar*, 50 μm. In the control embryoblast the apoptotic cells are marked by *arrows*.

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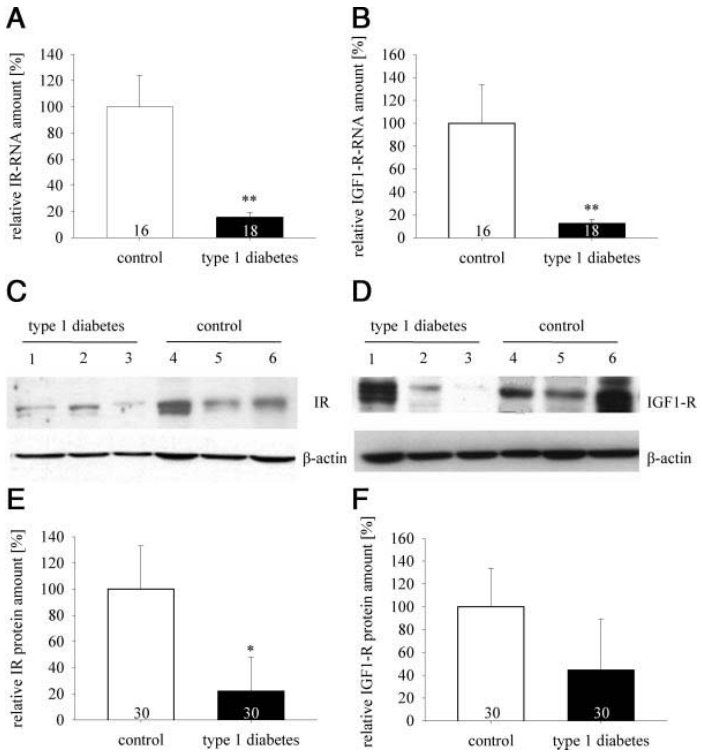

FIG. 5. IR and IGF-IR expression in blastocysts of type 1 diabetes rabbits. The mRNA transcripts for both receptors (A and B) were detected by real-time RT-PCR (mean ± SEM; n = 3). Differences in IR (C, E) and IGF-IR (D, F) protein expression were determined by Western blot (C, D) and densitometrical analyses (mean ± SEM; n = 3). The numbers of pooled blastocysts used for all analysis are inserted in the *bars*. The type 1 diabetic uterine milieu led to a significant reduction of receptor quantity (*, $P < 0.05$; **, $P < 0.01$) on mRNA (IR, IGF-IR) and protein level (only IR) compared with blastocysts derived from normoglycemic mothers (control).

To assess the apoptotic state of the blastocysts grown under type 1 diabetic conditions, the transcription level of the antiapoptotic gene *bcl-x(L)* and the number of apoptotic cells were quantified (Fig. 4). The RNA amount for the *bcl-x(L)* gene was reduced by 40% (Fig. 4A). Consistent with this decrease in gene expression was a 4.3-fold higher percentage of apoptotic cells in the embryoblast (Fig. 4, B and C). No differences were found in the averaged embryoblast cells from stage 1 blastocysts from diabetic mothers with 1974 ± 139 cells per embryoblast compared with controls with 2173 ± 180 cells per embryoblast ($P = 0.43$).

IR and IGF-IR expression in blastocysts from type 1 diabetic rabbits

The receptors for insulin (IR) and IGF (IGF-IR) are present in rabbit blastocysts at d 6 of gestation (15, 16). When exposed to a hyperglycemic milieu IR RNA and protein levels decreased dramatically by approximately 80% (Fig. 5, A and C). Also for IGF-IR a significant decrease in transcript numbers was found in all embryos, whereas the protein amounts where down-regulated only in two samples of three (with 10 pooled blastocysts each) (Fig. 5, B and D).

Metabolic target gene expression in blastocysts from type 1 diabetic rabbits

The gene expression of the metabolic key enzymes PEPCK and HK was analyzed by real-time RT-PCR. Compared with *in vivo*-derived d 6 blastocysts from normoglycemic controls, the RNA levels of both enzymes were significantly decreased in blastocysts from type 1 diabetic animals. The transcript numbers for PEPCK (Fig. 6A) and HK (Fig. 6B) were 50% and 60% lower, respectively.

IR, IGF-IR, and metabolic target gene expression in blastocysts cultured with 1, 10, or 25 mM glucose *in vitro*

To further analyze the hyperglycemic and hypoinsulinemic effects observed *in vivo*, blastocysts from control rabbits were cultured *in vitro* with varying glucose concentrations. Six-day-old blastocysts were cultured for 3 to 6 h in media containing glucose concentrations of 1, 10, or 25 mM, respectively, representing normal glucose concentration in uterine secretions (1 mM), standard *in vitro* levels (10 mM), and hyperglycemic development concentration (25 mM). Analyzed by real-time RT-PCR, transcripts of IR, IGF-IR, PEPCK, and HK were distinctly changed (Figs. 7 and 8). Increasing glucose concentrations up to 25 mM for 3 h caused a reduction in IR and IGF-IR expression by 43% and 58%, respectively, compared with blastocysts cultured in medium containing

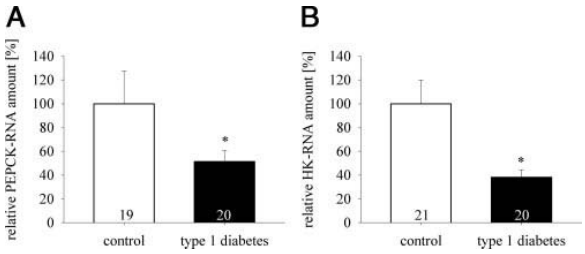

FIG. 6. PEPCK and HK expression in blastocysts from type 1 diabetes rabbits. The mRNA transcripts for both enzymes (A and B) were quantified by real-time RT-PCR (mean ± SEM; n = 3). Blastocysts were cultured in pools of three to four embryos, and transcript numbers were quantified in single blastocysts. The numbers of blastocysts per group are indicated in the *bars*. The type 1 diabetic uterine milieu led to a significant reduction in PEPCK and HK transcript numbers (*, $P < 0.05$).

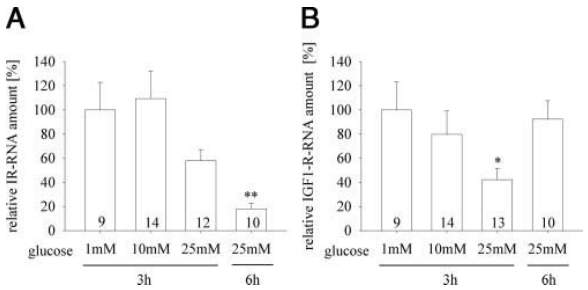

FIG. 7. IR and IGF-IR expression in rabbit blastocysts cultured *in vitro*. The blastocysts were cultured in pools of three to four embryos for 3 (3h) or 6 h (6h) in medium containing 1 mM, 10 mM, or 25 mM glucose. The mRNA transcripts in single blastocysts for IR (A) and IGF-IR (B) were quantified by real-time RT-PCR (mean ± SEM; n = 3). The numbers of blastocysts per group are indicated in the *bars*. A 3-h hyperglycemic milieu led to a significant reduction of IR transcripts, an effect which could be seen also after 6 h (**, $P < 0.01$). While IGF-IR mRNA was decreased by high glucose concentration after 3-h culture, it was restored after 6 h in 25 mM glucose.

1 mM glucose. A longer incubation for up to 6 h in 25 mM glucose led to an IR expression level down to only 18% compared with 1 mM glucose concentration (Fig. 7A). In contrast, the same conditions reincreased IGF-IR expression to the basic expression level observed in the 1 mM group (Fig. 7B).

A glucose-dependent change was also detectable for PEPCK and HK transcripts (Fig. 8). After culture for 3 h in high glucose concentrations the RNA amount for PEPCK was decreased with concomitantly increased HK transcripts. Culture for additional 3 h with 25 mM glucose did not change the PEPCK level, whereas HK transcript numbers showed a further 4-fold increase (Fig. 8, A and B). Insulin treatment of blastocysts cultured with 1 mM or 10 mM glucose significantly reduced the number of PEPCK transcripts numbers and elevated the HK RNA levels. In blastocysts from the 25 mM glucose group, neither 3 h nor 6 h insulin stimulation affected PEPCK or HK mRNA (Fig. 8, A and B).

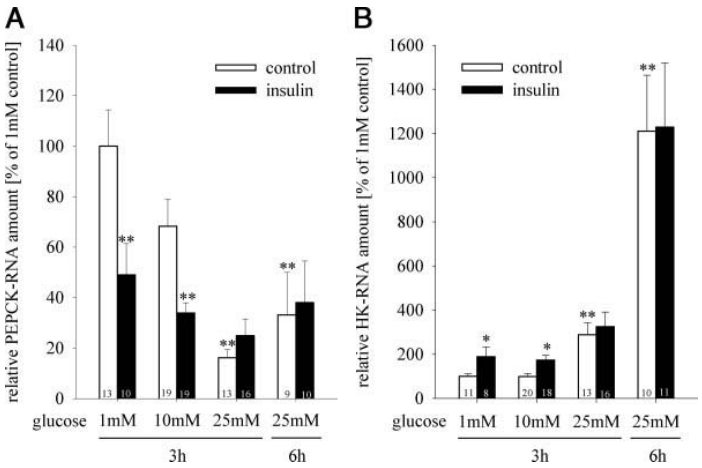

FIG. 8. PEPCK and HK expression in rabbit blastocysts cultured *in vitro*. The blastocysts were cultured in pools of three to four embryos for 3 (3h) or 6 h (6h) in medium containing 1 mM, 10 mM, or 25 mM glucose with (17 nM insulin, *black bars*) or without insulin (control, *white bars*). The mRNA transcripts for PEPCK (A) and HK (B) were quantified by real-time RT-PCR in single blastocysts (mean ± SEM; n = 3). The numbers of blastocysts per group are indicated in the *bars*. A 3-h culture with high glucose concentrations (25 mM) led to a significant reduction in PEPCK transcription. This down-regulation still persists after 6 h (6h) (**, $P < 0.01$). Insulin treatment in the 1-mM and 10-mM glucose groups significantly reduced the number of transcripts. In 25-mM glucose neither 3-h nor 6-h insulin stimulation affected the PEPCK mRNA. The opposite trend was seen for HK mRNA. It was elevated with increasing glucose concentration, most pronounced after 6-h culture time. An insulin treatment in the 1-mM and 10-mM glucose groups increased the RNA levels, whereas in less than 25 mM glucose no insulin effect was detectable.

Discussion

Maternal diabetes impairs ovulation and embryo development, a phenomenon which has been confirmed in several mammalian species [rats (32–34); mice (22, 23); rabbit, current study].

One hypothesis for the developmental effects of diabetes is the primary toxic effect of hyperglycemic glucose, leading as consequence to an accumulation of metabolites and programmed metabolic dysfunction. By Moley and co-authors (35, 36) a metabolic explanation for the developmental retardation seen in the preimplantation embryos from diabetic mice was supposed. In these studies only at dramatically high glucose concentrations accumulation of glucose in the embryo and developmental delay were observed, clearly disproving the hypothesis of a primary toxic effect of high glucose.

In the diabetic rabbit we could show that the uterine glucose concentration was 3-fold increased. In absolute concentrations, however, it was as low as 1.5 mmol/liter and therefore considerably lower than in hyperglycemic blood serum or in standard *in vitro* culture experiments.

So far, the down-regulation of IR and IGF-IR in embryos from diabetic mothers has not been described in other experimental models. Our findings strongly support the view that the disturbance in insulin-mediated glucose metabolism causes ramifications of embryo development. In a first step the decrease in the insulin concentration leads to an inhibition of the insulin-driven glucose metabolism which then results in an intracellular accumulation of nonmetabolized glucose as a major pathophysiological mechanism.

We could demonstrate that embryonic insulin sensitivity is heavily disturbed, as shown by the strongly reduced receptor expression. The disturbed glucose metabolism is proven by changes in key enzymes of glycolysis (HK) and gluconeogenesis (PEPCK) in the blastocyst. PEPCK is a rate-limiting enzyme of gluconeogenesis in the liver. Its gene expression is controlled at the transcriptional level and is induced by glucagon and glucocorticoids and inhibited by insulin (37–39). Earlier studies led to the assumption that gluconeogenesis is an essential metabolic pathway in the rabbit blastocyst (15). By inhibiting PEPCK gene transcription, glucose participates in a feedback control loop governing its production by gluconeogenesis. A related feedback regulation was observed for hexokinase. As a key enzyme in glycolysis, HK initiates the phosphorylation of glucose to glucose-6-phosphate, an intermediate for several principal metabolic pathways like glycogen synthesis, pentose phosphate pathway, and hexosamine biosynthesis. These pathways are of vital importance for the preimplantation embryo (40–42). In the adult liver, glucose is a well-known transcriptional regulator of lipogenic, glycolytic, and gluconeogenic enzymes (43).

In the rabbit blastocysts we could demonstrate the transcriptional regulatory capacity of glucose *in vitro* and *in vivo*. The concrete mechanisms involved need to be clarified. Conceivably the transcription factors C/EBPβ and FoxO1 could be responsible, which are on the one hand direct regulators of PEPCK expression (44, 45) and are on the other hand themselves modulated by glucose (46, 47). However, short-term exposure of the blastocyst to glucose mediates embryonic genes involved in glucose homeostasis, both positively and negatively. It is not unexpected that glucose participates in the regulation of its own production and utilization in the preimplantation embryo.

Elevated glucose levels in the uterine secretions leads to a decrease in embryonic PEPCK expression. The insulin stimulus, normally responsible for a decreased PEPCK expression, is missing in blastocysts from mothers with type 1 diabetes. This implicates that glucose is the more potent regulator for PEPCK in blastocysts than insulin. In case of HK the opposite effect was observed. In blastocysts from type 1 diabetic rabbits, HK transcription was down-regulated. The dramatic HK down-regulation can either be caused by the absence of an adequate insulin stimulus or the insensitivity of the blastocyst to IGFs.

The down-regulation of both key enzymes, HK and PEPCK, seems to be a complex reaction of the blastocyst to the type 1 diabetic situation of the mother. It cannot be explained by a simple metabolic adaptation. The down-regulation of both enzymes has consequences for the utilization of glucose as energy substrate and substrate in biosynthesis. The deficiency in glucose utilization is the most likely reason for the impaired preimplantation development of embryos from type 1 diabetic rabbits.

It is known from studies in various cell lines that a hyperglycemic condition leads to oxidative stress and induction of stress-activated signaling pathways, resulting in inflammation and apoptosis. Apoptosis in preimplantation embryos exposed to hyperglycemia has been shown in present study and in a previous study (48). The link between hyperglycemia, inflammation, and inactivation of glucose-dependent transcription is currently missing, but it is supposed that the modulation of intracellular phosphorylation events leads to the disruption of major glucose-dependent pathways, thus contributing to metabolic disorders, lipid accumulation, insulin resistance, diabetes, and the metabolic syndrome (49).

In preimplantation embryos insulin also acts as an important mitogenic factor mediating cell proliferation and initiation of gastrulation, and it prevents apoptosis (14, 30). Under physiological concentrations it exerts these effects mainly via the IR. Due to high homology, it is also able to bind to the IGF-IR (50, 51). The rabbit blastocyst, as other mammalian preimplantation embryos too (52–54), is not able to produce endogenous insulin. The transcription of the insulin gene is switched off as shown by RT-PCR (Fig. 2B). Preimplantation embryos rely on maternally provided insulin. This insulin was detectable with a significant staining in the embryoblast and trophoblast in rabbit blastocysts immediately after recovery from the uterus (Fig. 2B). The presence of insulin in blastocysts can be explained by transcytosis of the peptide from maternal blood through the uterine epithelium into the uterine secretions. Transcytosis of insulin has been shown in the oviduct and uterus in mouse embryos (52, 54). The maternal insulin was detectable in the cytoplasm of Em and Tr cells, indicating that the insulin protein was taken up into the embryonic cells. For insulin it is known that the aggregated hormone-receptor complex is internalized into endocytic vesicles (55, 56). In the cytoplasm ligand and receptor are sorted and insulin is targeted to lysosomes for degradation. Insulin degradation is inevitably linked to insulin action. All insulin-sensitive tissues degrade the hormone (57). This is apparently also applicable for the rabbit blastocyst.

An *in vitro* culture without insulin reduced the detectable insulin signal in blastocysts within 10 h compared with *in vivo* blastocysts (Fig. 2B), demonstrating the clearance of the insulin peptide within a fairly short time period. Blastocysts grown in diabetic mothers do not show insulin staining (Fig. 2B), indicating that the maternal plasma insulin level is directly correlated with the insulin amount in blastocysts provided by uterine secretions. Next to the loss of insulin, the signaling of the hormone is

impeded due to IR and IGF-IR down-regulation. Normally, in adult tissues, the receptor is up-regulated to sensitize cells for insulin when its concentration is low (58). In the type 1 diabetic situation, however, both receptors are down-regulated. The *in vitro* culture results clearly show that the high glucose concentration is the major factor responsible for decreased receptor expression. Furthermore, our results show that the time interval of hyperglycemia is not pivotal, as short (6 h *in vitro* culture; Fig. 7) and long-time exposure (diabetes; Fig. 5) result in a strongly reduced receptor expression. We consider this regulation not to be adaptive but rather a pathological response, as seen by the missing feedback of insulin-regulated PEPCK and HK transcription under hyperglycemic *in vitro* culture condition (Fig. 8).

As shown in the current study, type 1 diabetes during pregnancy has detrimental effects on glucose metabolism and differentiation of the blastocyst. The dysregulation of embryonic development due to maternal lack of insulin and hyperglycemia appears at a time when the woman is not yet aware of her pregnancy.

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Insulin growth factor adjustment in preimplantation rabbit blastocysts and uterine tissues in response to maternal type 1 diabetes

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ABSTRACT

Insulin-like growth factors (IGFs) are well-known regulators of embryonic growth and differentiation. IGF function is closely related to insulin action. IGFs are available to the preimplantation embryo through maternal blood (endocrine action), uterine secretions (paracrine action) and by the embryo itself (autocrine action). In rabbit blastocysts, embryonic IGF1 and IGF2 are specifically strong in the embryoblast (ICM). Signalling of IGFs and insulin in blastocysts follows the classical pathway with Erk1/2 and Akt kinase activation.

The aim of this study was to analyse signalling of IGFs in experimental insulin dependent diabetes (exp IDD) in pregnancy, employing a diabetic rabbit model with uterine hypoinsulinemia and hyperglycaemia. Exp IDD was induced in female rabbits by alloxan treatment prior to mating. At 6 days p.c., the maternal and embryonic IGFs were quantified by RT-PCR and ELISA.

In pregnant females, hepatic IGF1 expression and IGF1 serum levels were decreased while IGF1 and IGF2 were increased in endometrium. In blastocysts, IGF1 RNA and protein was approx. 7.5-fold and 2-fold higher, respectively, than in controls from normoglycemic females. In cultured control blastocysts supplemented with IGF1 or insulin in vitro for 1 or 12 h, IGF1 and insulin receptors as well as IGF1 and IGF2 were downregulated. In cultured T1D blastocysts activation of Akt and Erk1/2 was impaired with lower amounts of total Akt and Erk1/2 protein and a reduced phosphorylation capacity after IGF1 supplementation.

Our data show that the IGF axis is severely altered in embryo-maternal interactions in exp IDD pregnancy. Both, the endometrium and the blastocyst produce more IGF1 and IGF2. The increased endogenous IGF1 and IGF2 expression by the blastocyst compensates for the loss of systemic insulin and IGF. However, this counterbalance does not fill the gap of the reduced insulin/IGF sensitivity, leading to a developmental delay of blastocysts in exp IDD pregnancy.

1. Introduction

IGF1, IGF2 and insulin belong to the insulin-like growth factor (IGF) family. They play a fundamental role in embryo-maternal crosstalk and in early embryo development. The specific importance of IGF signalling pathways during early embryogenesis is pointed out by knockout studies for IGF1, IGF2 and IGF1R which all result in a reduced birth weight (40–60%) and an increased neonatal lethality (Baker et al., 1993; Liu et al., 1993). A lack of insulin typically caused by type 1 diabetes mellitus (T1D), leads to maternal subfertility and an increase in miscarriage rates and congenital malformations (Casson et al., 1997; Penney et al., 2003; Verheijen et al., 2005; Yang et al., 2006).

It is known that IGFs have the ability to compensate for each other in a distinct manner. Mode and mechanisms of compensation during early pregnancy and diabetic pregnancy, however, are not known. In the oviductal and uterine lumen, insulin reaches the preimplantation embryo via transcytosis from the maternal blood flow (Heyner et al., 1989; Schultz et al., 1992). As the embryo cannot produce insulin itself (Heyner et al., 1989; Lighten et al., 1997; Ramin et al., 2010; Rappolee et al., 1992; Schultz et al., 1992), it is dependent on maternal supply. In contrast, IGF1 and IGF2 are produced by the embryo itself and by maternal reproductive tissues (Lighten et al., 1997).

IGFs play an essential role not only in early embryo development, growth and differentiation but also in the implantation process. IGF1 has been shown to induce Akt phosphorylation in human endometrium. Akt phosphorylation was restricted to the

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glandular epithelium during implantation (Toyofuku et al., 2006). Activation of the PI3K-Akt signalling pathway seems to be essential for successful implantation by maintaining cell survival, trophoblast invasion and induction of extracellular matrix remodelling (Prast et al., 2008; Toyofuku et al., 2006).

Insulin, IGF1 and IGF2 act via the insulin (IR) and IGF1 receptor (IGF1R). In women physiological IGF1 concentrations of 8.0 and 10.9 nM were found in the Fallopian tube and in uterine secretions, respectively (Lighten et al., 1998). Human embryos supplemented with IGF1 in vitro (1.7 nM) showed an increase in blastocyst development and in the number of ICM cells by 19% and 59%, respectively (Lighten et al., 1998). Compared with healthy patients, diabetic patients show a decrease by 55% in serum IGF1 due to an increased GH level (Colao et al., 2008; Shishko et al., 1994). The predominant source of the circulating IGF1 is the liver, regulated by GH (Schwander et al., 1983), but a local IGF1 synthesis is seen in many tissues and can potentially compensate for a loss of systemic IGF1.

Recently, we have shown that the expression of IR and IGF1R is markedly down-regulated in blastocysts grown in diabetic rabbits (Ramin et al., 2010). The aim of current study was to further elucidate maternal and embryonic synthesis of IGFs and potential interactions during early pregnancy in an experimental insulin dependent diabetes (exp IDD) animal model.

2. Material and methods

2.1. Animals, embryo recovery and in vitro culture

2.1.1. In vivo model of experimental insulin dependent diabetes (exp IDD) in rabbit preimplantation development

Exp IDD was induced by alloxan treatment as described before (Ramin et al., 2010). Hyperglycaemia with a permanent blood glucose concentration of >14 mmol/L was established 36–48 h after alloxan injection. The blood glucose level was monitored two times a day and kept in the range of 14–25 mmol/L. Ketoacidosis was prevented by regular insulin supplementation (Insuman Rapid 40 IU/mL, Aventis, Frankfurt a.M., Germany) after feeding. Rabbits were held in diabetic conditions for 9–11 days before they were mated. All animal experiments were in accordance with the principles of laboratory animal care and had been approved by the local ethics committee (Landesverwaltungsamt Dessau, reference number 42502-2-812).

2.1.2. Embryo sampling

Blastocyst recovery was performed as described before (Navarrete Santos et al., 2000; Ramin et al., 2010). Briefly, blastocysts were collected from sexually mature rabbits which had been stimulated with 110 I.U. pregnant mare serum gonadotropin (PMSG, Intervet, Unterschleißheim, Germany) 3 days prior mating and with 75 I.U. human chorionic gonadotropin (hCG, Intervet, Unterschleißheim, Germany) after mating. Superovulation allowed embryos use from the same animals for in vivo and in vitro analyses from control and diabetic animals. The embryos were flushed from the uteri at day 6.0 post coitum (p.c.), washed two times in basal synthetic medium II (BSM, serum- and growth-factor-free) (Maurer, 1978), pooled and randomly divided amongst the experimental groups.

2.1.3. Embryo in vitro culture

In vitro culture was conducted to verify and strengthen findings from the in vivo studies. Short cultures of 10 min and 1 h and longer culture times of 12 h were performed with 6 day old blastocysts. They were cultured in groups of 4 to 8 in 4 mL BSM II medium ± supplementation of IGF1, IGF2 or insulin at 37 °C in a water saturated atmosphere of 5% O_2, 5% CO_2 and 90% N_2 in a water-jacketed incubator (Heracell 150i, Heraeus, Hanau, Germany). Insulin, IGF1 or IGF2 were pre-diluted in 100 μL culture medium and then used in 4 mL culture medium. Either 17 nM insulin (Sigma–Aldrich, Taufkirchen, Germany), 1.3 nM IGF1 (Sigma–Aldrich, Taufkirchen, Germany) or 13 nM IGF2 (Sigma–Aldrich, Taufkirchen, Germany) were added. These concentrations had been chosen according to physiological ranges (Chi et al., 2000) and have been employed in various other studies before (Navarrete Santos et al., 2000,2004b,2008; Ramin et al., 2010; Thieme et al., 2012). Controls were handled in the same manner without growth factors supplementation. PD98059 (20 μM) or LY294002 (20 μM) (Sigma–Aldrich, Taufkirchen, Germany) dissolved in DMSO (Sigma–Aldrich, Taufkirchen, Germany) were used for specific inhibition of the MAPKK (MEK1) or PI3K and added 30 min prior to IGF2 supplementation. IGF1 treatment was performed at the same day with Erk and Akt phoshorylation measured in corresponding blastocysts from control and exp IDD animals.

After culture the blastocysts were washed two times in ice cold phosphate buffered saline (PBS) and transferred into ice cold 0.05% polyvinylalcohol (PVA)/PBS buffer. For RNA isolation, the extracellular coverings were removed mechanically. To investigate spatial expression of IGF2 and to discriminate its effects in Tr and Em, the embryonic discs were microdissected from the trophoblast under a stereomicroscope (Navarrete Santos et al., 2004a). Isolated Em and Tr of single blastocysts were stored separately at −80 °C until processed for gene expression analysis.

2.2. IGF1-ELISA

For the measurement of the IGF1 concentration in EDTA plasma and in single blastocysts the *Active IGF1 ELISA* (Diagnostic System Laboratories Inc., Webster, USA) was used according to the manufacturer's protocol. Plasma samples were collected from control and diabetic females at day 6 p.c. and 50 μl plasma was used for analysis. Determination of embryonic IGF1 concentration was performed on single blastocysts. After measurement of blastocyst size (by diameter), the extracellular coverings were removed mechanically and the blastocysts were lysed in ice cold 50 μl lysis buffer by ultrasonication for 10 min.

IGF1 concentrations were quantified by an IGF1 standard curve in each ELISA. The absolute amount of embryonic IGF1 was related to the blastocyst surface (in mm^2) to correct for differences in blastocysts sizes.

2.3. RNA extraction, RT reaction

RNA extraction for blastocysts was performed as previously described (Navarrete Santos et al., 2004a) using Dynabeads mRNA DIRECT Kit (Dynal, Oslo, Norway) according to the manufacturer's instructions. The final volume of the cDNA reaction was adjusted with water to 100 μl for blastocysts and to 50 μl for separated embryoblast and trophoblast. Total RNA extraction from liver tissues were performed as published before (Navarrete Santos et al., 2008). RT-PCR for IGF1 and IGF2 was done as described before (Navarrete Santos et al., 2004a).

2.4. Cloning and sequencing of partial cDNA for rabbit IGF2

A new partial IGF2 (286 bp) cDNA was amplified from the rabbit kidney cell line RK13 using human primers. The sequence was identical with human IGF2 to 92% and 98% at the mRNA and the protein level, respectively, using the alignment BLASTN and BLASTX tool.

Table 1
Oligonucleotides.

Gene	Primer sequence	Length	Tm	Acc. No.
Rabbit GAPDH	fw: GCCGCTTCTTCTCGTGCAG rev: ATGGATCATTGATGGCGACAACAT	144 bp	60 °C	L23961
Rabbit IGF1	fw: TGGTGGATGCTCTCAGTTCGTGT rev: GCTGATACTTCTGAGTCTTGGGCA	237 bp	60 °C	NM_001082026.1
Human IGF2	fw: TTCCGATTGCTGGCCATCTCTG rev: TGTTTCCGCAGCTGTGACCTGG	308 bp	60 °C	NM_000612.4
Rabbit IGF2	fw: TGGAAGAACTTGCCCACGGAG rev: GCTGCATTGCTGCTTACCGC	286 bp	60 °C	JN825734.1
Rabbit IGF2R	fw: CGGCATGGCAACCTGTATGACC rev: TGTCGATGGTCGGGCAGATGTC	127 bp	60 °C	AF339157.1
Rabbit IGF1R	fw: CCCAAGCTCACGGTCATCACTG rev: ATGGGCTTCTCCTCCAAGGTCC	347 bp	60 °C	EF616472
Rabbit IR	fw: ACCGACTACCTGCTGCTGTT rev: TGACCAGCGCATAGTTGAAG	112 bp	60 °C	AY339877.1

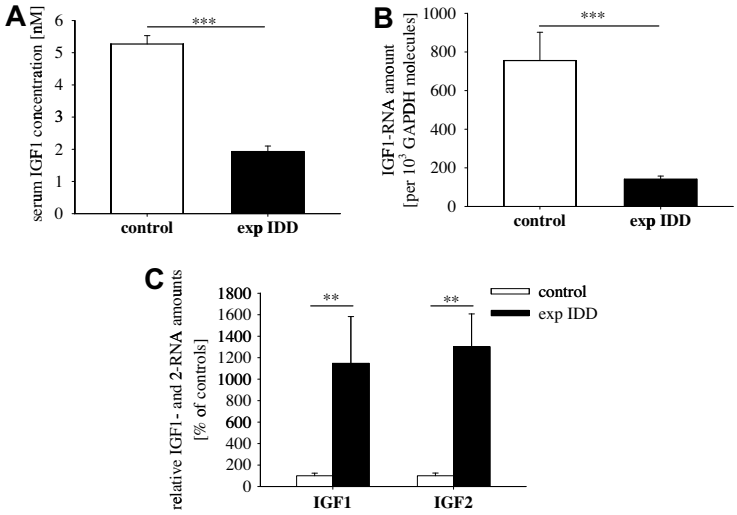

Fig. 1. IGFs in pregnant rabbits. (A) ELISA quantified IGF1 serum concentration and (B) hepatic IGF1 RNA expression are decreased, while (C) endometrial IGF1- and IGF2-RNA expression are increased in exp IDD animals at day 6 of pregnancy (mean ± SEM; $N = 3$; $n = 6$; $^{**}p < 0.01$; $^{***}p < 0.001$).

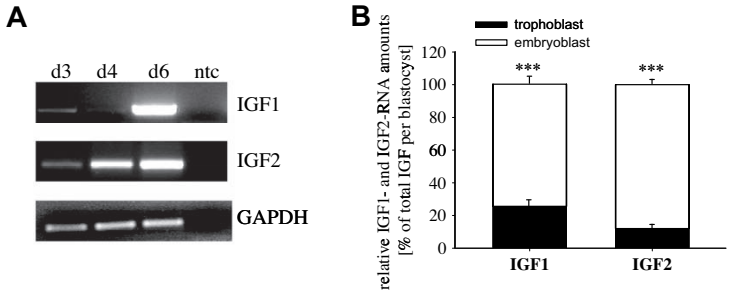

Fig. 2. IGFs expression in the embryoblast and trophoblast of rabbit blastocysts at day 6 (A) Embryonic expression (RT-PCR) of IGF1 and IGF2 was determined in an ontogenetic series at day 3 (morula), 4 (early blastocyst) and 6 p.c. (expanded blastocyst). (B) Relative amounts of IGF1 and IGF2 mRNA are quantified by real time RT-PCR in embryoblast and trophoblast. The expression level of both growth factors is clearly higher in the embryoblast. (mean ± SEM, $N = 3$, $n \geqslant 4$; $^{***}p < 0.001$).

2.5. Real time RT-PCR

Samples were analysed by real time RT-PCR as reported before (Navarrete Santos et al., 2008). The primers are given in Table 1. Each assay included duplicates of each cDNA sample and a no template control for each primer set. The expression of GAPDH RNA was used as reference for the amount of cDNA used per reaction. GAPDH levels of day 6 blastocysts from control and exp IDD animals were comparable with ct values for controls 17.67 ± 0.27 and exp IDD 17.41 ± 0.49 ($p = 0.6225$). In each real time RT-PCR, a calibration curve was included that was generated from serial dilutions (10^8–10^3 copies) of primer-specific DNA probes generated

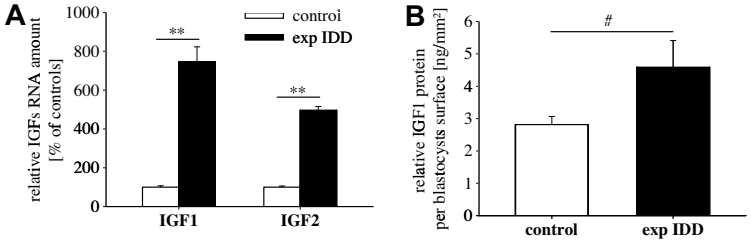

Fig. 3. IGFs mRNA expression and IGF1 protein amounts in blastocysts from exp IDD rabbits. (A) IGF1 and IGF2 transcript amounts were measured in blastocysts from exp IDD and control rabbits by real time RT-PCR. The transcript levels of both, IGF1 (7.5-fold) and IGF2 (5-fold), are increased in blastocysts from diabetic mothers. (B) The IGF1 protein was quantified by ELISA using single blastocysts from control and exp IDD rabbits and is increased under diabetic conditions. The IGF1 protein amount was normalized by the area of the blastocyst surface (mean ± SEM, $N = 3$, $n \geqslant 4$; $^{\#}p = 0.087$; $^{**}p < 0.01$).

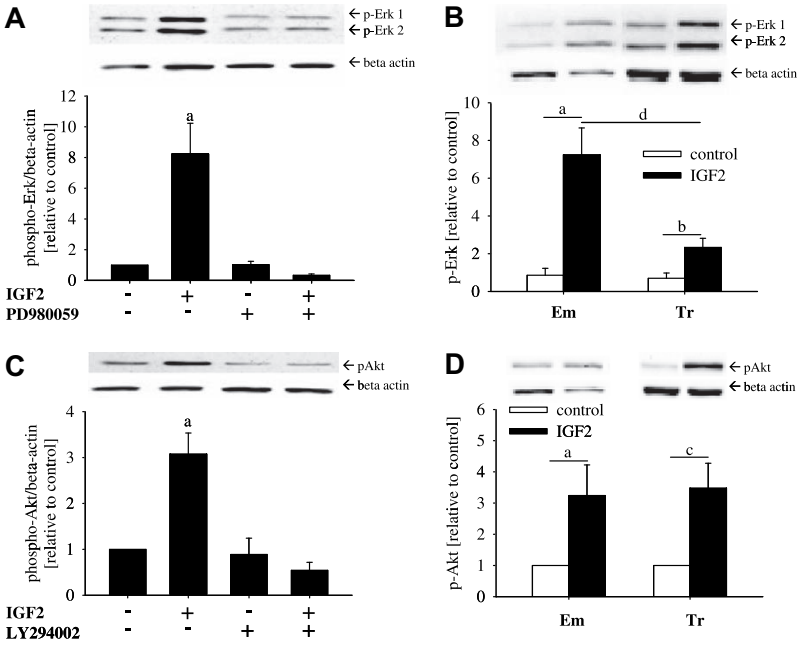

Fig. 4. IGF2 mediated activation of Erk1/2 (A and B) and Akt (C and D) in the rabbit blastocyst. Day 6 rabbit blastocysts were cultured in groups of 10 with or without 13 nM IGF2 for 10 min with/without the Erk or Akt specific inhibitors PD98059 (20 μM) or LY294002 (20 μM) 30 min prior to IGF2 supplementation. (A and B) The ratio of phosphorylated Erk1/2 and (C and D) Akt related to the total Erk1/2 and Akt amount for the same sample and Western blot were calculated with a strong activation by IGF2, while the use of PD98059 or LY294002 prevented the IGF2 mediated increase in phosphorylated Erk1/2 and Akt, respectively (mean ± SEM; one-way ANOVA, $a < 0.001$). The increase in Erk1/2 and Akt phosphorylation is present in both embryonic compartments. Detailed analysis of Erk1/2 (B) and Akt (D) phosphorylation by IGF2 in embryoblast (Em) ($a-p < 0.001$) and trophoblast (Tr) ($b-p = 0.019$ and $c-p = 0.004$) revealed an increase in both cell lineages, with a strong Erk1/2 phosphorylation in the embryoblast ($d-p = 0.003$, tow-way ANOVA with $p = 0.008$ (B) and $p = 0.012$ (D), mean ± SEM, $N = 3$, $n = 10$).

from cDNA plasmid clones. Analysis of the individual data therefore yielded values relative to these standards.

2.6. Protein preparation and immunoblotting

Protein isolation and Western blot analyses were performed with 20 μg protein. Phosphorylated und non-phosporylated Erk and Akt expression was investigated with an anti-phospho-Akt1 (S473) (mouse mAb, 1:1000, #4051), anti-Akt1 (mouse mAb, 1:500, #2967), anti-phospho-Erk1/2 (Thr202/Tyr204) (rabbit Ab, 1:1000, #4051) and anti-Erk1/2 (mouse mAb, 1:1000, #9107) antibody (all NEB, Frankfurt, Germany). After blotting, the nylon membranes were stained with Ponceau-S. Molecular weights were determined by comparison with PageRuler™ prestained molecular weight marker (Fermentas, St. Leon-Rot, Germany). Afterwards the immunoreactive signals were visualized by enhanced chemiluminescence detection (Millipore, Schwalbach, Germany) and quantified by ChemiDoc-It Imaging System (LTF Labortechnik, Wasserburg, Germany). The amounts of phosphorylated protein were evaluated by re-blotting with an antibody against the non-phosphosphorylated Erk1/2, Akt or the mouse monoclonal beta-actin antibody (1:40,000, Sigma–Aldrich, Taufkirchen, Germany) after stripping the membranes. Protein amounts were calculated as ratio of band intensities (pErk1/2 or pAkt vs. Erk1/2 or Akt vs. beta-actin, respectively) in the same blot in order to correct for differences in protein loading.

2.7. Statistics

If not stated otherwise, levels of significance between groups were calculated using student's *t*-test after proving normal distribution. Multiple comparisons were made by factorial variance

analysis (SigmaPlot v. 11.0). Data are expressed as mean value ± standard error (mean ± SEM). Levels of statistical significance are indicated as follows $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. *N* represents the number of experimental replicates, *n* the number of samples (e.g. blastocysts) per experiment.

3. Results

3.1. Maternal IGF1 at day 6 of pregnancy

IGF1 serum levels were clearly reduced in exp IDD rabbits. The plasma IGF1 level of 1.9 nM was decreased by 60% compared with normoglycemic controls (5.3 nM) (Fig. 1A). The hepatic IGF1 transcription, the major source of circulating IGF1, was decreased by 85% in the liver of exp IDD females at day 6 p.c. (Fig. 1B).

3.2. Expression pattern of IGF1 and IGF2 in the endometrium

In exp IDD animals a 11-fold and 13-fold increase in the endometrial IGF1 and IGF2 RNA expression level, respectively, was measured at gestational day 6 (Fig. 1C).

3.3. Embryonic IGF1 and IGF2 at day 6 of pregnancy

IGF1 and IGF2 transcripts were present in all investigated embryo stages from morula (d3), early blastocyst (4d) to expanded blastocyst at day 6 p.c. (Fig. 2A). IGF1 and IGF2 RNA amounts were quantified and analysed by real time RT-PCR in separated embryoblast and trophoblast. Transcript numbers of IGF1 and IGF2 in the embryoblast exceeded those in the trophoblast by approximately 4-fold (Fig. 2B).

In exp IDD blastocysts IGF2 transcript levels and IGF1 RNA and protein levels were clearly elevated. The rises in IGF1 and IGF2 RNA levels added up to 7.5 and 5-fold, respectively (Fig. 3A). IGF1 protein increased from 2.81 ± 0.25 ng/mm^2 (controls) to 4.59 ± 0.82 ng/mm^2 (1.6-fold increase) in exp IDD blastocysts. This increase was consistent and independent from blastocyst size (Fig. 3B). IGF2 could not be determined due to lack of a specific ELISA detecting rabbit IGF2 protein.

3.4. IGF2 activates Akt and Erk in rabbit blastocysts in vitro

Recently we have reported on Akt and Erk activation by insulin and IGF1 in day 6 p.c. rabbit embryos and their differential signalling in embryoblast and trophoblast cells (Navarrete Santos et al., 2008). Because of endometrial and embryonic increase in IGF2 expression in diabetic animals (Figs. 1 and 2), the IGF2 signalling was analyzed in preimplantation embryos, too. Therefore 6 day old blastocysts were stimulated with IGF2 (13 nM) in vitro.

Erk1/2 and Akt phosphorylation increased 8.3-fold and 3.1-fold, respectively, compared with untreated controls (Fig. 4A and C). Using the MEK1 specific inhibitor PD98059 and the PI3K specific inhibitor LY294002 30 min prior to IGF2 supplementation revealed the IGF2 specific signalling as the increase in Erk1/2 and Akt phosphorylation by IGF2 was diminished to control levels (Fig. 4A and C). To discriminate IGF2 mediated activity between embryoblast and trophoblast, these cell lineages were separately analysed after IGF2 treatment. The increase was 7-fold and 3.2-fold for phopspho-Erk1/2 (Fig. 4B) and phosphor-Akt (Fig. 4D), respectively, in the embryoblast and 2.3-fold and 3.3-fold in the trophoblast. Phosphorylation of Erk1/2 was significantly higher in the embryoblast than in the trophoblast. The activation of Erk and Akt by IGF1 and insulin has recently been shown by Navarrete Santos et al., 2008.

3.5. Kinase content and phosphorylation of Akt and Erk by IGF1 in blastocysts from exp IDD animals

The total amount of Akt and Erk protein in exp IDD blastocysts was reduced to 45% and 20%, respectively (Fig. 5A). The relative phosphorylation levels of both kinases closely resembled those in control blastocysts (Fig. 5B).

Following supplementation of 1.3 nM IGF1 in vitro, Akt and Erk phosphorylation was 6-fold and 4.5-fold higher in control blastocysts than in blastocysts from exp IDD animals, demonstrating a reduced sensitivity to IGF1 in exp IDD blastocysts (Fig. 5C).

3.6. IGF1, IGF2, IR and IGF1R expression in cultured blastocysts

Maternal exp IDD leads to marked decrease in IR and IGF1R expression in 6 day old rabbit blastocysts (Ramin et al., 2010). In this study, day 6 blastocysts were cultured with 17 nM insulin or 1.3 nM IGF1 for 1 h or 12 h in vitro. When analysed after 1 h

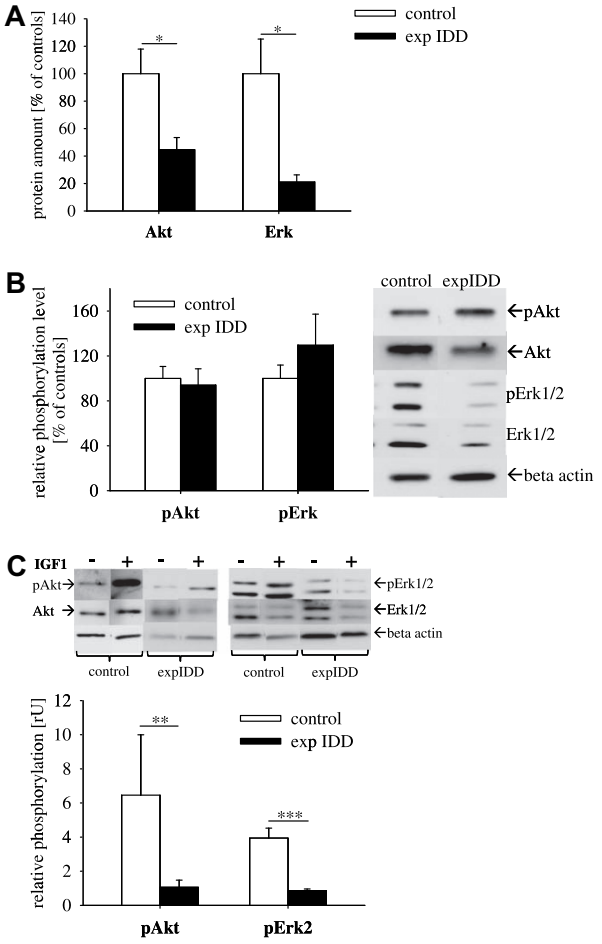

Fig. 5. Kinase activation by IGF1 in blastocysts from exp IDD animals. Total Akt and Erk protein (A) and their phosphorylation levels (B) were analyzed by western blot in in vivo grown blastocysts from exp IDD and normoglycemic rabbits at day 6 p.c. There was no change in the ratio of phospho-Akt/Akt and phospho-Erk1/2/Erk1/2 but in the total level of the protein ($^{*}p < 0.05$). (C) In vitro cultured blastocysts from exp IDD and normoglycemic rabbits supplemented with 1.3 nM IGF1 for 10 min show a clearly lower increase in the ratio of phospho-Akt/Akt and phospho-Erk2/Erk2 compared to blastocysts from control animals (mean ± SEM; $N = 3$; $n = 10$; tow-way ANOVA, $^{**}p = 0.01$; $^{***}p < 0.001$).

in vitro culture, insulin had decreased IGF1 and IGF2 mRNA levels by 55% while IGF1 supplementation decreased the ligands differently by 70% and 50%, respectively (Fig. 6A/B). Like in vivo, both receptors were downregulated. After 12 h culture, IGF1R RNA expression was decreased by 70% by IGF1 and insulin, and IR RNA by 50% by insulin and 65% by IGF1 (Fig. 6C and D), closely reflecting receptor downregulation in vivo in exp IDD mothers.

4. Discussion

The preimplantation embryo is a sensitive target of IGFs. Except for insulin, the whole range of the insulin/IGF ligand and receptor system is expressed during preimplantation development. These hormones are known key players in embryo growth and differentiation and synchronise and balance the crosstalk between mother and embryo during early pregnancy. Disturbances of the insulin/IGF axis by diabetes mellitus or other metabolic diseases result in embryonic loss and miscarriages.

In general IGF homoeostases is regulated by the growth hormone and the local synthesis in tissues. During pregnancy the IGF1 serum level is increased by 3-fold (third trimester) compared to the serum level of a non-pregnant women. Systemic IGF2 levels do not change (Gargosky et al. 1990). During gestation in the rabbit the IGF2 and IGF1 serum levels increase from 4 to 810 nM (IGF2) and from 23 to 38 nM (IGF1), respectively, from day 0 to 23 (Nason et al., 1996). The increase in both growth factors is closely correlated with implantation and formation of a functional placenta (Nason et al., 1996). In this study, in exp IDD rabbits, we show a remarkable increase in IGF1 and IGF2 production by the embryo and endometrium to compensate locally, in a paracrine fashion, for the dramatic loss of circulating insulin (Ramin et al., 2010) and IGF1 levels (Fig. 1).

Unlike insulin, the IGFs are present in most tissues and cell types. The liver is the major source for systemic IGFs (Schwander et al., 1983). This has been shown for IGF1 by a liver specific knock-down (Yakar et al., 1999) with a 75% drop in systemic IGF1. If ALS (IGF1-IGFBP3-ALS), a factor essential for IGF1 synthesis, was additionally eliminated in these mice IGF1 serum level decreased to 5% (Yakar et al., 2002). It is important to emphasise that the relevant IGFs for reproduction are produced locally in the uterus, creating a micromilieu sufficient for IGF action and to secure pregnancy. These IGFs act paracrine and compensate for the systemic loss. In diabetic women, this local safeguard regulation cannot be detected by measurement of serum IGFs.

In the rabbit the IGF expression profiles of the embryoblast and trophoblast (Fig. 2B) show a distinct pattern with a 4 to 5-fold higher expression of both growth factors in the embryoblast. IGFs are known as "GF of the embryoblast/ICM" (Harvey and Kaye, 1992; Kaye, 1997; Navarrete Santos et al., 2008; Pantaleon et al., 1997). This view is supported by current findings, indicating a higher local requirement of IGF1 and IGF2 activation in the embryoblast than in the trophoblast. These important growth factors are not only produced by the blastocyst itself but cross the trophectoderm via transcytosis from the uterine lumen, as shown for IGF1 by electron microscopy (Smith et al., 1993). In this study we show that IGF2 is a key player in ICM/embryoblast development, too, by activating the mitogenic Erk pathway to a greater extend in embryoblast than in trophoblast cells (Fig. 4B).

The specific importance of IGF1 and IGF2 has been demonstrated in knockout models. Whereas an IGF1 knockout results in a reduced viability at birth and growth, in developmental defects of the lung, brain, skeleton, muscle and in infertility of the offspring (Butler and LeRoith, 2001; Powell-Braxton et al., 1993; Rother and Accili, 2000), an IGF2 knockout leads to a reduced birth weight and infertility (DeChiara et al., 1990). IGF2 knockout offspring are able to catch up weight postnatally. This suggests a stronger impact of IGF1 than IGF2 on early embryonic developmental failures (Baker et al., 1993; Rother and Accili, 2000). As seen in our study expression of IGFs in the preimplantation period can be altered in specific metabolic conditions with a potential impact of both studied IGFs on long-term development.

As shown in current study the "insulin gap" in exp IDD can be bridged during pregnancy. Maternal exp IDD affects both parts of IGF signalling, receptor and ligand expression, in the embryo and endometrium. While IR and IGF1R (Ramin et al., 2010); Fig 6) are down-regulated in exp IDD, expression of both ligands is increased (Fig. 2). The sensitivity for intracellular downstream activation is diminished by 55% and 80% of total available Akt and Erk in

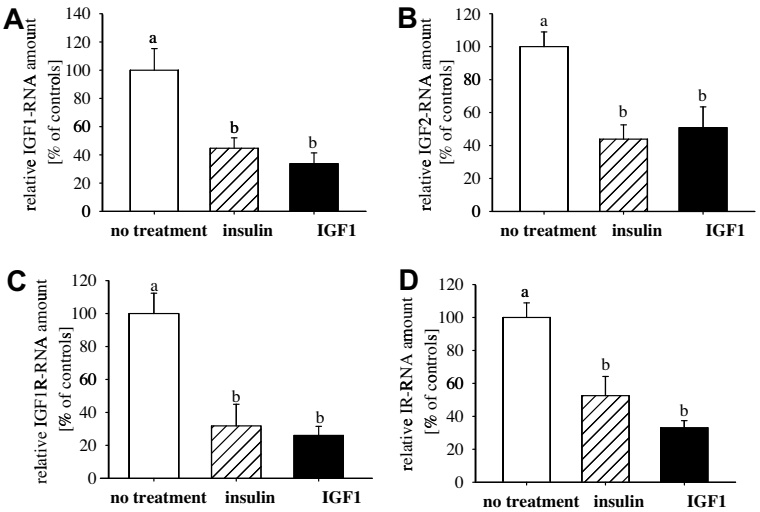

Fig. 6. Ligand (IGF1 and IGF2) and receptor (IR and IGF1) expression in cultured rabbit blastocysts. IGF1, IGF2, IR and IGF1R RNA were measured by real time RT-PCR after 12 h (A and B) and short time 1 h (C and D) in vitro exposure to insulin or IGF1. The transcript numbers of non-treated controls were set to 100%. The expression of IGF1 (A) and IGF2 (B) are decreased by 55% by insulin and by 70% and 50% by IGF1, respectively (mean ± SEM; $N = 2$; $n = 8$; one-way ANOVA with $a–p < 0.001$). Insulin and IGF1 decreased (C) IGF1R RNA by 70% and (D) IR RNA by 50% (insulin) and 65% (IGF1), respectively (mean ± SEM; $N = 3$; $n = 10$; one-way ANOVA with $a–p < 0.001$).

blastocysts from diabetic mothers (Fig. 5A). Although the proportion of active phosphorylated to non-phosphorylated protein is the same in blastocysts from control and exp IDD mothers (Fig. 5B). Exp IDD blastocysts have an impaired metabolic and developmental competence due to the lower content of Akt and Erk. Experimental evidence for this view has recently been given by a delay in development and mesoderm initiation, an increase in apoptotic cells in the embryonic disc and a decrease in the expression of enzymes related to glucose homeostasis (HK2 and PEPCK) reported for exp IDD blastocysts by Ramin et al. (2010) and Thieme et al. (2012). To further elucidate the responsiveness of blastocysts from exp IDD mothers we have treated exp IDD blastocysts with physiological concentrations of IGF1 (1.3 nM). Exp IDD blastocysts show a lower capacity of IGF1 to activate Akt and Erk (Fig. 5C). The increase in IGF1 and IGF2 ligands can therefore be seen as an adaptation to gap growth factor activity in blastocysts during diabetic pregnancy.

In this context it is important to note that the ability to compensate for kinase activity is different between Erk1 and Erk2. Erk2 activation contributes to trophoblast formation in the mouse and proliferation of polar trophectoderm cells (Saba-El-Leil et al., 2008). However, Erk1 is unable to compensate for a lack in Erk2 activation for trophoblast formation (Saba-El-Leil et al., 2008), while disturbances in Erk1 do not result in any developmental phenotype (Pagès et al., 1999). A disturbed Erk/MAPK activation has been shown to result in defective placenta development (Rossant and Cross, 2001). Erk activation can be seen as one of the most important proliferative signals to promote cell cycle progression during whole embryonic development (Li et al., 2011).

5. Conclusion

A direct link may exist between the disarranged IGF/insulin axis with a lack in kinase activation, disturbed activation of Wnt signalling responsible for mesoderm and heart development in early embryogenesis, and the reduced insulin and IGF1 receptor expression (Ramin et al., 2010). A disarranged Wnt signalling repertoire (ligand, receptors and intracellular signalling molecules) in foetal hearts by maternal diabetes has been shown in mice (Pavlinkova et al., 2008,2009). In the rabbit insulin has the capacity to initiate mesoderm formation via Wnt3a and Wnt4, two of the most potent factors for mesoderm induction, and the Erk signalling pathway in vitro. Wnt3a expression is down regulated in gastrulating blastocysts from exp IDD (Thieme et al., 2012).

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cAMP-Responsive Element Binding Protein: A Vital Link in Embryonic Hormonal Adaptation

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The transcription factor cAMP responsive element-binding protein (CREB) and activating transcription factors (ATFs) are downstream components of the insulin/IGF cascade, playing crucial roles in maintaining cell viability and embryo survival. One of the CREB target genes is adiponectin, which acts synergistically with insulin. We have studied the CREB-ATF-adiponectin network in rabbit preimplantation development in vivo and in vitro. From the blastocyst stage onwards, CREB and ATF1, ATF3, and ATF4 are present with increasing expression for CREB, ATF1, and ATF3 during gastrulation and with a dominant expression in the embryoblast (EB). In vitro stimulation with insulin and IGF-I reduced CREB and ATF1 transcripts by approximately 50%, whereas CREB phosphorylation was increased. Activation of CREB was accompanied by subsequent reduction in adiponectin and adiponectin receptor (adipoR)1 expression. Under in vivo conditions of diabetes type 1, maternal adiponectin levels were up-regulated in serum and endometrium. Embryonic CREB expression was altered in a cell lineage-specific pattern. Although in EB cells CREB localization did not change, it was translocated from the nucleus into the cytosol in trophoblast (TB) cells. In TB, adiponectin expression was increased (diabetic 427.8 $\pm$ 59.3 pg/mL vs normoinsulinaemic 143.9 $\pm$ 26.5 pg/mL), whereas it was no longer measureable in the EB. Analysis of embryonic adipoRs showed an increased expression of adipoR1 and no changes in adipoR2 transcription. We conclude that the transcription factors CREB and ATFs vitally participate in embryo-maternal cross talk before implantation in a cell lineage-specific manner. Embryonic CREB/ATFs act as insulin/IGF sensors. Lack of insulin is compensated by a CREB-mediated adiponectin expression, which may maintain glucose uptake in blastocysts grown in diabetic mothers. **(*Endocrinology* 154: 2208–2221, 2013)**

A distinct activation pattern of transcription factors is required for normal development and survival of mammalian embryos. Growth hormones and metabolic factors like glucose control transcription factor activity. The family of insulin and IGFs plays a fundamental role in early embryo development, mediating mitogenic, antiapoptotic, and anabolic effects (1, 2). IGF-I and IGF-II are produced by the embryo and by reproductive tissues in the human (3), rabbit (4, 5), and mouse (6, 7). The production of IGF-I in bovine preimplantation embryos is controversially discussed with publications reporting IGF-I by the embryo (7) or not (8, 9). Insulin has to be provided by the mother, because the embryo cannot produce insulin itself in mammals (3, 4, 6, 7). Insulin and IGFs can partly compensate for each other. So far, few attempts have been made to investigate mechanisms of insulin compensation and replacement during early pregnancy (5).

Adiponectin is the most abundantly secreted adipokine with a 1000 times greater serum concentration than that of others. Adiponectin exerts its biological function by

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Abbreviations: adipoR, adiponectin receptor; ATF, activating transcription factor; BSM, basal synthetic medium; CRE, cAMP responsive element; CREB, CRE-binding protein; CREM, CREB modulator protein; EB, embryoblast; exp IDD, experimentally induced insulin-dependent diabetes; GAPDH, glyceraldehyde-3-phosphate; IGF-IR, IGF-I receptor; IR-A, insulin receptor isoform A; 3-OMG, 3-*O*-methyl-d-[1-^{3}H]glucose; p.c., postcoitum; qPCR, quantitative PCR; TB, trophoblast.

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binding to the adiponectin receptor (adipoR)1 or adipoR2. Both receptors are transmembrane proteins and located in embryoblast (EB) inner cell mass and trophoblast (TB) cells (10). Adiponectin is an important insulin-sensitizing adipokine (11–14). Adiponectin production and functional signaling has previously been shown in rabbit and murine preimplantation embryos (10, 15, 16, for review see Ref. 17). Adiponectin has profound effects on glucose metabolism by enhancing embryonic glucose uptake and translocation of the solute carrier family 2 (facilitated glucose transporter), member 4 (also known as glucose transporter 4) from the cytosol to the cell membrane.

Overexpression of adiponectin improves insulin sensitivity, and down-regulation of adiponectin goes along with insulin resistance and obesity (11, 18–20). The expression of adiponectin and its receptors are regulated by several extracellular signals and hormones, including IGF-I, insulin, and TNFα (21, 22). A potential mediator for the transcriptional regulation of adiponectin is the cAMP responsive element (CRE)-binding protein (CREB) transcription factor (23–25).

CREB acts as a downstream effector in many signaling pathways. These pathways are mainly regulated in response to growth factors, stress signals, intracellular Ca^{2+}, and specific peptides. So far, more than 300 different signals are known to activate CREB. The rate-limiting step of CREB activity is the phosphorylation of CREB at the residue Ser133 (26). A phosphorylation of CREB is essential for dimerization and binding to CREB-binding protein. After binding to the CRE element, the transcription of target genes is activated.

Activating transcription factor (ATF)1 and CREB modulator protein (CREM) are members of the CREB/ATF family. Both factors, ATF1 and CREM, can form heterodimers with CREB and induce transcription (27, 28). Furthermore, CREB and ATF1, sharing an overall 70% sequence homology, are expressed ubiquitously, whereas CREM is predominantly expressed in neuroendocrine tissue (27, 28).

Previous studies failed to detect CREM in the preimplantation embryo but showed a strong coexpression of CREB and ATF1 during mouse preimplantation development (29, 30). A deletion of CREB and ATF1 results in embryonic death before implantation (29). During embryo development, CREB and ATF1 can compensate for each other. However, ATF1 possesses a lower transcriptional activity than CREB due to a lack of the glutamine-rich domain (31). The phosphorylation of CREB and its nuclear localization has first been observed in the 2-cell embryo stage of the mouse at the onset of embryonic genome activation (30). In *Xenopus laevis*, CREB regulates cell specification in early development (32). An inhibition of CREB at blastula and early gastrula stages as well as at the beginning of neurulation has deleterious effects on embryogenesis with malformations, such as microcephaly and spina bifida (33)

The functional properties of other family members, ATF3 and ATF4 (also called CREB-2), are poorly understood, particularly in early embryo development. Both transcription factors can function as transcriptional activators or repressors. The ATF3 knockout mouse does not exhibit any distinguishable phenotype (34). However, mice deficient in ATF4 are 50% smaller than their wild-type counterparts and suffer from a variety of developmental defects (35–37). ATF4 acts as a negative regulator of insulin secretion and insulin sensitivity in liver, muscle, and fat (38, 39). The ATF4−/− mutant mouse is resistant to diet-induced and age-dependent obesity and diabetes (37).

We investigated the CREB-ATF network during preimplantation embryo development in the rabbit (40). We have characterized the expression and localization of CREB and the ATFs in rabbit blastocyst gastrulation in normal development and under experimentally induced insulin-dependent diabetes (exp IDD). Furthermore, we have stimulated blastocysts with insulin and IGFs in vitro. Our results show that insulin and IGF-I down-regulate adiponectin and adipoR1 expression in vitro. A maternal diabetes mellitus type 1 increases embryonic adiponectin and adipoR1 expression mediated by CREB and ATF transcription factors.

Materials and Methods

Embryo recovery

Embryos were collected from sexually mature rabbits stimulated with 110 IU pregnant mare serum gonadotropin sc (Intervet, Unterschleißheim, Germany) 3 days before mating. After mating, 75 IU human choriongonadotropin was injected iv (Intervent). Mating and embryo recovery were performed as described (41). On days 3, 4, and 6 postcoitum (p.c.), embryos were flushed from oviducts or uteri, respectively, washed 3 times with PBS, and randomly divided among the experimental groups. On day 6 p.c., gastrulation stages can be reliably discriminated in the rabbit, because implantation starts on day 6 18 hours, ie, half a day later (30).

In vitro culture

To study the effects of insulin and IGF-I on CREB, adiponectin, and ATF expression, day 6 blastocysts were cultured in groups of 5-10 at 37°C in a water-saturated atmosphere of 5% O_2, 5% CO_2, 90% N_2 in a water-jacketed incubator (HERAcell 150i, Heraeus; Thermo Fisher Scientific, Bonn, Germany). Blastocysts were precultured for 2 hours in serum- and insulin-free basal synthetic medium (BSM) (42). Afterwards, either 17nM

insulin (Invitrogen, Karlsruhe, Germany), 1.3nM IGF-I, or 13nM IGF-II (Sigma, Taufkirchen, Germany) was added to the culture medium, and the culture was continued for different time intervals (10 min to 12 h). The concentrations comply with physiological ranges measured within the Fallopian tube and uterine cavity in the human (3, 43) and rhesus (43) and have been employed in various other studies before (4, 44–46). They are known to specifically activate IGF-I receptor (IGF-IR) (IGF-I), insulin receptor isoform A (IR-A) and IGF-IR (IGF-II), and IR-A and IR-B (insulin) (47).

Controls were cultured without insulin, IGF-I, or IGF-II but otherwise treated identically. Phosphorylation of CREB was analyzed after culture in the presence or absence of insulin, IGF-I, or IGF-II for 10 minutes. To analyze transcriptional regulation, blastocysts were cultured with or without insulin or IGF-I for 6 and 12 hours, respectively.

The influence of glucose on adiponectin transcription was analyzed in blastocysts cultured with 0mM, 10mM, or 25mM glucose. Day 6 p.c. blastocysts were cultured in groups of 5-6 for 6 hours.

Alloxan treatment

exp IDD was induced in 18- to 20-week-old female nonpregnant rabbits by alloxan (Sigma) treatment as described before (4). Rabbits were hold in diabetic conditions with permanent blood glucose concentrations of more than 14 mmol/L by regular insulin supplementation 4 times per day (Insuman Rapid and Lantus; Sanofi-Aventis, München, Germany), started at the second day after alloxan treatment. The blood glucose level was monitored with MediSense Precision Xceed Diabetes Management System (Abbott, Wiesbaden, Germany) 2 times per day. Therefore, fresh blood was collected at 10 AM and 6 PM by puncturing the vena auricularis lateralis and tested for glucose concentration by a commercial test strip.

All animal experiments were conducted in accordance with the principles of laboratory animal care, and the experimental protocol had been approved by the local ethical commission of the Landesverwaltungsamt Dessau (reference number 42502-2-812).

RNA isolation and cDNA synthesis

mRNA of single blastocysts was extracted with Dynabeads Oligo$(dT)_{25}$ (Invitrogen) and subsequently used for cDNA synthesis. All protocol procedures were carried out according to the manufacturer's instructions, with modification described previously by Tonack et al (48).

Reverse transcription-polymerase chain reaction

RT-PCR amplification was carried out with 0.5 μL cDNA from single blastocysts in a 25 μL volume containing 200μM each deoxyribonucleotide, 2.5 U *Taq* polymerases, and specific oligonucleotides for CREB, ATF1, ATF2, ATF4, adiponectin, and glyceraldehyde-3-phosphate (GAPDH) (primers listed in Table 1). Nucleotide sequence for rabbit CREB and ATF1 were determined using human primers for amplification of rabbit liver cDNA. The amplification was done for 40 cycles (94°C for 45 sec, 60°C for 45 sec, and 72°C for 60 sec). Resulting PCR products were separated by electrophoresis on 2% agarose gel and stained with ethidium bromide.

RT-qPCR

Real-time analyses (RT-quantitative PCR [qPCR]) were performed as duplicates by using the Applied Biosystems StepOnePlus System (Applied Biosystems, Darmstadt, Germany) with a no template control for each primer set as described in Thieme et al (5). The nucleotide sequences of the primers used in this study are listed in Table 1. GAPDH was simultaneously quantified as endogenous control, and target gene expression was normalized to that of GAPDH in each sample. GAPDH was shown to be unaffected by the treatment, because no variation in the absolute GAPDH levels compared with GAPDH RNA levels in blastocysts from healthy rabbits were observed (5). Analysis of the individual data therefore yielded values relative to these standards. Data are expressed as percentage relative to control blastocysts.

Protein preparation and immunoblotting

Protein preparation, quantification, and Western blotting were performed with 8-10 blastocysts as described in Fischer et al (10). For Western blot analysis, 25 μg total protein lysates were subjected to sodium dodecyl sulfate-polyacrylamide electrophoresis and electrotransferred to nitrocellulose membranes. For detection of phospho-CREB, CREB and β-actin membranes were blocked in Tris-buffered saline containing 0.1% (vol/vol) Triton X-100 with 3% (wt/vol) nonfat dry milk at room temperature for at least 1 hour. For ATF1 and ATF3 detection,

Table 1. Primers used for RT-PCR and RT-qPCR

Gene name	GenBank number	Temperature (°C)	Fragment (bp)	Sequence 5′→3′
Adiponectin	DQ334867	60	158	fw: cctggtgagaagggtgaaaa rev: gctgagcggtagacataggc
ATF1	NM_005171	60	197	fw: caacctggttcagcagttc rev: tttctgccccgtgtatcttc
ATF3	XM_002717521	60	163	fw: cgctggtgtttgaggatttt rev: ctgactccagtgcagacgac
ATF4	LOC100339383	60	193	fw: gcgagaagctggagaagaag rev: tccagcaggtccttgaggta
CREB	NM_004379.3	60	169	fw: gtatgcacagaccacggatg rev: tgcaggctgtgtaggaagtg
GAPDH	L23961	60	144	fw: gccgcttcttctcgtgcag rev: atggatcattgatggcgacaacat

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membranes were blocked in Tris-buffered saline containing 0.1% (vol/vol) Triton X-100 with 3% (wt/vol) BSA for 1 hour. The primary antibody was incubated at 4°C overnight. Antibodies were as following: phospho-CREB (no.9196, 1:1000; Cell Signaling Technology, Beverly, Massachusetts), CREB (no. 9104, 1:1000; Cell Signaling Technology), ATF1 (sc-243, 1:1000; Santa Cruz Biotechnology, Inc, Santa Cruz, California), ATF3 (sc-81189, 1:500; Santa Cruz Biotechnology, Inc), β-actin (A-5441, 1:40 000; Sigma), and antimouse IgG conjugated to horseradish peroxidase (115-036-003, 1:45 000; Dianova, Hamburg Germany). The amounts of phosphorylated proteins were evaluated by stripping the membranes and reblotting with the nonphosphorylated protein antibody. The amount of phosphorylated CREB relative to CREB was measured in each sample by image analysis. Protein phosphorylation was calculated as the ratio of band intensities (phosphorylated protein vs nonphosphorylated protein) in the same blot to correct for differences in protein loading.

Immunohistochemical localization of CREB, ATFs, and adiponectin

Blastocysts were washed twice in ice-cold PBS and fixed in 4% (wt/vol) paraformaldehyde at 4°C overnight. Preparation and immunohistochemical protocol were performed as described in Fischer et al (10). Antibodies for CREB (Cell Signaling Technology) and ATF1 and ATF3 (both Santa Cruz Biotechnology, Inc) were diluted 1:400 in 3% (wt/vol) BSA/PBS. For adiponectin detection, the antibody (ab 22554; Abcam, Cambridge, Massachusetts) was diluted 1:100 in (wt/vol) BSA/PBS. The secondary antibody Dako EnVision + System-HRP labeled Polymere antimouse (1:1 in PBS, K4001; Dako, Glostrup, Denmark) and diaminobenzidine (WAK-Chemie Medikal, Steinbac, Germany) were used for detection.

For immunofluorescence detection, a secondary antibody conjugated with fluoresceinthiocyanate (1:250 dilution) was used. The nuclei were counterstained with Hoechst for 5 minutes. All steps were performed within the same experiment, examined microscopically during the same session, using identical microscope and camera settings.

Adiponectin ELISA

Adiponectin concentrations in embryonic tissue and blastocysts fluid were measured by ELISA (AdipoGen, San Diego, California). All protocol procedures were carried out according to the manufacturer's instructions. To measure serum adiponectin concentration, blood samples were collected with S-Monovetten (Sarstedt, Nümbrecht, Germany), left to coagulate for at least 30 minutes, and centrifuged for 10 minutes by 4°C and 1000*g*. The supernatant was stored at −80°C until use. Endometrium samples were collected by opening the uterus on the antimesometrial side. Endometrium was removed mechanically from the myometrium with a scalpel and stored at −80°C for subsequent protein preparation and analysis by ELISA. Flushed blastocysts were washed 3 times with ice-cold PBS. Blastocyst stage and size were determined. Blastocysts were then placed on a dry watch glass. Extracellular coverings were removed mechanically, blastocysts were punctured, and the effluent cavity fluid was collected. Blastocyst fluids were stored at −80°C until use. Blastocysts were then separated into TB and EB for further analyses (45). EBs and TBs of single blastocyst samples were stored in PBS at −80°C for subsequent protein preparation and analysis by ELISA. In vitro-cultured blastocysts were washed twice in ice-cold PBS and stored in radioimmunoprecipitation buffer at −80°C until use. Adiponectin concentrations were quantified in duplicate and compared with an internal adiponectin standard (0.1-32 ng/mL).

Glucose transport studies

Glucose uptake was measured as previously described (10). After preculture for 2 hours, embryos were washed 3 times in glucose-free BSM II media and transferred into 600-μL pulse droplets, kept strictly at 37°C for 3 minutes. The glucose-free pulse medium contained 0.3mM 3-*O*-methyl-d-[1-^{3}H]glucose (3-OMG) (37 GBq/L; Amersham, Piscataway, New Jersey) and 25mM 3-OMG (Sigma-Aldrich, Munich, Germany). Uptake was stopped after 3 minutes by transferring the embryos through 4 washes of ice-cold, glucose-free BSM II media. The diameters of the embryos were recorded using a calibrated ocular micrometer. Radioactivity of individual embryos was determined in a Packard 1600TR liquid scintillation analyzer. Uptake of 3-OMG is expressed as nanomoles per minute per surface area (cm^2).

Statistics

All data are expressed as means ± SEM. Levels of significance between groups were calculated by factorial ANOVA with Bonferroni adjustment. $P < .05$ was considered statistically significant. All experiments were conducted at least 3 times.

Results

Expression of CREB in rabbit embryos

In 3-day-old morulae, the transcription of CREB was not detectable, but it was present in early blastocysts on day 4 p.c. (Figure 1A). Blastocysts at day 6 showed an increase in CREB transcript levels during gastrulation from stage 1 to stage 3 compared with stage 0 (Figure 1B). A corresponding increase in CREB protein amount from stage 1 to stage 2 was verified by Western blotting (Figure 1, C and D). Using the same anti-CREB antibody for immunohistochemical detection, CREB was localized in both EB and TB with the most prominent localization in the nuclei of EB cells (Figure 1E).

Activation of CREB by insulin, IGF-I, and IGF-II in in vitro-cultured blastocysts

Transcriptional activation of CREB is commonly associated with phosphorylation at residue Ser133 and nuclear translocation. To clarify whether CREB can be activated by insulin and IGFs, we stimulated blastocysts with 17nM insulin, 1.3nM IGF-I, or 13nM IGF-II for 10 minutes in vitro. The antibody used in the phosphorylation assay detected specifically CREB phosphorylated at Ser133 (phospho-CREB Ser133). Supplementation led to an increased amount of phosphorylated CREB (Figure 1G). The rela-

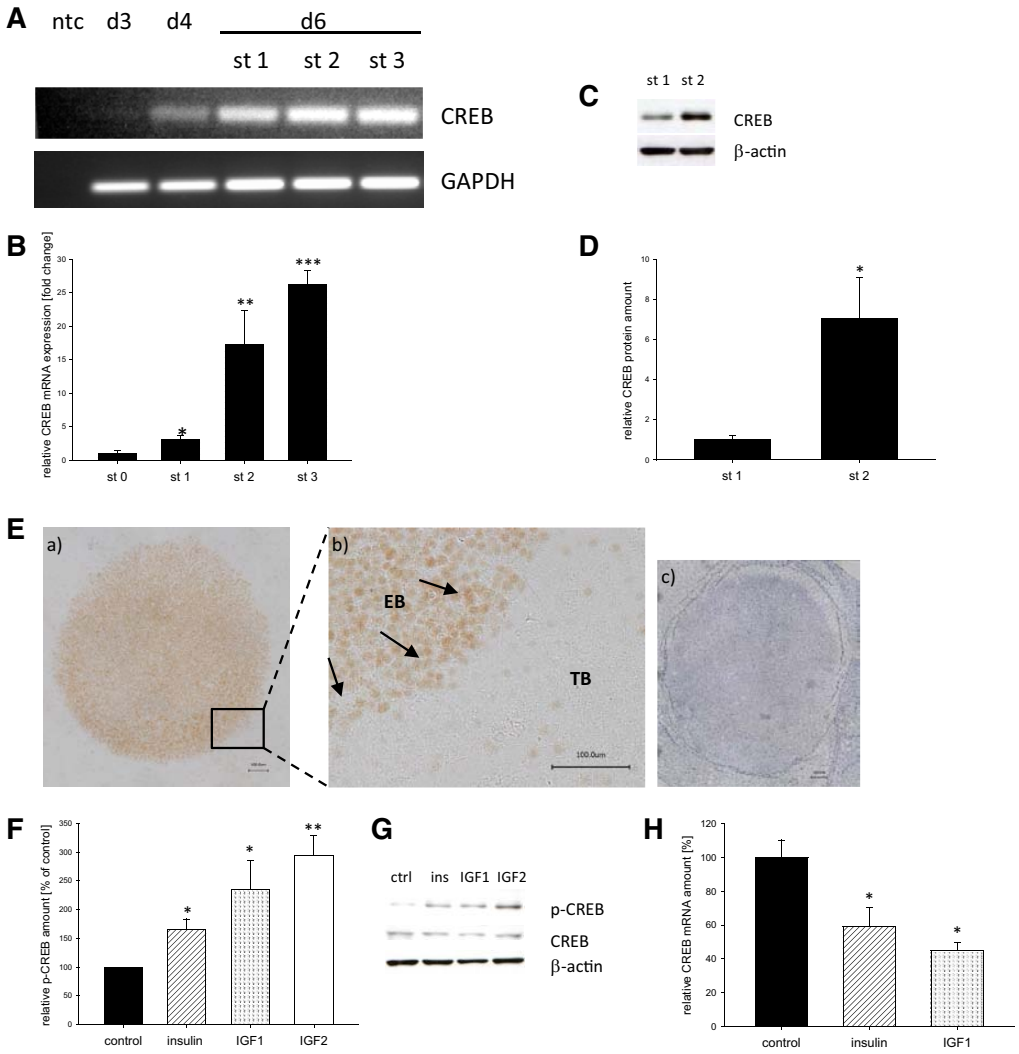

Figure 1. Expression of CREB in rabbit preimplantation embryos. (A) Transcripts of CREB were detected in day 3, 4, and 6 p.c. embryos and blastocysts in gastrulation stage 1 (st 1), stage 2 (st 2), and stage 3 (st 3). A probe without cDNA was used as negative control (ntc). Internal control was the expression of GAPDH in all probes. (B) Transcript amount of CREB was increased during gastrulation. The amount of CREB mRNA was measured by RT-qPCR in 9 blastocysts per group in 3 independent experiments (N = 3, n = 9). For normalization, RT-qPCR was performed for the housekeeping gene GAPDH. The results are shown as mean ± SEM (*$P < .05$; **$P < .01$; ***$P < .001$). (D) Relative CREB protein amount in day 6 p.c. blastocysts was analyzed by Western blotting with an anti-CREB antibody and related to β-actin (mean ± SEM; N = 3, n = 10; *$P < .05$). A representative Western blotting for CREB is shown in C. (E) Immunohistochemical analysis of CREB revealed a prominent localization of CREB in the nucleus of EB cells (a) (with a higher magnification; b). The CREB protein was visualized by peroxidase-diaminobenzidine reaction (brown color). A control reaction is shown in c. In vitro stimulation of blastocysts with 17nM insulin (ins), 1.3nM IGF-I, or 13nM IGF-II led to a significantly higher phospho-CREB amount (*$P < .05$; **$P < .01$). The phosphorylation of CREB was analyzed by Western blotting with antiphospho-CREB antibody and set in relation to total CREB protein. A representative Western blotting for CREB is shown (G). Results of 3 stimulation experiments (N= 3, n = 10; mean ± SEM) are shown in F. (H) RT-qPCR analysis of long-time (12 h) in vitro-stimulated blastocysts revealed a reduced amount of CREB RNA. Culture of blastocysts was performed in groups of at least 5 blastocysts with or without 17nM insulin and 1.3nM IGF-I and with N = 3, n ≥ 5. The results are shown as mean ± SEM (*$P < .05$; **$P < .01$). ctrl, control.

tive increase in phosphorylation is shown in Figure 1F. The in vitro stimulation with insulin or IGF-I for 12 hours led to a reduction in CREB RNA amounts (Figure 1H), indicating a negative feedback loop in embryos.

Expression of ATFs in preimplantation embryo

ATF1 is highly related to CREB and can compensate its absence. Starting on day 4 p.c., ATF1 expression increased in all embryonic stages investigated (Figure 2, A and B).

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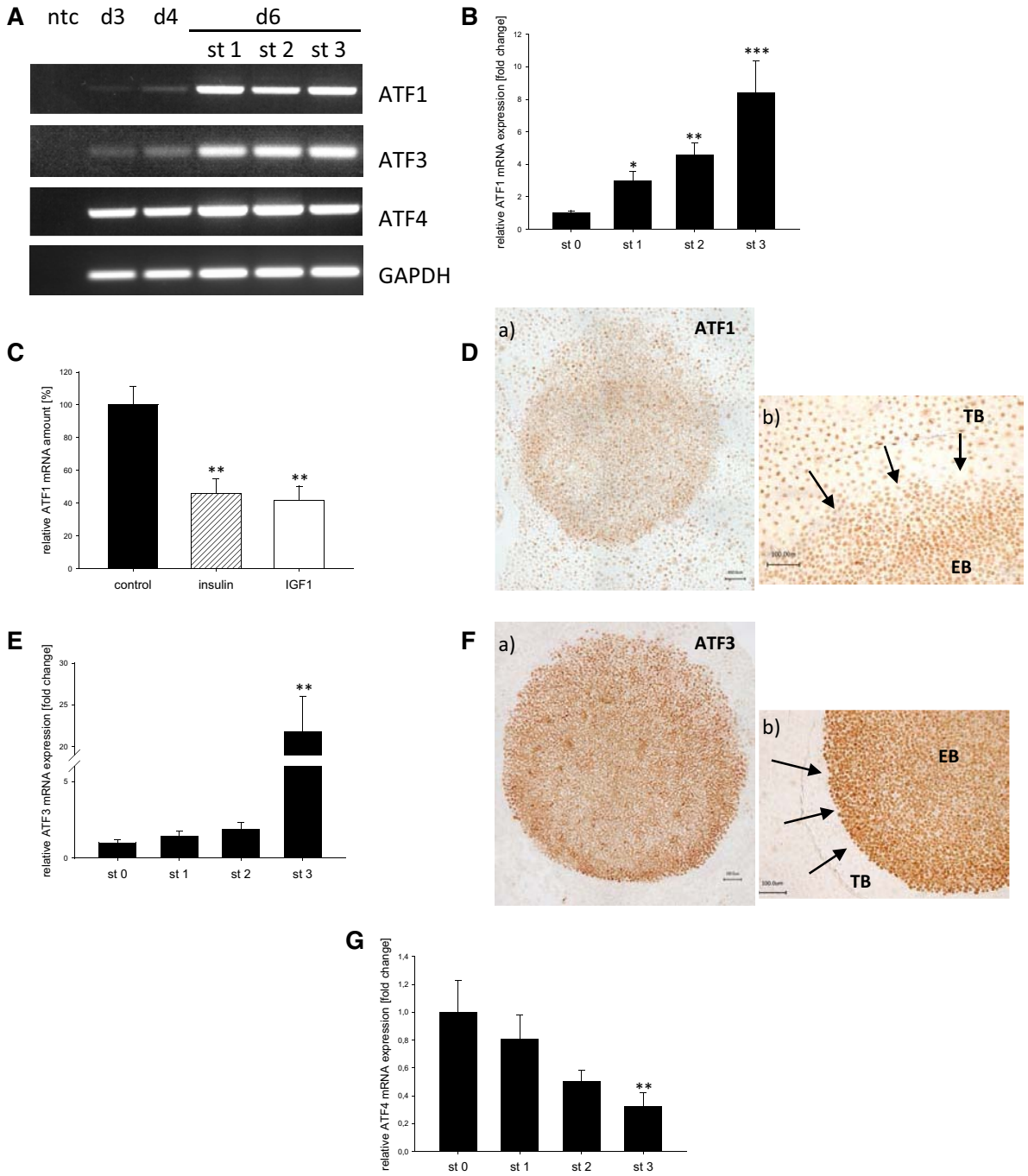

Figure 2. Expression of ATF family in rabbit preimplantation embryos. (A) Analysis by RT-PCR shows an expression from day 4 to 6 p.c. for ATF1 and from day 3 to 6 p.c. for ATF3 and ATF4. Blastocysts were staged in stage 1 (st 1), 2 (st 2), or 3 (st 3). A probe without cDNA was used as negative control (ntc). GAPDH was used as internal control. mRNA levels of ATF1 (B), ATF3 (E), and ATF4 (G) were quantified on day 6 p.c. by RT-qPCR. The experiment was performed from 9 blastocyts per stage in (N = 3, n = 9). Expression of ATF1, ATF3, and ATF4 was normalized to GAPDH, and results are shown as mean ± SEM relative to stage 0 (*$P < .05$; **$P < .01$; ***$P < .001$). (C) Long-time in vitro stimulation with or without insulin or IGF-I was performed in groups of at least 5 blastocysts in each treatment and repeated in 3 independent replicates (N = 3, n ≥ 5). Expression of ATF1 in the nontreated group (control) was set 100%. Culture with insulin or IGF-I led to a decreased ATF1 expression compared with the corresponding nonstimulated control (*$P < .05$; **$P < .01$). Immunohistochemical analysis of ATF1 revealed an expression in nuclei of EB cells (a) (with a higher magnification, b). ATF3 is shown in F (a) (with a higher magnification, b). Contrary to CREB and ATF3 (EB staining), ATF1 was also localized in the nuclei of TB cells (D).

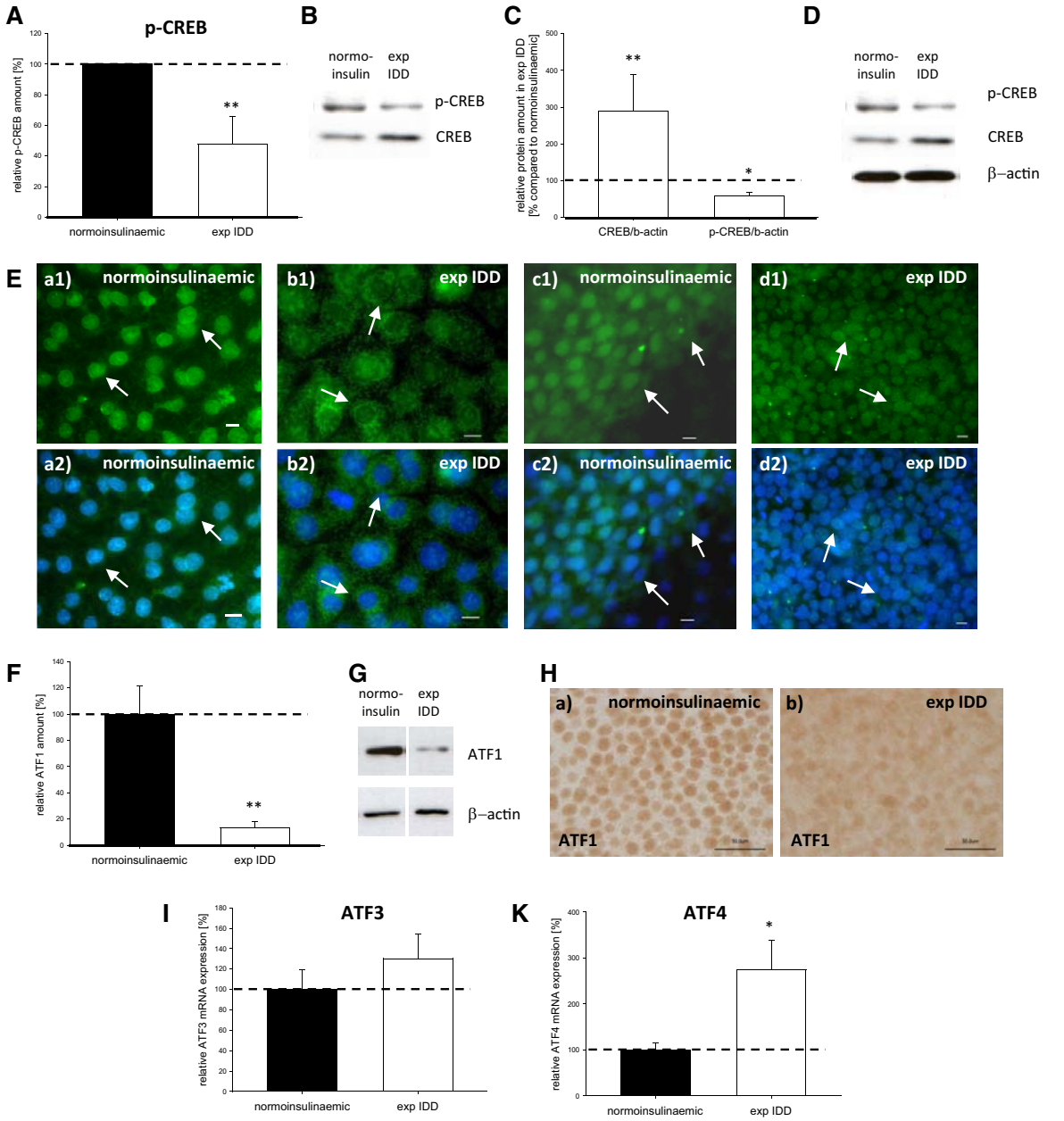

Figure 3. Expression pattern of the CREB family in blastocysts from diabetic rabbits. (A) The amount of phosphorylated CREB was quantified by Western blotting with an antiphospho-CREB-specific antibody in relation to nonphosphorylated protein. Results are shown as mean ± SEM (N = 3, n = 10). The amount of phosphorylated CREB was significantly lower in blastocysts from diabetic rabbits (exp IDD) (*$P < .05$). (B) Representative Western blotting. (C) Amounts of CREB protein in blastocysts from normoinsulinaemic and diabetic rabbits (exp IDD) were quantified by Western blotting and calculated in relation to β-actin (mean ± SEM; N = 3, n = 10). In blastocysts from diabetic rabbits (exp IDD), CREB amount was significantly increased (*$P < .05$). A representative Western blotting is shown (D). (E) Immunofluorescent detection of CREB (green color) in blastocysts from normoinsulinaemic (a) and exp IDD (b) rabbits show weaker nuclear localization in TB of diabetic blastocysts. In the EB, CREB was localized in the nuclei of blastocysts from normoinsulinaemic (c) and diabetic (d) (exp IDD) rabbits. Nuclei were counterstained with Hoechst (blue) (a2, b2, c2, and d2). Arrows indicate cells with a cytoplasmatic localization (b1 and b2) or a nuclear localization (a1, a2, c1, c2, d1, and d2) of CREB. (F) Quantification of ATF1 protein revealed a significant decrease in blastocysts from diabetic rabbits (exp IDD) (**$P < .01$). Western blotting was performed by using a specific anti-ATF1 antibody and set in relation to β-actin amount in the same probes (mean ± SEM; N = 3, n = 10). (G) Representative Western blotting. (H) Immunohistochemical detection of ATF1 (brown color) showed a weaker nuclear localization in the EB of blastocysts from diabetic rabbits (exp IDD) (H). The relative amounts of ATF3 (I) and ATF4 (K) mRNA were measured by RT-qPCR in at least 8 blastocysts per group. The results are shown as mean ± SEM (*$P < .05$).

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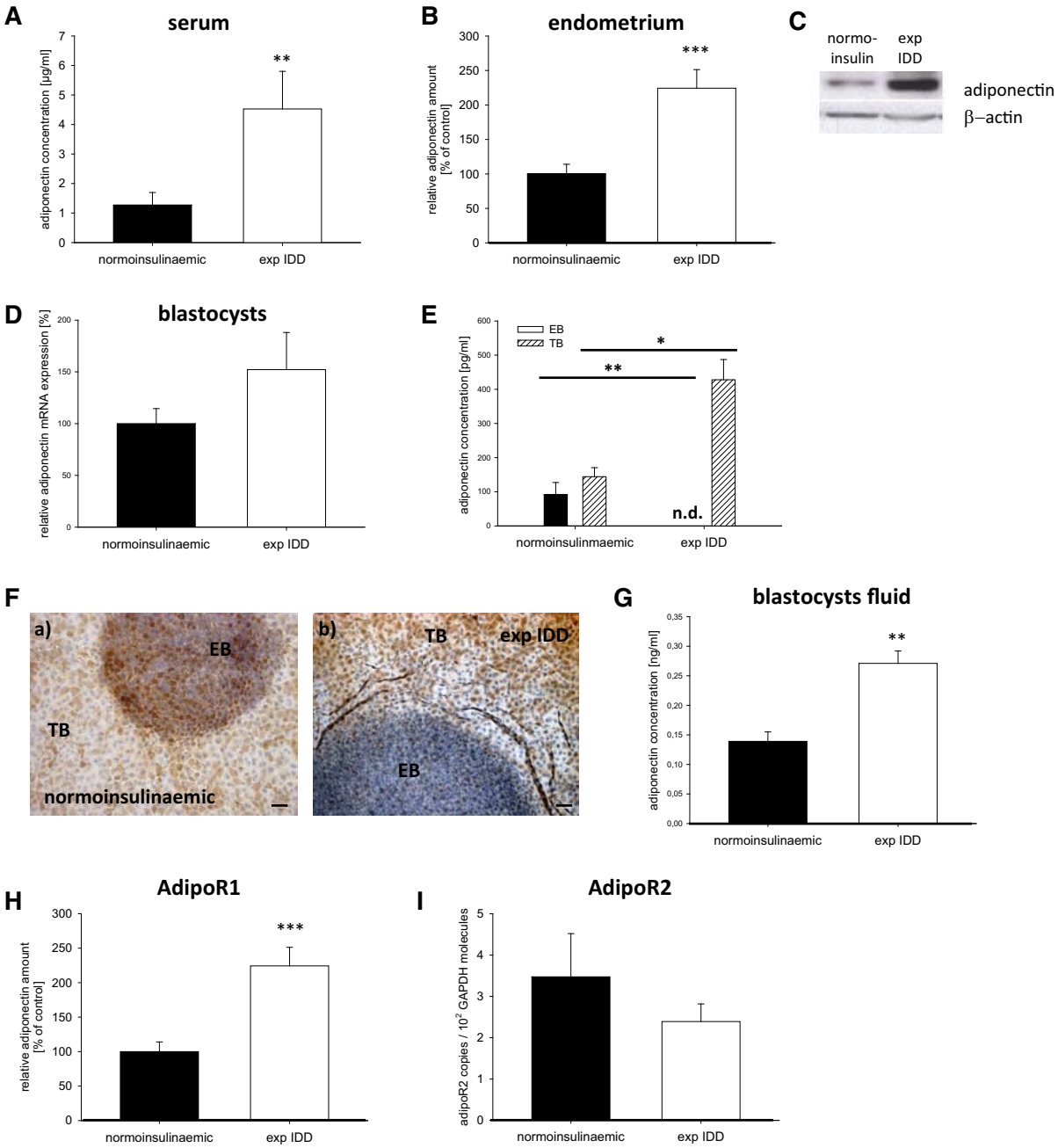

Figure 4. Adiponectin expression in normoinsulinaemic and diabetic rabbits. (A) In the serum of normoinsulinaemic and diabetic rabbits (exp IDD), adiponectin concentration was determined by ELISA (mean ± SEM; N = 3, n = 11; $^{**}P < .01$). (B) Western blot analysis revealed increased amounts of adiponectin in endometrium of diabetic rabbits (exp IDD) (mean ± SEM; N = 3, n = 10; $^{***}P < .001$). (C) Representative Western blotting. Expression of adiponectin in the endometrium of normoinsulinaemic rabbits was set to 100%. (D) mRNA amount of adiponectin was quantified by RT-qPCR in blastocysts from normoinsulinaemic and diabetic (exp IDD) rabbits (N = 3, n = 9). (E) Adiponectin protein amount was quantified by ELISA in EB and TB of single blastocysts from normoinsulinaemic and exp IDD rabbits (n.d., not detectable; mean ± SEM; N = 3, n ≥ 8; $^{*}P < .05$, $^{**}P < .01$). (F) Immunohistochemical detection of adiponectin (brown color) showed a diminished nuclear localization in the EB and a more intensive staining in the TB of blastocysts from diabetic rabbits (exp IDD). (F) Representative examples (a, in blastocyst from a normoinsulinaemic rabbit; b, from an exp IDD rabbit). (G) Adiponectin concentration was analyzed by ELISA in blastocyst fluid of single blastocysts from normoinsulinaemic and exp IDD rabbits (mean ± SEM; N = 3, n ≥ 8; $^{**}P < .01$). adipoR1 and adipoR2 were quantified by RT-qPCR with an increased adipoR1 RNA (H) and no changes in adipoR2 RNA in blastocysts from diabetic rabbits (exp IDD) (I). The experiments were performed from 9 blastocysts per stage (mean ± SEM, N = 3, n = 9; $^{***}P < .001$).

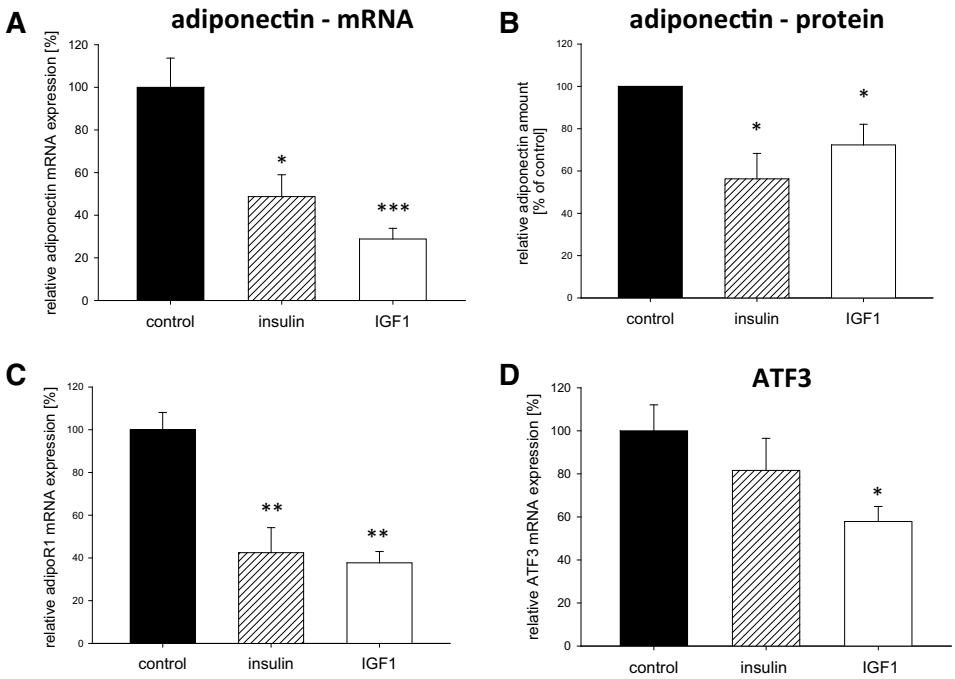

Figure 5. Regulation of adiponectin, ATF3, and ATF4 by insulin and IGF-I. (A) Adiponectin RNA was quantified by RT-qPCR in single blastocysts after a 12-hour culture with 17nM insulin or 1.3nM IGF-I. Stimulation with insulin or IGF-I led to a decreased adiponectin amount compared with the corresponding nonstimulation control (set 100%) (N = 3, n ≥ 5; $*P < .05$, $***P < .001$). (B) Adiponectin protein concentration was determined in blastocysts after a 12-hour culture with insulin or IGF-I and compared with the nontreated control (set 100%). Adiponectin concentration were determined by ELISA (mean ± SEM; N = 3, n ≥ 9; $*P < .05$). (C) Expression of adipoR1 was quantified after a 12-hour culture with or without 17nM insulin or 1.3nM IGF-I. Compared with the corresponding nonstimulated controls (set 100%) the expression of adipoR1 was significantly decreased (N = 3, n ≥ 5; $**P < .01$). Expression of ATF3 (D) and ATF4 (E) was analyzed by RT-qPCR after in vitro stimulation with or without insulin or IGF-I in groups of at least 5 blastocysts in each treatment and repeated in 3 independent replicates (N = 3, n ≥ 5). Transcriptions of ATF3 and ATF4 were quantified in blastocysts after culture for 6 and 12 hours, respectively. Amounts in the nontreated group (control) were set 100%. mRNA level of ATF4 in EB and TB of normoinsulinaemic and diabetic (exp IDD) rabbits was quantified by RT-qPCR (F). The experiment was performed from 5 blastocysts per stage (mean ± SEM; N = 3, n = 5; $*P < .05$). Adiponectin transcription level was analyzed after a 6-hour culture in media containing 0mM, 10mM, or 25mM glucose. The amounts of adiponectin mRNA levels in 0mM group were set 100%. No effect on adiponectin transcription level was observed (mean ± SEM; N = 3, n ≥ 5).

The quantification of ATF1 mRNA in in vitro-stimulated embryos (17nM insulin or 1.3nM IGF-I) showed a decrease in transcript levels (Figure 2C), as seen for CREB too. The ATF1 protein was also localized in the nucleus of embryonic cells, but unlike CREB, the staining had the same intensity in both cell lineages (Figure 2D).

ATF3 acts as important negative regulator of adiponectin expression in obesity and type 2 diabetes mellitus. As shown in Figure 2A, ATF3 was detectable in all investigated embryo stages (d 3; d 4, early blastocyst) with an increased expression in stage 3 blastocysts compared with stage 0 (Figure 2E). The ATF3 protein was mainly localized in nuclei of EB cells, with TB cells being hardly stained (Figure 2F).

Unlike CREB, which activates transcription of CRE promoters, the transcription factor ATF4 specifically represses CRE-dependent transcription. Transcription of ATF4 was detected in all stages during preimplantation embryo development (Figure 2A). However, in contrast to CREB and ATF1/3, the transcript level of ATF4 was decreasing during blastocyst development (Figure 2B). An antibody to determine the ATF4 localization is not commercially available.

Expression of CREB and ATFs in blastocysts from diabetic rabbits

In blastocysts grown in diabetic mothers (exp IDD), CREB activation was reduced by 50% (Figure 3, A and B). The CREB protein amount was increased (Figure 3C, left column, and D), confirming the regulatory role of insulin on CREB expression in vivo. However, the total amount of phosphorylated CREB was significantly decreased (Figure 3C, right column, and D). In the TB of diabetic blastocysts, CREB was mainly localized in the cytosol, indicating its inactivity (Figure 3E, normoinsulinaemic a1+a2 and exp IDD b1+b2). In the EB, CREB was still located in the nucleus (Figure 3E, normoinsulinaemic c1+c2 and exp IDD d1+d2). Contrary to CREB, ATF1 expression was dramatically decreased in blastocysts grown under diabetic conditions (Figure 3, F and G). Furthermore, in

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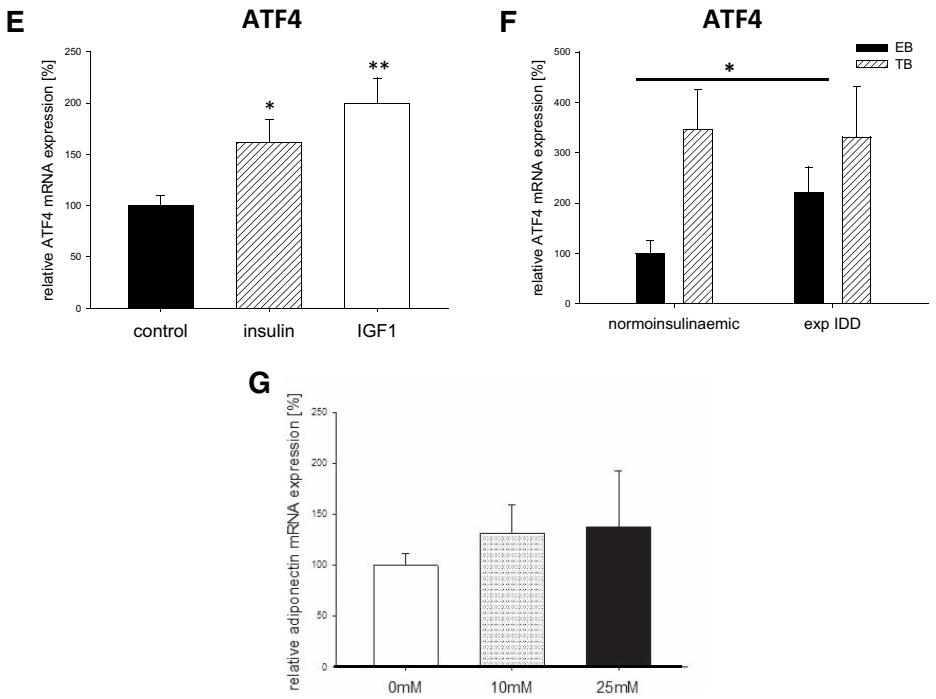

Figure 5. Continued.

the EB of diabetic blastocysts, ATF1 lost its nucleus-specific staining (Figure 3H). No changes on mRNA transcription were observed for ATF3 (Figure 3I), whereas ATF4 expression was significantly increased in blastocysts from diabetic rabbits (Figure 3K).

Adiponectin and receptor (adipoRs) expression in blastocysts from diabetic rabbits

Serum (Figure 4A) and endometrial adiponectin (Figure 4, B and C) were increased in diabetic rabbits. In blastocysts grown in diabetic females, transcriptional changes of adiponectin did not reach statistical significance (Figure 4D). Analysis of adiponectin protein, however, revealed a cell lineage-specific adiponectin distribution pattern. In EBs from diabetic blastocysts, adiponectin was not detectable (Figure 4E), whereas the adiponectin level in the TB was profoundly increased (Figure 4E). Immunohistochemical staining was clearly increased in the TB, whereas the EB almost completely lost its specific staining (Figure 4F). In blastocyst fluid, the adiponectin level was significantly increased (Figure 4G). Compared with corresponding controls, adipoR1 was increased (Figure 4H), whereas no changes in adipoR2 RNA transcript levels were observed (Figure 4I).

Insulin and IGF-I regulate adiponectin, adipoR1, ATF3, and ATF4 in vitro

To further investigate the compensatory role of adiponectin for insulin in blastocysts (Figure 4), we analyzed the influence of insulin and IGF-I supplementation on adiponectin and adipoR1 expression in vitro. After culture of blastocysts for 12 hours with 17nM insulin or 1.3nM IGF-I, a significant decrease in adiponectin transcript levels and protein amounts (Figure 5, A and B) and adipoR1 mRNA levels (Figure 5C) were observed.

It is known that CREB down-regulates adiponectin transcription by the intermediate activation of ATF3, which in turn represses expression of adiponectin (21). An in vitro stimulation with insulin or IGF-I for 1 hour had no effect (data not shown), whereas stimulation for 12 hours led to a decreased expression of ATF3 (Figure 5D). However, the transcript levels of ATF4, acting as a specific repressor of CRE-dependent transcription, were increased after a 6-hour in vitro stimulation (Figure 5E). To confirm the view that ATF4 acts as a potential negative regulator of adiponectin expression, we analyzed the expression of ATF4 in a cell compartment-specific manner. As shown in Figure 5F, the expression of ATF4 was significantly increased in the EB of blastocysts from diabetic mothers, whereas no difference in ATF4 transcript levels were detectable in the TB, confirming the inhibitory role of ATF4 on adiponectin expression.

Finally, blastocysts were cultured in vitro for 6 hours in culture medium containing 0mM, 10mM, or 25mM glucose. As shown in Figure 5G, adiponectin transcript levels were not altered by the different glucose concentrations studied, supporting our hypothesis that adiponectin re-

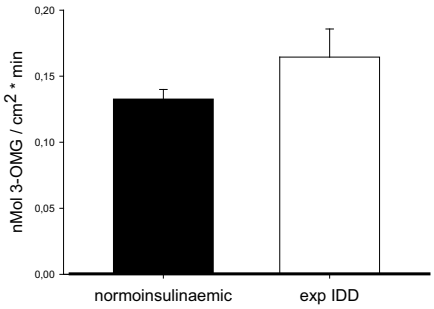

Figure 6. Uptake of 3-OMG in normoinsulinaemic and diabetic rabbit blastocysts. Glucose transport was measured by 3-OMG uptake during a 3-minute pulse period. Data are presented as the mean ± SEM and are expressed as nanomoles of 3-OMG per minute per surface area (cm^2) (see Materials and Methods). Blastocysts grown in a diabetic environment showed the same uptake of 3-OMG as the corresponding controls (N = 3, n = 28).

pression is related to the lack of insulin rather than to hypo- or hyperglycaemia.

Glucose uptake in blastocysts from normoinsulinaemic and diabetic rabbits

To test whether the lack of maternal insulin affects glucose uptake, we analyzed the rate of 3-OMG uptake in normoinsulinaemic and diabetic blastocysts. No difference in 3-OMG uptake was measured (Figure 6), implying a compensatory role of increased adiponectin levels for glucose uptake under hypoinsulinaemic conditions.

Discussion

Although CREB has been described as an important regulator of early embryo development, its specific function in this phase of development is still unknown. Our data show that CREB and the ATF family members mediate the embryo's adaptation to insulin by regulating adiponectin synthesis. Blastocysts react within 10 minutes with an insulin- and IGF-dependent CREB phosphorylation after stimulation in vitro. Insulin and IGFs were applied in physiological concentrations, ie, in an optimal range for ligand binding and induction of biological effects (43, 47, 49). Other groups have found an IGF-I-dependent CREB phosphorylation after 10 minutes via the MAPK and p38 MAPK pathways (50–53). We have previously demonstrated that insulin activates MAPK also in rabbit blastocysts (44, 45). A functional signaling cascade in blastocysts is indicated by a negative feedback loop (mechanism) for CREB, as shown by the reduced expression of CREB after a 12-hour in vitro stimulation with insulin and IGF-I.

Patients with type 1 diabetes and patients with a genetically defective IR show increased serum adiponectin levels (54). Blüher et al (55) found an elevated plasma adiponectin level in mice lacking IRs in adipocytes. Hyperinsulinaemia selectively down-regulates the high molecular weight form of adiponectin (56). In blastocysts, the insulin/IGF-dependent CREB activation of adiponectin has been demonstrated in vitro and in vivo. In our in vivo model of an experimentally induced diabetes mellitus type 1, an elevated adiponectin level was measured in maternal serum, endometrium, and in blastocyst cavity fluid. In 3T3-L1 preadipocytes, insulin led to a reduced adiponectin expression in vitro (22). It is noteworthy that EB and TB cells of diabetic blastocysts showed a distinct different adiponectin regulation. The difference can be explained by different insulin and IGFs signaling in both cell lineages (Figure 7). Insulin and IGFs act through their receptors IGF-IR and IR (IR-A and IR-B). The two IR isoforms, resulting from alternative splicing of the primary transcript (57), are expressed in a distinctly diverse pattern in both cell lineages in rabbit blastocysts (45). IR-A, the binding domain for insulin and IGF-II, is mainly expressed in the EB, whereas IR-B is the only detectable isoform in the TB (44, 45). IGF-IR is predominantly present in the EB and to a lower extent in TB cells (45). Phosphorylation of Erk induced by insulin occurs in the EB and to a lower extent in the TB, and IGF-I exclusively activates Erk/MAPKs in the EB (45). Further analysis revealed that IGF-I and IGF-II are highly in-

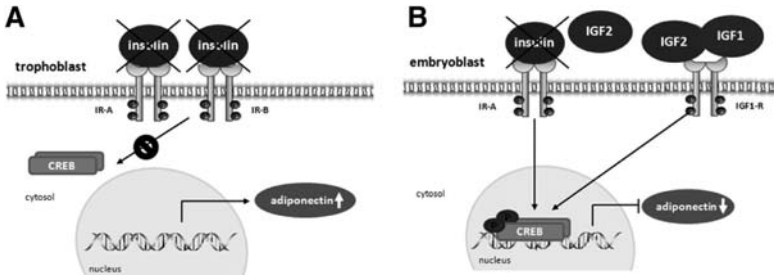

Figure 7. Cell lineage-specific molecular mechanisms of embryonic hormonal adaptation to a diabetic uterine environment Expression of IGF-I and IGF-II is highly increased in blastocysts and endometrium from diabetic rabbits. We interpret this as a compensatory mechanism to cover the lack of insulin normally provided by the mother. However, EB and TB differ in their responsiveness to IGFs due to distinct differences in IR and IGF-IR expression patterns (45). (A) Rabbit blastocysts barely express IGF-IR in the TB. Therefore, IGFs cannot compensate for the lack of insulin in these cells in diabetic blastocysts, leading to CREB inactivity as shown by its cytoplasmic localization. In this case, CREB is not able to inhibit adiponectin production resulting in higher adiponectin levels in TB cells in diabetic blastocysts. (B) The increased production of IGF-I and IGF-II and sensitivity of EB cells towards IGFs (expression of IGF-IR and IR-A) sustain CREB activation and nuclear localization, leading to a down-regulated adiponectin synthesis. Adiponectin was not detectable in EB cells in diabetic blastocysts.

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creased in blastocysts and endometrium of rabbits with an experimentally induced diabetes mellitus type 1, most likely as part of a compensatory mechanism to cover the lack of insulin normally provided by the mother (5). Because IGF-I and IGF-II are considered as growth factors of the EB in rabbits (45, 46) and mice (58, 59) and insulin is depleted in diabetic mothers (4), a cell lineage-specific regulation of the CREB-mediated adiponectin expression is likely. In the EB of blastocysts grown under diabetic conditions, the increased production of IGFs sustains activation and nuclear localization of CREB, correlating closely with the drop in adiponectin level observed in current study (Figure 7B). In TB cells, however, both factors, IGF-I and IGF-II, were not able to compensate for the lack of insulin due to absence of the IGF-IR and CREB signaling in these cells (Figure 7A). Although IGFs may maintain glucose uptake in EB cells, adiponectin may replace insulin in TB cells, securing embryo development in diabetic mothers.

CREB regulates the transcription of target genes directly and indirectly. In rabbit blastocysts, adiponectin transcript levels are decreased by CREB activation, posing the question regarding potential CREB inhibitors. A potential candidate is ATF3 (23, 60). Noteworthy, in our in vitro experiments, ATF3 was also down-regulated by insulin and IGF-I. No differences in ATF3 transcript level and localization were detectable in blastocysts from diabetic rabbits, arguing against ATF3. In contrast, ATF4 represses specifically the CRE-dependent transcription of target genes. Insulin and IGF-I led to increased amounts of ATF4 transcripts. Transcript levels of ATF4 were increased in blastocysts from diabetic rabbits, particularly in the EB. Therefore, it is tempting to propose a role for ATF4 in adiponectin gene expression in rabbit blastocysts. It is noteworthy, however, that the levels of adiponectin are not affected in ATF4−/− or in mice overexpressing ATF4 (38). The specific role(s) of ATF(s) in hormonal adaptation of blastocysts still needs to be clarified.

Adiponectin exerts its biological function by binding to adipoR1 or adipoR2. Both receptors are mainly expressed in the TB but also in the EB of rabbit blastocysts (10, 61). These results were confirmed by Kim et al (15) in mouse blastocysts, demonstrating adipoR1 and adipoR2 in EB and TB cells. A transcriptional regulation of adipoR1 by insulin has already been shown in rat and mouse tissues (62–64). However, the insulin effect on adipoRs depends on the cell type. In muscle cells, insulin down-regulates adipoR1 and adipoR2 expression, whereas in fat cells, adipoR2, but not adipoR1, is up-regulated (64). We could show insulin- and IGF-I-dependent decreased adipoR1 transcription levels in blastocysts grown in vitro. These results are supported by increased adipoR1 levels in blastocysts developed in vivo in a hypoinsulinaemic uterine milieu. No differences in adipoR2 transcription levels were observed. This suggests that insulin may have different functions in the regulation of adipoR1 and adipoR2. Therefore the interplay of both receptors has to be kept in mind when downstream signaling of adipoRs in preimplantation embryo is assessed.

In conclusion, maternal diabetes alters the hormonal sensitivity of the embryo during preimplantation development. Our results demonstrate that hypoinsulinaemia elevates adiponectin levels and the expression of adipoR1 in blastocysts. This effect is caused by insulin/IGF, because no difference in adiponectin transcript levels were observed after high and low glucose administration. Furthermore, we found an insulin- and IGF-I-dependent adipoR1 transcription. The regulation of adiponectin and adipoR expression may be part of an embryonic adaptation process, compensating for the lack of maternal insulin to maintain embryonic glucose metabolism (10). This view is supported by fact that no difference in glucose uptake was measured in blastocysts from normoinsulinaemic and diabetic rabbits. Thus, CREB-regulated embryonic adiponectin expression may be a functional connecting link between maternal insulin supply and embryonic metabolic adaptation. Not only adiponectin, but also adipoR1, is negatively regulated by insulin. In diabetes, this failsafe system may compensate for the loss of insulin and helps to maintain embryo development.

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Maternal Diabetes Leads to Unphysiological High Lipid Accumulation in Rabbit Preimplantation Embryos

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According to the "developmental origin of health and disease" hypothesis, the metabolic set points of glucose and lipid metabolism are determined prenatally. In the case of a diabetic pregnancy, the embryo is exposed to higher glucose and lipid concentrations as early as during preimplantation development. We used the rabbit to study the effect of maternal diabetes type 1 on lipid accumulation and expression of lipogenic markers in preimplantation blastocysts. Accompanied by elevated triglyceride and glucose levels in the maternal blood, embryos from diabetic rabbits showed a massive intracellular lipid accumulation and increased expression of fatty acid transporter 4, fatty acid–binding protein 4, perilipin/adipophilin, and maturation of sterol-regulated element binding protein. However, expression of fatty acid synthase, a key enzyme for de novo synthesis of fatty acids, was not altered in vivo. During a short time in vitro culture of rabbit blastocysts, the accumulation of lipid droplets and expression of lipogenic markers were directly correlated with increasing glucose concentration, indicating that hyperglycemia leads to increased lipogenesis in the preimplantation embryo. Our study shows the decisive effect of glucose as the determining factor for fatty acid metabolism and intracellular lipid accumulation in preimplantation embryos. **(*Endocrinology* 155: 1498–1509, 2014)**

The global rise in the prevalence of diabetes mellitus is paralleled by an alarming increase in diabetic pregnant women. Currently, in the western world 3% to 10% of pregnancies are affected (1). Diabetes mellitus is associated with abnormalities in lipid metabolism such as hypertriglyceridemia and low levels of high-density lipoprotein (2). Maternal metabolic disorders are well known risk factors affecting fertility and embryonic and fetal development.

Early embryos are able to store intracellular lipids in the form of lipid droplets (3). Lipids play an important role in energy metabolism during early embryo development by serving as an energy source and influencing properties and functions of biological membranes, cell-cell interactions, cell proliferation, and intercellular and intracellular transport (for a review, see Ref. 4). The size and number of lipid droplets are considered as markers of embryo vitality (5, 6). Embryos cultured with serum abnormally accumulate cytoplasmic lipids, probably as a result of incorporation of lipoproteins from the serum (6–8). An increased amount of intracellular lipids reflects poor embryo quality and developmental potential. Pig and bovine embryos contain the highest amounts of lipids. This observation correlates with the lowest survival rate after cryopreservation (9–12). Mouse embryos with low amounts of lipid droplets are not as sensitive toward adverse effects of vitrification as embryos from other species (13).

Lipid droplets are coated by lipid droplet–associated proteins (PLIN1–4). They regulate the assembly, maintenance,

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Abbreviations: cSREBP, cytoplasmic sterol-regulated element binding protein; expIDD, experimental insulin-dependent diabetes; FABP, fatty acid–binding protein; FASN, fatty acid synthase; FATP, fatty acid transporter; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HRP, horseradish peroxidase; nSREBP, nuclear sterol-regulated element binding protein; PLIN, lipid droplet–associated protein; pc, post coitum; PVA, polyvinyl alcohol; qPCR, quantitative PCR; SREBP, sterol-regulated element binding protein.

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and composition of lipid droplets, as well as lipolysis and lipid efflux (14–16). The best known are perilipin and adipophilin, also referred as PLIN1 and PLIN2. Adipophilin expression has been documented previously from day 2 post coitum (pc) onward in rabbit preimplantation embryos (17).

The first step in long-chain fatty acid utilization is their uptake across plasma membranes. This process involves 2 components, passive diffusion through the lipid bilayer and protein-facilitated transfer. In early embryos, the fatty acid uptake is poorly understood. However, a facilitated transport is possible, as indicated by the expression of fatty acid transport protein (FATP) 4 and fatty acid–binding protein (FABP) 4 in mouse blastocysts (18) and in human trophoblast cells (19, 20).

The expression levels of FATP4 correlate with measures of obesity and insulin resistance (21). FABP4, also known as adipocyte fatty acid–binding protein (AFABP) or aP2, affects intracellular lipid metabolism by transporting fatty acids to the nucleus for transcriptional regulation, to mitochondria for β-oxidation, and to lipid droplets for storage (22, 23). New evidence from population studies and experimental animal models indicates that serum FABP4 is a powerful new risk marker for predicting obesity, insulin resistance, and dyslipidemia (24, 25). However, the exact secretion mechanism of FABP4 is still not clear.

A source of intracellular fatty acids is de novo synthesis. Fatty acid synthase (FASN), the key enzyme in de novo lipogenesis, coordinates together with acetyl-CoA carboxylase energy homeostasis by converting excess food intake into lipids for storage (26, 27). $FASN^{-/-}$ null mutant mice die during preimplantation development, demonstrating the important role during early embryonic development (28). The promoter region of *FASN* contains binding sites for the transcription factor sterol-regulated element binding protein (SREBP) 1 (29). SREBPs are synthesized as precursor proteins that remain bound to the endoplasmic reticulum. After activation, SREBP is processed and translocated to the nucleus (30, 31). The mature nuclear SREBP activates the transcription of genes regulating synthesis and uptake of fatty acids, triglyceride, and cholesterol (31, 32). Currently, 3 different isoforms have been described: SREBP1a and SREBP1c, derived from a single gene by alternative transcription start sites, and SREBP2 (33). SREBP-overexpressing transgenic animals showed an accumulation of cholesterol and/or fatty acids in liver cells (32, 34, 35), indicating that the SREBP1 isoforms are more directed forward activation of fatty acid biosynthesis, whereas SREBP-2 is more specific for controlling cholesterol biosynthesis.

Despite the increasing incidence of metabolic disorders in women of reproductive age, few attempts have to be made to analyze the maternal influence on embryonic lipid metabolism (36–38). Therefore, we have investigated intracellular lipid accumulation and expression of key lipogenic markers (such as perilipin, adipophilin, FATP4, FABP4, FASN, and SREBP1) in response to hyperglycemia in vivo caused by an induced maternal diabetes mellitus type 1 and hyperglycemia created in vitro in rabbit preimplantation embryos.

Materials and Methods

Alloxan treatment

Experimental insulin-dependent diabetes (expIDD) was induced in mature 18- to 20-week-old female nonpregnant rabbits by alloxan treatment (Sigma-Aldrich) as described before (39). Rabbits were held in the diabetic condition with permanent blood glucose concentrations of >14 mmol/L by regular insulin supplementation 4 times/d (Insuman Rapid and Lantus; sanofi aventis), started on the second day after alloxan treatment. The blood glucose level was monitored with a MediSense Precision Xceed Diabetes Management System (Abbott) 2 times/d. For that, fresh blood was collected at 10:00 AM and 6:00 PM by puncturing the vena auricularis lateralis and tested for glucose concentration by a commercial test strip.

All animal experiments were conducted in accordance with the principles of laboratory animal care, and the experimental protocol had been approved by the local ethics commission of the Landesverwaltungsamt Dessau (reference no. 42502-2-812).

Embryo recovery

Embryos were collected from sexually mature rabbits stimulated with 110 IU of pregnant mare serum gonadotropin sc (Intervet) 3 days before mating. After mating, 75 IU of human chorionic gonadotropin was injected iv (Intervet). Mating and embryo recovery were performed as described previously (40). On days 3, 4, and 6 pc embryos were flushed from oviducts or uteri, respectively, washed 3 times with PBS and randomly divided among the experimental groups. On day 6 pc, gastrulation stages can be reliably discriminated in the rabbit because implantation starts on day 6 18 hours, ie, half a day later (41). Only blastocysts from gastrulation stage 1 and 2 were used for further analyses.

Embryo in vitro culture

To study the effects of different glucose concentrations on perilipin/adipophilin, FABP4, FATP4, and FASN expression and intracellular lipid accumulation, day 6 blastocysts were cultured in groups of 6 to 10 at 37°C in a water-saturated atmosphere of 5% O_2, 5% CO_2, and 90% N_2. Blastocysts were cultured for 6 hours in serum- and insulin-free BSM II medium (42) with 0, 10 (standard culture medium), and 25 mM glucose (hyperglycemic conditions), respectively.

Quantification of triglycerides in blood samples

Triglyceride concentrations in blood samples were measured by a commercial kit (DiaSys Triglycerides FS; Diagnostic Systems). All procedures were performed according to the manufacturer's instructions. To measure the serum triglyceride concentration, blood samples were collected with the S-Monovette system(Sarstedt), left to coagulate for at least 30 minutes, and centrifuged for 10 minutes at 4°C and 1000 × *g*. The supernatant was stored at −80°C until use.

Oil Red O staining of rabbit blastocysts

Blastocysts were washed twice in ice-cold PBS and fixed in 4% (wt/vol) paraformaldehyde for 10 min. The fixed blastocysts were washed again in ice-cold PBS and transferred to 0.05% (wt/vol) polyvinyl alcohol (PVA)/PBS. Blastocyst coverings were removed mechanically. The embryonic disk was mechanically dissected with surgical forceps and scissors (Fine Science Tools GmbH) (39) and used immediately for Oil Red O staining. Oil Red O stock solution (0.5 mg of Red Oil O [Sigma-Aldrich] dissolved in 100 mL of isopropanol) was diluted 3:2 with distilled water. To remove the precipitate, the working solution was filtered using a filter with a pore diameter of 20 μm (BD Biosciences).

For Oil Red O staining, the embryonic disc was incubated in filtered Oil Red O solution for 2 hours. After staining the lipid droplets, the Oil Red O staining solution was removed by washing the embryonic disc for 3 hours in 0.05% (wt/vol) PVA/PBS. Subsequently, the embryonic disk was embedded on Superfrost slides (Menzel Gläser) using 4.8 g of Mowiol reagent (Calbiochem, Germany) dissolved in 12.0 g of glycerol (Merck). Embryonic disks were examined by light microscopy (BZ 8000; Keyence).

Preparation of a single-cell suspension

For the separation of blastocysts to single-cell suspensions, the gentleMACS-m-neural-tissue kit (Neural Tissue Dissociation Kit; Miltenyi Biotec) was used. Blastocyst coverings were removed mechanically, and blastocysts were transferred in a 1.5-mL tube. The preheated papain enzyme mix 1 provided with the kit was given directly to the blastocysts and incubated according to the manufacturer's protocol, except that the incubation step was performed at room temperature. Enzyme mix 2, prepared according to the manufacturer's protocol, was added directly to the sample. The specimen was incubated for 15 minutes at room temperature on a rotator.

To remove cell aggregates from the cell suspension, a preseparation filter with a pore diameter of 40 μm (BD Biosciences) was used. After a washing step, the cells were resuspended in ice-cold 0.05% (wt/vol) PVA/PBS. Nile Red stock solution (100 μg/mL; Sigma-Aldrich) was prepared in dimethyl sulfoxide and stored in the dark. For Nile Red staining, the dye was added at a final concentration of 0.1 μg/mL. The cells were then incubated on ice for 5 minutes, centrifuged, and washed with 0.05% (wt/vol) PVA/PBS. Afterward they were resuspended in a volume of 0.05% (wt/vol) PVA/PBS and kept on ice in the dark until flow cytometry analysis.

Flow cytometry

All measurements were performed on a BD FACS Vantage (BD Biosciences) equipped with 3 lasers (excitation wavelengths: 488, 633, and 351nm) as described previously (43). Cell debris, doublets, and aggregates were excluded from analysis. Blastocyst cells without Nile red staining served as a negative control for flow cytometry analysis. Measurements and data analysis were performed with FACSDiva (version 5.0.3; BD Biosciences). Nile Red specifically stains intracellular lipid droplets, excites at 485 nm, and emits at 525 nm. For sorting, a 90-μm nozzle was used, and the sorting rate was no more than 1000 events per second.

The visualization by dot plot and histogram shows 2 different cell populations R1 (P1) and R2 (P2). To prove that region R1 contains embryoblast and R2 contains trophoblast cells, we sorted both cell populations and used them subsequently for RT-quantitative PCR (qPCR) analyses for cytokeratin 18 and cdx2, 2 trophoblast markers (44). Cytokeratin 18 and cdx2 were highly expressed in the R2 cell population, demonstrating that these cells were almost exclusively trophoblast cells (Figure 1I).

The analysis of the percentage of cells located in R3 (cell debris or putative dead cells) revealed no significant differences between the normohyperglycemic and hyperglycemic groups. This result suggests that in our experiments increased cell death in the hyperglycemic group compared with that in the control group can be excluded.

RNA isolation and cDNA synthesis

mRNA of single blastocysts was extracted with Dynabeads oligo$(dT)_{25}$ (Invitrogen) and subsequently used for cDNA synthesis. All protocol procedures were performed according to the manufacturer's instructions, with modifications described previously (45).

RT-PCR

RT-PCR amplification was performed with 0.5 μL of cDNA from single blastocysts in a 25-μL volume containing 200 μM concentrations of each dNTP, 2.5 U of Taq polymerases, and specific oligonucleotides for adipophilin, FABP4, FATP4, FASN, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (primers are listed in Supplemental Table 1 published on The Endocrine Society's Journals Online web site at http://end.endojournals.org). The nucleotide sequence for rabbit FASN was determined using human primers for amplification of rabbit liver cDNA. The amplification was done for 40 cycles (94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 60 seconds). The resulting PCR products were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide. The PCR products were sequenced and analyzed as described previously (46). Sequence homology was proven by using the alignment BLASTN tool.

RT-qPCR

Amounts of FAS, FATP4, FABP4, adipophilin, and GAPDH cDNA were determined by real-time qPCR with SYBR Green detection by using the StepOnePlus System (Applied Biosystems) with a no template control for each primer set (described in Ref. 47). The nucleotide sequences of the primers used in this study are listed in Supplemental Table 1. The amount of cDNA per sample was normalized by referring to the GAPDH gene. GAPDH was shown to be unaffected by the treatment because no variation was observed between the absolute GAPDH levels and GAPDH RNA levels in blastocysts from healthy rabbits (47). qPCRs for target and reference genes were always run in duplicate from the same cDNA dilution taken from the same RT reaction.

The absolute amount of the transcripts was calculated along with a standard (calibrator) sample. As standards we used defined concentrations of plasmid standards constructed for the gene of interest and GAPDH. For all genes investigated a partial sequence was amplified from rabbit tissues. Purified PCR products were ligated into pGEM-T with subsequent transformation in XL1Blue competent bacteria (described in Ref. 47).

Results are given in molecules of the target gene per GAPDH molecule. Statistical analysis was performed on the amounts of molecules. Fold changes were calculated when the results are expressed as percentages.

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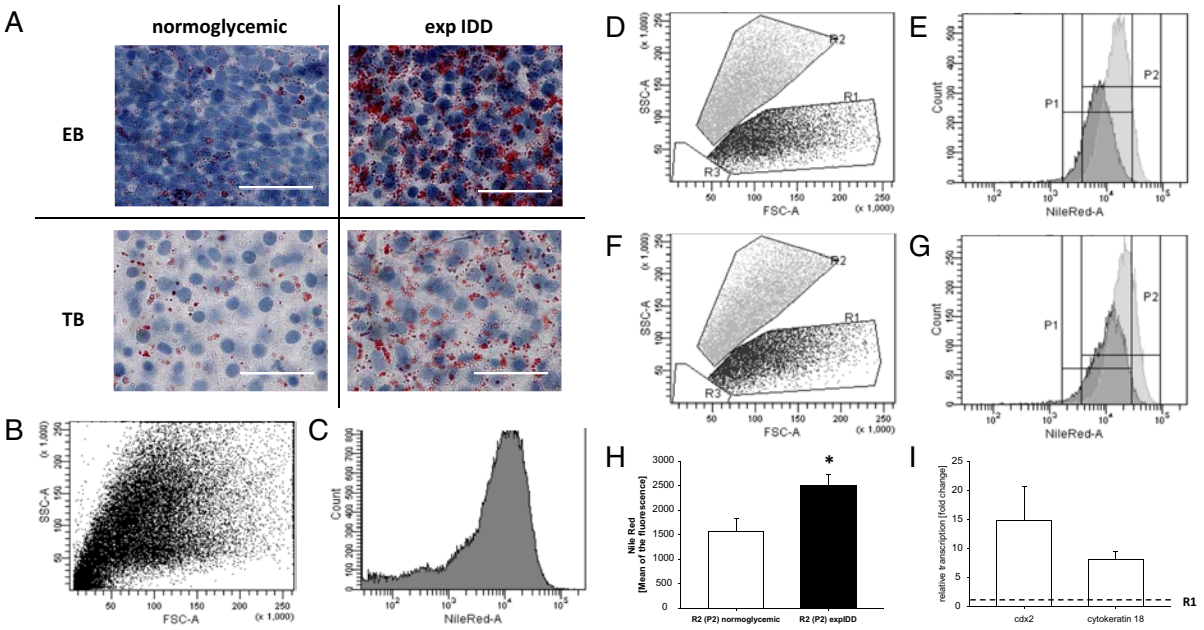

Figure 1. Oil Red O staining and Nile Red flow cytometry analyses of blastocyst cells from diabetic rabbits. A, Blastocysts derived from healthy and diabetic (expIDD) rabbits were stained with Oil Red O, marking intracellular lipid droplets red in trophoblast (TB) and embryoblast (EB) cells (scale bar corresponds to 50 μm). Nuclei were counterstained with hematoxylin in blue. B–G, For flow cytometry analysis blastocyst cells were stained with Nile Red. A representative analysis is shown. Blastocyst cells were visualized on a dot plot of forward scatter (FSC) vs side scatter (SSC) (B, D, and F) or in a Nile Red histogram (C, E, and G). The visualization by dot plot, using the parameters granularity and cell size, indicated 2 different cell populations, region R1 with embryoblast cells and R2 with trophoblast cells, respectively. For further analyses, blastocyst cells were gated in region R1 and R2. Cell debris and death cells were excluded (region R3). Blastocyst cells were gated in region R1 and R2 from normoglycemic (D) and diabetic (F) rabbits. Nile Red absorbance of blastocyst cells from regions R1 and R2 is shown for normoglycemic (E) and for diabetic (G) rabbits in P1 and P2, respectively. H, Nile Red mean fluorescence in R2 (P2) of normoglycemic and diabetic cells determined in 3 independent experiments is shown. Blastocyst cells were sorted in region R1 and R2 and subsequently used for RT-qPCR analysis of trophoblast markers cdx2 and cytokeratin18. I, Results are shown as relative amounts and fold change of R1 (means ± SEM; N = 4, n ≥ 18). R2 sorted cells were identified as trophoblast cells with 10- and 5-fold higher cdx2 and cytokeratin 18 RNA amounts, respectively, than those in the R1 cells.

Protein preparation and immunoblotting

Protein preparation, quantification, and Western blotting were performed with 8 to 10 blastocysts as described previously (48). For Western blot analysis, 2.5 μg of total protein lysates was subjected to SDS-polyacrylamide electrophoresis and electrotransferred to nitrocellulose membranes. For detection of FASN, SREBP1, and β-actin, membranes were blocked in Tris-buffered saline containing 0.1% (vol/vol) Triton X-100 (0.1% TBST) with 3% (wt/vol) nonfat dry milk at room temperature for at least 1 hour. For perilipin detection, membranes were blocked in 0.1% TBST with 3% (wt/vol) BSA for 2 hours. The primary antibody was incubated at 4°C overnight. The following antibodies were used as follows perilipin (1:1000; Sigma-Aldrich), FASN (1:1000; Santa Cruz Biotechnology), SREBP1 (1:1000; Active Motif), β-actin (1:40 000; Sigma-Aldrich), anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (1:15 000; DAKO Cytomation), and anti-mouse IgG conjugated to HRP (1:45 000; Dianova). The protein amount was calculated as the ratio of band intensities (perilipin protein or FASN protein vs β-actin protein, respectively) in the same blot to correct for differences in protein loading.

Immunohistochemical localization of FABP4 and FASN

Blastocysts were washed twice in ice-cold PBS and fixed in 4% (wt/vol) paraformaldehyde at 4°C overnight. The sample was prepared, and the immunohistochemical protocol was performed as described previously (48). Antibodies for FABP4 and FASN (both Santa Cruz Technology) were diluted 1:100 and 1:500 in 3% (wt/vol) BSA/PBS, respectively. The secondary antibody Dako EnVision+ System HRP-labeled polymer anti-mouse (1:1 in PBS) and diaminobenzidine (WAK-Chemie Medikal) were used for detection.

All steps were performed within the same experiment, and samples were examined microscopically during the same session, using identical microscope and camera settings (BZ 8000; Keyence).

FABP4 ELISA

FABP4 concentrations in blastocyst fluids were measured by ELISA (MyBioSource). All protocol procedures were performed

Table 1. Mean Nile Red Fluorescence Intensity From Normoglycemic and Diabetic (expIDD) Blastocyst Cells in Region R1 (P1) and Region R2 (P2)

	Nile Red (Mean ± SEM)	*P* Value
R1 (P1) normoglycemic	951 ± 280	.6
R1 (P1) expIDD	1093 ± 202	
R2 (P2) normoglycemic	1566 ± 267	.03
R2 (P2) expIDD	2503 ± 233	

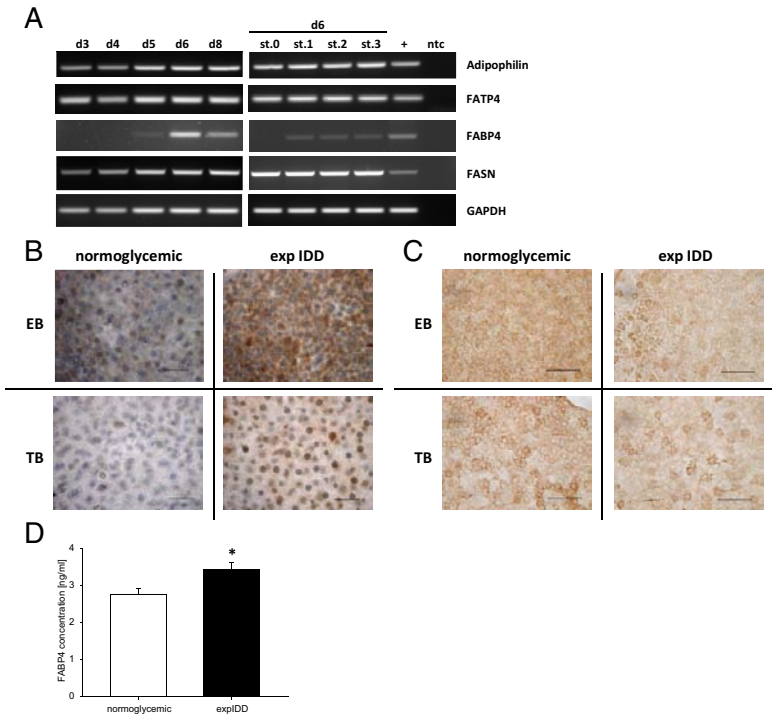

Figure 2. Expression patterns of the lipogenic markers adipophilin, FATP4, FABP4, and FASN in rabbit preimplantation embryos. A, RT-PCR was performed with specific primers for adipophilin, FATP4, FABP4, and FASN on rabbit morulae at day (d) 3, on blastocysts at days 4, 5, 6, and 8 pc, and on 6-day-old blastocysts of different gastrulation stages with stage (st) 0, 1, 2, and 3. Transcripts of adipophilin, FATP4, and FASN were detected in all analyzed developmental stages. FABP4 transcription was measurable from gastrulation stage 1 (st 1) onward. A probe without cDNA was used as negative control (ntc). As a positive control, a liver cDNA probe was used (+). The internal control was the expression of GAPDH in all probes. The FABP4 and FASN proteins were detected by immunohistochemical analysis and visualized by a peroxidase-diaminobenzidine reaction (brown color) (scale bar corresponds to 50 μm). B, In blastocysts from healthy controls (normoglycemic), FABP4 was localized in the cytosol and nucleus of embryoblast cells (EB). The trophoblast cells (TB) were not or less stained for FABP4. In blastocysts from diabetic rabbits (expIDD), the embryoblast and trophoblast cells showed more intense staining for FABP4, especially in the nuclei of both cell lineages. Nuclei were counterstained in blue with hematoxylin. C, Immunohistochemical localization revealed FASN in the cytosol of embryoblast and trophoblast cells. No difference in staining and localization was found between blastocysts from diabetic and normoglycemic rabbits. D, FABP4 concentrations were analyzed by ELISA in blastocyst fluids of single blastocysts from normoglycemic and expIDD rabbits (means ± SEM; N = 3; n ≥ 9; *, $P < .05$).

according to the manufacturer's instructions. Flushed blastocysts were washed 2 times with ice-cold PBS. Blastocyst stage and size were determined. Blastocysts were then placed on a dry watch glass. Extracellular coverings were removed mechanically, blastocysts were punctured, and the effluent cavity fluid was collected. Blastocyst fluids were stored at −80°C until use. FABP4 concentrations were quantified in duplicate and compared with an internal FABP4 standard (0.625–50 ng/mL).

Statistics

All data are expressed as means ± SEM. Levels of significance between groups were calculated by factorial ANOVA with Bonferroni adjustment. A value of $P < .05$ was considered statistically significant. All experiments were conducted at least 3 times.

Results

In previous studies, we demonstrated that the increases in maternal blood glucose concentrations correlate with increased glucose levels in the uterine secretions (39). Here, we focused on the lipid content and lipogenic markers to test whether a diabetic disorder during early pregnancy would affect the embryonic fatty acid metabolism.

Elevated triglyceride levels in female rabbits with expIDD at day 6 pc

In blood samples of diabetic (expIDD) pregnant rabbits, triglyceride levels were increased approximately 6-fold (3.55 ± 0.43 mM; mean ± SEM) compared with that in the healthy controls (0.57 ± 0.05 mM) [number of independent experiments used for blastocysts (N) = 3; total number of blastocysts used for analysis (n) = 9; $P < .001$], respectively.

Accumulation of intracellular lipid droplets in normoglycemic and diabetic (expIDD) blastocysts

The effect of alloxan-induced maternal diabetes mellitus type 1 on intracellular lipid accumulation was examined by Oil Red O staining. Blastocysts from diabetic rabbits showed a higher accumulation of intracellular lipids in the embryoblast and trophoblast, indicated by the higher amount of red-stained lipid droplets (Figure 1A). To further support these data, we used a quantitative method to determine the cellular lipid content based on Nile Red staining and flow cytometry analyses. In Figure 1, B to G, the results of a representative flow cytometry analysis are illustrated. Blastocyst cells were visualized by dot plot and histogram. A higher Nile Red fluorescence intensity was found in blastocyst cells from diabetic rabbits in both cell populations R1 (P1) and R2 (P2). However, in contrast to the R2 (Figure 1I and Table 1), the difference in the Nile Red fluorescence mean in the R1 between normoglycemic and diabetic blastocysts was not significant (data not shown). The mean Nile Red absorbance is given in Table 1.

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Table 2. Relative Protein Amount of Lipogenic Markers in Blastocysts from Normoglycemic and Diabetic (expIDD) Rabbits

Protein	Normoglycemic	expIDD	*P* Value
Perilipin	100.0 ± 3.5	136.8 ± 10.5	.048
FASN	100.0 ± 6.8	105.3 ± 1.2	.473
nSREBP1	100.0 ± 7.2	107.2 ± 5.2	.437
cSREBP1	100.0 ± 12.7	76.9 ± 5.8	.138
Ratio: nSREB1/ cSREBP1	0.51 ± 0.03	0.70 ± 0.03	.005

Results are given relative to protein amounts in blastocysts from healthy rabbits (normoglycemic) (means ± SEM; N = 3; n = ≥10).

Adipophilin and perilipin expression in preimplantation embryos from diabetic rabbits

Intracellular lipid droplets are coated by the lipid droplet–associated proteins perilipin and adipophilin. The transcription of adipophilin was detectable in the rabbit preimplantation embryos from day 3 old morulae onward in all analyzed days and stages (Figure 2A). Transcription levels in blastocysts (day 6 pc stage 1) from diabetic rabbits (expIDD) were significantly increased compared with those from normoglycemic rabbits (Table 2). Quantification of adipophilin in the embryoblast and trophoblast revealed that the increase in the adipophilin amount resulted from transcriptional upregulation, mainly in embryoblast cells (Table 4). In addition, the perilipin protein amount was significantly increased in blastocysts from diabetic rabbits (Table 3).

FATP4 and FABP4 expression in preimplantation embryos from diabetic rabbits

FATP4 and FABP4 are expressed in rabbit blastocysts. Whereas FATP4 transcription was detectable in all investigated embryonic stages, starting on day 3 pc (Figure 2A), FABP4 expression was only measurable in expanded and gastrulating blastocysts on day 6 pc, from stage 1 onward and on day 8 pc (Figure 2A). Analysis of FABP4 transcription, however, revealed a cell lineage–specific FABP4 distribution pattern with the main expression in the embryoblast (Figure 2B). Only a few cells stained positive for FABP4 in the trophoblast.

In diabetic conditions, the transcription levels for both FATP4 and FABP4 were increased in blastocysts (Table 2). FATP4 mRNA was increased in the embryoblast and trophoblast (Table 4). For FABP4, the RNA amount was increased only in the embryoblast (Table 4). Compared with embryos from healthy rabbits, blastocysts from diabetic mothers showed more intense FABP4 staining in both cell lineages, with the most prominent localization in the nuclei (Figure 2B).

Because FABP4 can be secreted from cells and is present in the circulation, we analyzed the FABP4 concentration in blastocyst fluid. Analysis by ELISA showed that FABP4 is present in blastocyst fluid and that the amount is increased in the cavity fluid from diabetic blastocysts (Figure 2D).

FASN expression in preimplantation embryos from diabetic rabbits

The key enzyme of de novo lipogenesis, FASN, was detectable form day 3 old morulae (d3) onward in all analyzed stages (Figure 2A). Neither FASN transcription (Table 2) nor protein (Table 3) amounts were significantly different in blastocysts from diabetic rabbits. In addition, no alterations of FASN transcription levels in embryoblasts and trophoblasts from normoglycemic and diabetic rabbits were detectable. However, a cell lineage–specific transcription was observed (Table 4), with higher transcripts in the embryoblast. FASN protein was localized in both embryoblast and trophoblast cells with a cytoplasmic localization (Figure 2C). In blastocysts from diabetic rabbits, no differences in staining intensity or in cellular localization were observed.

SREBP1 expression in preimplantation embryos from diabetic rabbits

Under hyperglycemic conditions, no changes in total SREBP1 protein amounts were observed (Table 2). However, the ratio of cytoplasmic (cSREBP1) and mature, nuclear SREBP1 (nSREBP1) was influenced. Blastocysts from diabetic rabbits had relatively more SREBP1 located in the nucleus (based on the total SREBP1 protein amount and the ratio of nSREBP1 to cSREBP1) compared with that of blastocysts from normoglycemic rabbits (Table 2), indicating a higher transcriptional regulation by SREBP1 in blastocysts from diabetic rabbits.

Table 3. Relative mRNA Amount of Lipogenic Target Genes in Blastocysts from Normoglycemic and Diabetic (expIDD) Rabbits

mRNA	Normoglycemic	expIDD	%	*P* Value
Adipophilin	51.76 ± 15.28	167.78 ± 3.99	287.4 ± 29.9	.020
FABP4	4.45 ± 2.03	9.91 ± 2.27	222.6 ± 51.1	.041
FATP4	855.4 ± 124.1	1382.4 ± 179.6	188.9 ± 23.2	.038
FASN	0.66 ± 0.18	0.38 ± 0.06	58.7 ± 10.1	.195

Results are given as molecules and as a percentage of transcription levels in normoglycemic blastocysts (means ± SEM; N = 3; n = ≥10).

Table 4. Relative mRNA Amount of Lipogenic Target Genes in Embryoblast and Trophoblast From Normoglycemic and Diabetic (expIDD) Rabbits

	Embryoblast		Trophoblast	
mRNA	**Normoglycemic**	**expIDD**	**Normoglycemic**	**expIDD**
Adipophilin	100.0 ± 8.3	131.3 ± 11.8[a]	118.1 ± 6.6	125.1 ± 17.7
FABP4	100.0 ± 17.1	293.3 ± 71.2[a]	14.0 ± 3.5[b]	9.9 ± 1.8[b]
FATP4	100.0 ± 6.4	147.2 ± 15.3[a]	92.8 ± 10.4	148.6 ± 15.1[a]
FASN	100.0 ± 23.5	91.4 ± 23.4	25.9 ± 6.7[b]	15.8 ± 2.3[b]

Results are given as a percentage of the amount in the embryoblast from healthy rabbits (normoglycemic) (means ± SEM; N = 3; n = ≥10).

[a] Amount significantly different between control and expIDD ($P < .05$).

[b] Amount significantly different between embryoblast and trophoblast ($P < .05$).

Glucose-dependent expression of lipogenic genes

To clarify whether glucose can regulate the expression of lipogenic genes, we cultured blastocysts without glucose (0 mM) and with 10 mM (standard culture media) or 25 mM (hyperglycemia in vitro) for 6 hours in vitro. The quantification of adipophilin and FABP4 transcript levels and perilipin protein showed increased expression of all markers in blastocysts cultured under hyperglycemic conditions (Figure 3, A, B, C, and E). No significant differences were measured for the FATP4 transcript levels in response to the various glucose concentrations (Figure 3D). In contrast to diabetic conditions in vivo, the FASN protein amount was increased in blastocysts cultured with 10 and 25 mM compared with that in blastocysts cultured with 0 mM, respectively (Figure 3, F and G). Total protein amounts of SREBP1 were increased in a glucose-dependent manner with a higher accumulation of mature nSREBP1 in the nucleus (Figure 3, H–K).

Glucose-dependent accumulation of lipid droplets

Excess energy intake leads to an accumulation of intracellular lipids. To analyze whether a high concentration or the lack of glucose influences the amount of lipid droplets in embryos, we cultured blastocysts (day 6 pc) from normoglycemic and diabetic rabbits for 6 hours with either 0, 10, or 25 mM glucose and afterward stained intracellular lipid droplets with Oil Red O. Blastocyst cells from both normoglycemic and diabetic (Figure 4) rabbits showed a glucose-dependent accumulation of intracellular lipids. High glucose concentrations led to a higher amount of lipid droplets compared with 0 and 10 mM in blastocysts from normoglycemic rabbits. The lowest amount of lipid droplets was found in blastocysts cultured with 0 mM. A comparable glucose-dependent increase in lipid droplets was observed in blastocysts from diabetic rabbits. However, the amount of lipid droplets in each group (0–25 mM) was higher in diabetic blastocysts than in the corresponding controls.

Discussion

Since the initial observation by Mills in 1982 (49), a great number of studies have shown the determining effect of maternal hyperglycemia on pregnancy outcome, offspring malformation, and the risk of later life diseases (39, 50–55). Adverse effects of high glucose concentrations have been shown by in vitro and in vivo culture experiments, such as retarded embryo development and increased apoptosis (39, 55–63). The current study shows for the first time the direct influence of maternal diabetes in vivo and hyperglycemia in vitro on embryonic lipid storage. One central finding of this study is the highly increased amount of intracellular lipid droplets in blastocysts from diabetic rabbits. Embryos with higher amounts of cytoplasmic lipids are more sensitive to chilling and cryopreservation (64) and have a lower developmental efficiency (10, 11). Therefore, the increased amount of lipid droplets in blastocysts may contribute to the adverse effects of maternal diabetes on preimplantation embryo development.

The massive lipid accumulation in blastocysts from diabetic rabbits raises the question of whether the embryo produces lipids from excess maternal glucose supply or by an increased transport of fatty acids into the cell. Type 1 diabetes mellitus is not only characterized by high serum glucose concentrations but also by increased serum triglyceride concentrations. These phenomena are closely reflected by hyperglycemia and hypertriglyceridemia in the diabetic rabbit model. Diabetes mellitus is associated with maternal and fetal dyslipidemia, which manifests as high plasma triglyceride concentrations, elevated concentrations of nonesterified fatty acids, increased concentrations of low-density lipoprotein cholesterol, and decreased levels of high-density lipoprotein cholesterol (20, 65–68). An increase in fatty acids enhances the formation of lipid droplets and expression of adipophilin and FABP4 (20, 69–71). Therefore, it is likely that the diabetes-associated hyperlipidemia is a potent regulator of embryonic lipid metabolism. This view is supported by the observation

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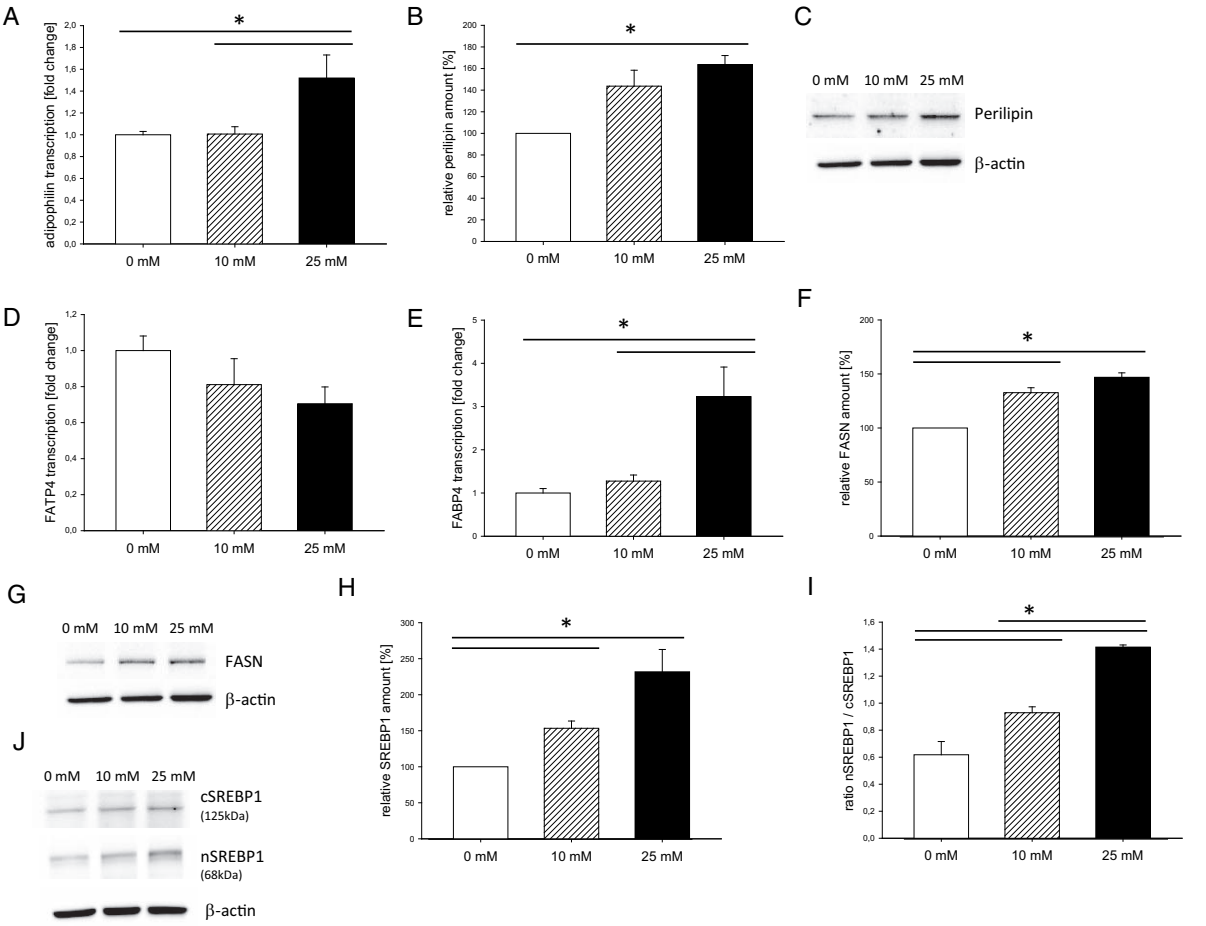

Figure 3. Relative expression of lipogenic genes in rabbit blastocysts cultured in vitro with 0, 10, or 25 mM glucose. A, In vitro culture of blastocysts for 6 hours with 0, 10, or 25 mM glucose led to a significantly higher adipophilin transcription level in blastocysts of the 25 mM group (*, $P < .05$). Blastocysts were cultured in groups of at least 5 blastocysts (N = 3; n ≥ 5). mRNA levels were analyzed by RT-qPCR, and results are shown as means ± SEM (*, $P < .05$). B and C, Relative perilipin amounts after 6 hours of cultivation without or with 10 or 25 mM glucose were analyzed by Western blotting with anti-perilipin antibody and set in relation to total β-actin. A representative Western blot for perilipin is shown in panel C. Results of 3 independent stimulation experiments (N = 3, n ≥ 9; means ± SEM) are shown in panel B. The group without glucose (0 mM) was set at 100%. D and E, RT-qPCR analysis of FATP4 (D) or FABP4 (E) after in vitro culture (for 6 hours) of blastocysts with 0, 10, or 25 mM shows a distinctly different expression pattern. In vitro culture with 10 or 25 mM or without glucose was performed in groups of at least 5 blastocysts in each treatment and repeated in 3 independent replicates (N = 3; n ≥ 5). Results are shown as means ± SEM (*, $P < .05$). F and G, FASN protein amount was analyzed by Western blot and the 0 mM group (control) was set at 100% (*, $P < .05$). Results (F) and a representative Western blot (G) are shown. H–K, Relative SREBP1 amounts after 6 hours of cultivation without or with 10 or 25 mM glucose were analyzed by Western blotting with anti-SREBP1 antibody, which specifically detects cSREBP1 (125 kDa) and nSREBP1 (68 kDa). Culture was performed in groups of at least 5 blastocysts in each treatment and repeated in 3 independent replicates (N = 3; n ≥ 5). H, Relative SREBP1 protein amounts (nSREBP1 and cSREBP1) in relation to those in the 0 mM group, which was set at 100%. I, Ratio of nSREBP1 vs cSREBP1. J, Representative Western blot for SREBP1.

that both normoglycemic and diabetic blastocysts cultured for 6 hours in vitro showed a lower amount of lipid droplets than that of their in vivo counterparts, implying that metabolic factors other than glucose might play a role, too. Furthermore, FASN expression was not altered in blastocysts from diabetic rabbits. Because lipogenesis encompasses fatty acid and subsequent triglyceride synthesis, it is tempting to speculate that the unchanged FASN expression in vivo is the result of an increased lipid supply by the diabetic mother and the increased expression of FABP4 and FATP4 in their blastocysts (Figure 5). On the other hand, it is also possible that the 3-fold increase in glucose in uterine secretions (20) is not high enough to regulate FASN expression. Finally, the increased FABP4 amount in the cavity fluid from diabetic blastocysts may play a pivotal role by trafficking free fatty acids to blastocyst cells.

In our in vitro model, we can create a hyperglycemic situation independent of any triglyceride influence. In the case

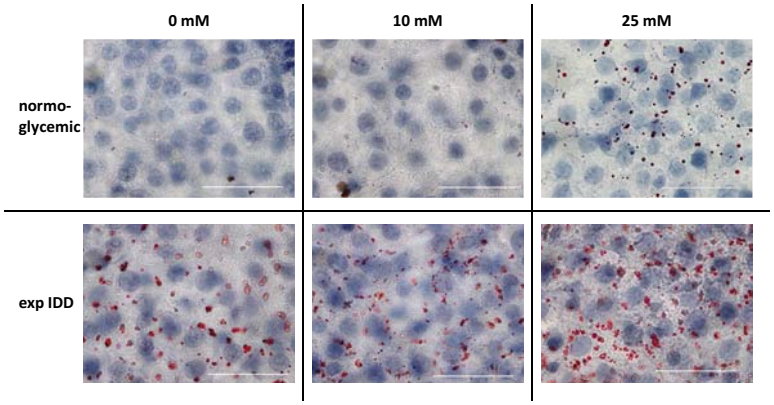

Figure 4. Intracellular lipid accumulation in blastocysts cultured in vitro with 0, 10, and 25 mM glucose. Six-day-old blastocysts were removed from healthy (normoglycemic) and diabetic (expIDD) rabbits and cultured in vitro with 0, 10, or 25 mM glucose for 6 hours. Then lipid droplets were stained red with Oil Red O (red dots). Nuclei were counterstained blue with hematoxylin. Representative pictures from trophoblasts are shown (scale bar corresponds to 50 μm). Culture was performed in groups of at least 4 blastocysts in each treatment and repeated in 3 independent replicates (N = 3; n ≥ 4).

of an oversupply of glucose in vitro, the embryo shows a glucose-dependent regulation of lipogenic marker genes, including FASN (Figure 5). We interpret these findings as increased de novo lipogenesis, the first step of lipogenesis. However, FATP4 expression was not affected in vitro because the blastocysts were not supplied with lipids from the outside. Glucose can stimulate de novo lipogenesis via several mechanisms. First, glucose can be converted into lipids for storage (72). Furthermore, embryos cultured with high glucose concentrations show a slightly increased uptake of palmitic acid and an enhanced uptake of arachidonic acid (36). That high glucose and enhanced fatty acid uptake increase the accumulation of lipid droplets has also been shown in renal tubal epithelium and pancreatic β-cells (73–76). The up-regulation of FASN was SREBP1 dependent (74–76). We found a glucose-dependent accumulation of lipid droplets, a higher nuclear SREBP1 amount in vitro and an increased nSREBP1/cSREBP1 ratio in vivo. Therefore, SREBP1 could belong to the connecting link between glucose and lipid storage in preimplantation embryos.

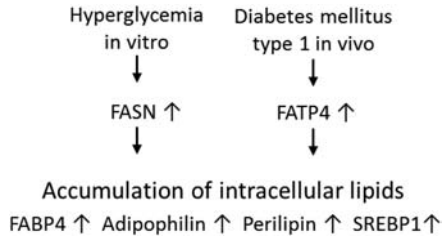

Figure 5. Schematic mechanism of intracellular lipid accumulation in rabbit blastocysts in diabetic conditions in vivo and hyperglycemia in vitro. In the preimplantation embryo, hyperglycemia leads to a nonphysiological lipid accumulation with increased expression of the adipogenic markers adipophilin, perilipin, FABP4, and SREBP1. In in vivo and in vitro conditions, the embryonic gene regulation differed only in FATP4 or FASN with increases in FATP4 in vivo and FASN in vitro.

Several studies clearly show that glucose is a main source and key signal for SREBP1 induction and cleavage of SREBP1 precursor protein in a dose-dependent manner (77–79), as shown by our in vitro experiments, too. This effect is not due to osmotic properties, because glucose could not be replaced by the glucose analogs 2-deoxyglucose or 3-*O*-methyl glucopyranose (77, 79). SREBP1 activity is necessary not only for full induction of de novo fatty acid synthesis but also for intracellular lipid droplet accumulation by increasing cholesterol biogenesis and activation of the low-density lipoprotein receptor (80–82), leading to increased lipogenesis.

The present results are particularly important with respect to possible short- and long-term consequences of in vitro biotechnologies such as assisted reproductive technologies, in which embryos are cultured for various times with high glucose concentrations before transfer. Experimental evidence suggests that culture conditions influence normal development and may contribute to prenatal and/or postnatal disorders (83–86). We show for the first time that high glucose concentrations in vitro lead to a higher lipid accumulation and an elevated expression of key lipogenic target genes, indicating that the glucose concentration is critical for metabolic set points of embryonic cells. Embryonic cells adapt to changes in glucose concentration in the surrounding milieu and may retain the information during later differentiation, as has been shown in embryonic stem cells (87).

However, we cannot rule out the possibility that factors other than hyperglycemia or hyperlipidemia may also influence intracellular lipid accumulation and lipogenic gene expression. Insulin and IGFs, which are known to be regulated by diabetic developmental conditions (39, 47), are potent regulators of FASN expression and enzyme activity (88–90), FABP4 expression (91, 92), and SREBP1 transcription and maturation (78, 93, 94). Therefore, the altered insulin/IGF system and/or a potential interplay between insulin/IGFs, hyperlipidemia, and hyperglycemia has to be kept in mind, when the underlying mechanism of aberrant embryonic lipid storage in diabetic blastocysts is discussed.

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In conclusion, maternal diabetes alters the concentration of a variety of maternal nutrients, which in turn modifies the metabolic uterine environment for the developing embryo. Exposure to such an altered nutrient profile can disrupt normal development or, less dramatically, change embryonic metabolism. We demonstrated that embryonic lipid storage is altered under induced maternal diabetes mellitus in the rabbit. Intracellular lipid accumulation and expression of key genes for lipid storage (perilipin and adipophilin), fatty acid transport, and metabolism (FATP4 and FABP4) and lipogenesis (SREBP1) are increased. This effect can be explained in part by the fact that hyperglycemia in vitro increases the expression of lipogenic target genes and the amount of intracellular lipid droplets. Because preimplantation embryo development is one of the most critical periods in an individual's life, future health trajectories may be (mis)programmed with severe consequences later in life.

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REPRODUCTION
RESEARCH

Accumulation of advanced glycation end products in the rabbit blastocyst under maternal diabetes

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Abstract

Diabetes mellitus (DM) during pregnancy is one of the leading causes of perinatal morbidity and birth defects. The mechanism by which maternal hyperglycemia, the major teratogenic factor, induces embryonic malformations remains unclear. Advanced glycation end products (AGEs) are known to accumulate during the course of DM and contribute to the development of diabetic complications. Employing a diabetic rabbit model, we investigated the influence of maternal hyperglycemia during the preimplantation period on AGE formation (pentosidine, argpyrimidine, and N^{ε}-carboxymethyllysine (CML)) in the reproductive tract and the embryo itself. As a consequence of type 1 DM, the AGE levels in blood plasma increased up to 50%, correlating closely with an AGE accumulation in the endometrium of diabetic females. Embryos from diabetic mothers had increased protein-bound CML levels and showed enhanced fluorescent signals for AGE-specific fluorescence in the blastocyst cavity fluid (BCF). The quantification of CML by HPLC–mass spectrometry (MS/MS) showed a higher amount of soluble CML in the BCF of blastocysts from diabetic rabbits (0.26 ± 0.05 μmol/l) compared with controls (0.18 ± 0.02 μmol/l). The high amount of AGEs in blastocysts from diabetic mothers correlates positively with an increased *AGER* (receptor for AGE (*RAGE*)) mRNA expression. Our study gives alarming insights into the consequences of poorly controlled maternal diabetes for AGE formation in the embryo. Maternal hyperglycemia during the preimplantation period is correlated with an increase in AGE formation in the uterine environment and the embryo itself. This may influence the development of the embryo through increased AGE-mediated cellular stress by RAGEs.

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Introduction

Approximately 7% of pregnancies are complicated due to diabetes mellitus (DM; American Diabetes Association 2013). Increasing obesity rates are a serious risk factor for type 2 DM and gestational DM (American Diabetes Association 2013). DM during pregnancy is of great concern as it is a major cause of perinatal morbidity and mortality (Combs & Kitzmiller 1991, Greene 1999). Although our understanding and management of DM have improved during the last decades, diabetic pregnancies are still reported to have numerous adverse effects (Combs & Kitzmiller 1991, Aberg *et al.* 2001, Eriksson *et al.* 2003, Corrigan *et al.* 2009). Hyperglycemia is considered as a major teratogenic factor for congenital malformation, although other associated factors such as ketone bodies, branched amino acids, and triglycerides have also been shown to exert adverse effects on the developing embryo (Eriksson *et al.* 2000). However, it is not yet clear in which way maternal hyperglycemia affects prenatal embryo development. There is upcoming evidence that advanced glycation end products (AGEs) might play a critical role in diabetic pregnancies.

AGEs are a complex group of compounds formed via non-enzymatic reactions between reducing sugars and N-terminal amino groups on proteins, lipids, and nucleic acids. End-stage products of the protein glycation can be divided into fluorescent AGEs (such as argpyrimidine), non-fluorescent AGEs (such as N^{ε}-carboxymethyllysine (CML)), and cross-linking compounds (such as pentosidine). Owing to the intrinsic fluorescence of some AGEs, plasma and tissue fluorescence can be used as markers for AGE accumulation (Goh & Cooper 2008, Bos *et al.* 2011). Formation and accumulation of AGEs are related to aging as well as to prolonged hyperglycemia and oxidative stress resulting from DM (Sell *et al.* 1991, Lee & Cerami 1992, Dyer *et al.* 1993, Brownlee 1995).

AGEs are identified to play a role in the development of diabetic complications such as diabetic nephropathy, cardiomyopathy, atherosclerotic disease, peripheral neuropathy, and ocular disease (Ahmed & Thornalley 2007,

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Nass *et al.* 2007). The post-translational modification of proteins by reducing sugars alters their biological structure and function and leads not only to a loss of molecular function but also to a reduced degradation of these damaged proteins. An additional proposed mechanism of AGE-induced damage is the release of reactive oxygen species, particularly superoxide and hydrogen peroxide by AGEs (Carubelli *et al.* 1995, Ortwerth *et al.* 1998). AGEs are able to activate intracellular cascades by binding specific receptors, for example, the receptor for AGEs (RAGEs). AGE–RAGE interactions induce a broad spectrum of signaling mechanisms such as p21ras, Erk1/2 MAP kinases (MAPKs), p38 and SAPK/JNK MAPKs, Rho GTPases, PI3K, and the JAK/STAT pathway (Bierhaus *et al.* 2005, Rouhiainen *et al.* 2013). Downstream consequence is the activation of nuclear factor κB (NFκB) that results in the release of pro-inflammatory mediators such as free radicals and cytokines (Berbaum *et al.* 2008). AGER (RAGE) has been shown to be expressed in the rabbit blastocyst, at mRNA and protein levels (Ott *et al.* 2014).

Women with gestational DM have significantly higher serum AGE levels compared with healthy controls, whereas women with types 1 and 2 DM, in good medical supervision, show normal AGE levels (Buongiorno *et al.* 1997). There is a strong relationship between mothers and neonates regarding AGEs. In women with gestational DM, high levels of AGEs and advanced oxidation protein products (AOPPs) are also detectable in the umbilical blood of their neonates. Both, diabetic mother and neonate, showed higher AGE and AOPP levels compared with healthy controls (Boutzios *et al.* 2013). Elevated AGE levels in women with gestational DM are associated with pregnancy complications such as birth asphyxia, congenital malformations, or stillbirth (Guosheng *et al.* 2009). The harmful effects of AGEs after implantation and placentation are likely to threaten the embryo/fetus too, as maternal hyperglycemic blood is connected to the blood system of the embryo. However, this study demonstrates that the preimplantation period is also of great importance on AGE formation, especially in mothers with poorly controlled preexisting DM.

The preimplantation period is a critical ontogenetic stage in embryo development and highly vulnerable for teratogenesis. At this period, the embryo is most sensitive to its surrounding milieu, especially to deregulations by external stimuli (Watkins *et al.* 2008). In this study, we investigated the influence of a poorly controlled maternal type 1 DM on AGE formation in preimplantation embryos and in the reproductive tract employing a rabbit model. As the rabbit blastocyst implants at day 6.8 post coitum (p.c.), we recovered the blastocysts before, i.e. at day 6.0 p.c. At this time, the rabbit blastocyst is covered by an extraembryonic mucin layer (Fischer *et al.* 1991, Herrler *et al.* 2002). However, this layer does not interfere with glucose uptake and metabolism *in vivo* and *in vitro* (Fischer *et al.* 2010, Ramin *et al.* 2010, Schindler *et al.* 2013). We show that DM not only is critical for maternal metabolism but also affects the AGE accumulation and *AGER* mRNA levels in the developing embryo even before implantation.

Materials and methods

Alloxan treatment and allocation of samples

All animal experiments were performed in accordance with the principles of laboratory animal care and the experimental protocol was approved by the Local Ethical Commission of the 'Landesverwaltungsamt Dessau' (reference number: 42502-2-812).

Experimental type 1 DM was induced by alloxan (Sigma–Aldrich) treatment as described previously (Ramin *et al.* 2010). Rabbits were allowed to eat *ad libitum*. The blood glucose level of females with type 1 DM increased 1 day after alloxan administration and was kept in a range between 20 and 30 mmol/l by insulin supplementation (Huminsulin, basal (NPI), Lilly, Gießen, Germany; three times per day). The duration for the poorly controlled type 1 DM was ∼10 days before mating and during the 6 days of pregnancy. On average, diabetic rabbits had a 4.5-fold higher blood glucose concentration in comparison to the normoglycemic reference group (27.6±0.5 and 6.2±0.1 mmol/l, $P<0.001$; Fig. 1).

Follicle growth was stimulated by s.c. injection of 110 IU pregnant mare's serum gonadotropin (Intervet, Unterschleißheim, Germany) and ovulation was ensured by i.v. injection of 75 IU human chorionic gonadotropin (Intervet) after mating with fertile males. Samples from diabetic and normoglycemic rabbits were obtained 6 days after mating (p.c.). Rabbits were killed by an overdose of pentobarbital (Sigma–Aldrich) and exsanguination. Later, we obtained maternal blood, maternal tissues, and the blastocysts. The blastocysts were flushed out of the uteri and washed three times with PBS to avoid contamination of blastocyst samples with uterine tissue.

Protein preparation of blastocysts and AGE detection by slot blot analyses

For protein extraction, a group of eight to ten blastocysts from at least three mothers were randomly pooled, dissolved in RIPA

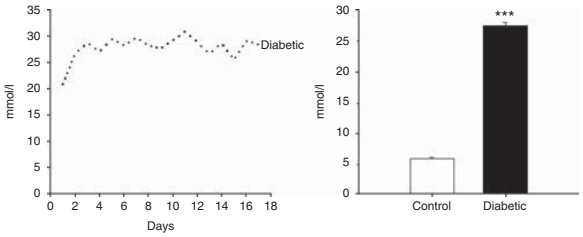

Figure 1 (A) Blood glucose levels after alloxan injection (day 0) in rabbits. The blood glucose level was monitored using commercially available blood glucose test strips three times a day. Data are shown as an average of nine animals with three measured data each per day (mean±S.E.M., ***$P<0.001$).

lysis buffer (PBS, 1% vol/vol NP-40, 0.5% wt/vol sodium deoxycholate, and 0.1% wt/vol SDS), and homogenized with a syringe (Omnifix 40 Duo, Braun, Melsungen, Germany). After incubation on ice for 30 min, the samples were centrifuged at 13 000 ***g*** for 20 min. The supernatant was stored at −80 °C until use for slot blot analysis.

Slot blot analyses were performed with 25 µg protein. Denatured protein samples (heated for 10 min at 80 °C) were spotted onto a nylon membrane (GE Healthcare, München, Germany) using a slot blot apparatus (Biostep, Rabenau, Germany). The protein load was determined by Ponceau S staining. After blocking with 5% milk/TBS–T for 1 h, the membrane was incubated with monoclonal mouse antibodies against CML, argpyrimidine, or pentosidine (1:100, Biologo, Kassel, Germany), respectively, overnight at 4 °C. Samples were rinsed three times with TBS–T for 5 min and subsequently incubated with a secondary goat anti-mouse IgG for 1 h (Dianova, Hamburg, Germany). The immunoreactive signal was visualized by ECL detection (Millipore, Schwalbach, Germany) and quantified by Fusion FX7 and the corresponding software Fusion 15.18. Protein modification rate was calculated as the ratio of protein load (Ponceau) and slot intensity by antibody reaction.

Protein preparation of maternal tissues and AGE detection by slot blot analyses

After flushing out the blastocysts, we dissected the uterine tissue. Protein isolation was carried out either with the entire uteri or with separated endometria. For the latter, the endometrium was scraped mechanically from the myometrium using sharp scalpels. Grinded uteri and scraped endometria were mixed with RIPA and homogenized with precellys (Peqlab, Erlangen, Germany). Slot blot analyses were performed in the same way as for blastocysts. AGE accumulation in blood was determined using EDTA–plasma. Slot blot analysis with uterine proteins was performed in the same way as for blastocysts.

Immunohistochemical localization of AGEs in blastocysts

The immunohistochemical analysis was performed with single blastocysts as described previously (Schindler *et al*. 2014). Embryonic discs were incubated with mouse MABs against CML, argpyrimidine, and pentosidine (1:100, Biologo). The nuclei were counterstained with hematoxylin. Embryonic discs were assessed using a light microscope (BZ 8100E, Keyence, Neu-Isenburg, Germany). The specificity of immunostaining of the secondary antibody reaction was proven by the absence of signals in sections processed after omission of the primary antibody.

Immunohistochemical localization of AGEs in ovary and uterus

Ovarian and uterine tissues were fixed in Bouin's solution, embedded in paraffin, and sectioned at 5 µm. Sections were mounted on silanized slides and deparaffinized at 60 °C overnight. Later, sections were rehydrated through a series of graded alcohols. Endogenous peroxidases were inhibited by incubating the slides with 3% H_2O_2 in methanol. After blocking in 10% goat serum for at least 1 h, the sections were incubated with the mouse MABs against CML, argpyrimidine, or pentosidine (1:100, Biologo) at 4 °C overnight. Samples were washed with TBS–T and incubated with the HRP-conjugated secondary goat anti-mouse IgG (Dako, Hamburg, Germany). The AGE-modified proteins were visualized by the peroxidase–diaminobenzidine reaction. The nuclei were counterstained with hematoxylin. Analysis was performed as described for blastocysts.

Quantification of AGER *mRNA in the rabbit blastocyst*

mRNA of single blastocysts was extracted using Dynabeads Oligo (dT) 25 (Invitrogen) and subsequently used for cDNA synthesis. The nucleotide sequence for rabbit *AGER* was determined using human primers for amplification of rabbit lung cDNA. The obtained rabbit *AGER* primers are as follows: sense, GCTACTGCTCCACCTTCTGG and antisense, GCAGTCAGAGCTGATGGTGA (ref. LOC100343142). The amount of *AGER* transcripts was determined by real-time quantitative PCR (RT-qPCR) using the Applied Biosystems StepOnePlus System (Applied Biosystems). The entire protocol for mRNA quantification and RT-qPCR has been described previously (Schindler *et al*. 2013).

AGE fluorescence in the blastocyst cavity fluid

To obtain the blastocyst cavity fluid (BCF), single blastocysts were washed twice with ice-cold PBS and placed on a Petri dish. The remaining PBS was removed and the blastocyst was punctured using a syringe. The escaping BCF was taken in an Eppendorf tube and stored at −80 °C. The AGE fluorescence was determined in BCF using a Synergy MX 200 microplate reader (BioTek, Bad Friedrichshall, Germany). Then, 3 µl of the undiluted BCF were analyzed in a black Take 3 Micro-Volume Plate (BioTek). PBS was used as a control. Fluorescence emission spectra were recorded at excitation wavelengths of 330 and 360 nm. The maximum emission for the tested excitation wavelengths were found at 405 and 440 nm respectively.

CML quantification in the BCF by HPLC/mass spectrometry

CML was synthesized according to the literature (Glomb & Monnier 1995). The identity of the reference compound was verified by nuclear magnetic resonance experiments. Furthermore, the elemental composition was confirmed by accurate mass determination. Up to seven BCF of at least two animals were pooled. The pooled BCF was diluted 1:10 with ultrapure water. The HPLC apparatus (Jasco, Gross-Umstadt, Germany) consisted of a pump (PU-2080 Plus) with a degasser (LG-2080-02) and a quaternary gradient mixer (LG-2080-04), a column oven (Jasco Jetstream II), and an Autosampler (AS-2057 Plus). Chromatographic separations were performed on a stainless steel column packed with RP-18 material (VYDAC CRT, no. 218TP54, 250× 4.6 mm, RP 18, 5 µm, Hesperia, CA, USA) using a flow rate of 1.0 ml/min. The mobile phase used was water (solvent A) and methanol/water (7:3 (v/v), solvent B). To both solvents (A and B), 1.2 ml/l heptafluorobutyric acid was added. Analysis was

performed at 35 °C column temperature using isocratic elution at 98% of A/2% of solvent B. Mass spectrometric detection was conducted on a API 4000 Q Trap LC/mass spectrometry (MS/MS) system (AB Sciex, Darmstadt, Germany) equipped with a turbo ionspray source using electrospray ionization in the positive mode: sprayer capillary voltage, 2.5 kV; nebulizing gas flow, 50 ml/min; heating gas, 60 ml/min at 550 °C; and curtain gas, 40 ml/min. The multiple reaction monitoring mode was used, utilizing collision-induced dissociation of the protonated molecule with compound-specific orifice potential (50 V) and fragment-specific collision energies (CEs). CML (*m/z* 205.1→130.2 (CE 17), 84.1 (CE 46), and 159.1 (CE 15)) was detected at the retention time of $t_R = 6.0$ min.

Quantification was performed by the standard addition method. Briefly, increasing concentrations of an authentic reference compound by factors of 0.5, 1, 2, and 3×the concentration of the analyte in the sample were added to separate aliquots of the sample after workup procedure. The aliquots were analyzed, and a regression of response vs concentration was used to determine the concentration of the analyte in the sample. Calibration using this method resolves potential matrix interferences.

All samples were analyzed in a single batch to exclude inter-assay variations. Intra-assay coefficient of variation values (CV = 9%) were determined by repeated analyses of a BCF sample ($n = 5$). In addition, the limit of detection (LOD = 4.8 pmol/ml) and the limit of quantification (LOQ = 14.5 pmol/ml) with all steps of the analysis included were estimated according to the German standard method DIN 32645: 2008-11 ($n = 5$, confidence level $P = 0.95$, and $k = 3$).

Determination of glyoxal and methylglyoxal in plasma and blastocysts

The quantification of glyoxal (GO) and methylglyoxal (MGO) was achieved mainly as described by Espinosa-Mansilla *et al.* (2007). Standard curves of GO and MGO (40% aqueous solution, Sigma–Aldrich) were obtained by preparing serial dilution of GO and MGO (2700, 900, 300, 100, and 0 nM) in HPLC-grade water (Millipore). The rabbit plasma proteins and cell lysate proteins of the embryos were precipitated using trifluoroacetic acid (1/10 of the sample volume). After 10 min of incubation on ice, the samples were centrifuged at 13 000 ***g*** for 10 min. To 100 µl of the supernatant, HPLC-grade water was added (final volume, 1 ml) followed by the addition of 0.125 ml ammonium chloride (0.5 M; pH 10.0) and 2.5 ml of 5,6-diamino-2,4-hydroxypyrimidine sulfate (0.75 mM). The mixture was incubated for 90 min at 60 °C with constant shaking. Subsequently, citrate buffer (10 mM; pH 6.0) was added to a final volume of 25 ml. For the analysis, 10 µl of the samples were injected (ZORBAX, Eclipse XDB-C18 4.6×150 mm 5-micron column; Agilent, Oberhaching, Germany) and then separated by gradient elution with solvents A (3% acetonitrile) and B (97% citrate buffer) at a flow rate of 0.8 ml/min.

Statistical analysis

To obtain statistically funded data, we repeated the animal experiment at least three times ($n = 3$). In each animal experiment, we had nine diabetic and six healthy rabbits. We pooled tissue samples and blastocysts from each individual experiment from at least three animals. Levels of significance between groups were calculated using Student's *t*-test after proving normal distribution (SigmaPlot v. 11.0). The Mann–Whitney *U* rank sum test was used when a normal distribution was not guaranteed. Data are expressed as mean ± S.E.M. The levels of statistical significance were $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Results

Determination of α-dicarbonyls and AGEs in the plasma of female rabbits with type 1 DM

As the majority of AGEs *in vivo* appear to be formed from α-dicarbonyls (Brownlee 1995, Rabbani & Thornalley 2012), we measured the plasma level of GO and MGO by HPLC. The AGE precursor MGO was not altered, whereas GO (control 398 ± 31 nM and diabetic 522 ± 59 nM) was tendentially enhanced (Fig. 2A). To determine the AGE status of the diabetic rabbits, we investigated various AGEs by slot blot analysis with specific antibodies against pentosidine, CML, and argpyrimidine respectively. All of the analyzed protein modifications showed a considerable increase in the plasma probes of diabetic rabbits (Fig. 2B and C).

Determination of AGE modifications in the reproductive tract of female rabbits with type 1 DM

The constitution of the uterus tissue is crucial for the course of pregnancy. The endometrium, a dynamic mucosa adjacent to the myometrium of the uterus, is important for the implantation process. Argpyrimidine,

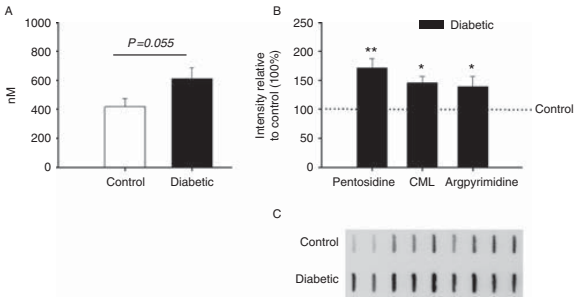

Figure 2 (A) Concentration of glyoxal (GO) in diabetic and non-diabetic rabbits. GO was quantified by HPLC (mean ± S.E.M.; $N = 3$, $n = 3$; P = as indicated). (B) Relative amount of protein-bound pentosidine, N^{ε}-carboxymethyllysine (CML), and argpyrimidine in the plasma of rabbits after being diabetic for ~2 weeks compared with the non-diabetic control (set 100%). The quantification was performed by slot blot analysis (mean ± S.E.M.; $N = 3$, $n = 9$; $^*P < 0.05$ and $^{**}P < 0.01$). The AGE amount is related to the protein load (Ponceau S staining). (C) A representative slot blot is shown for pentosidine.

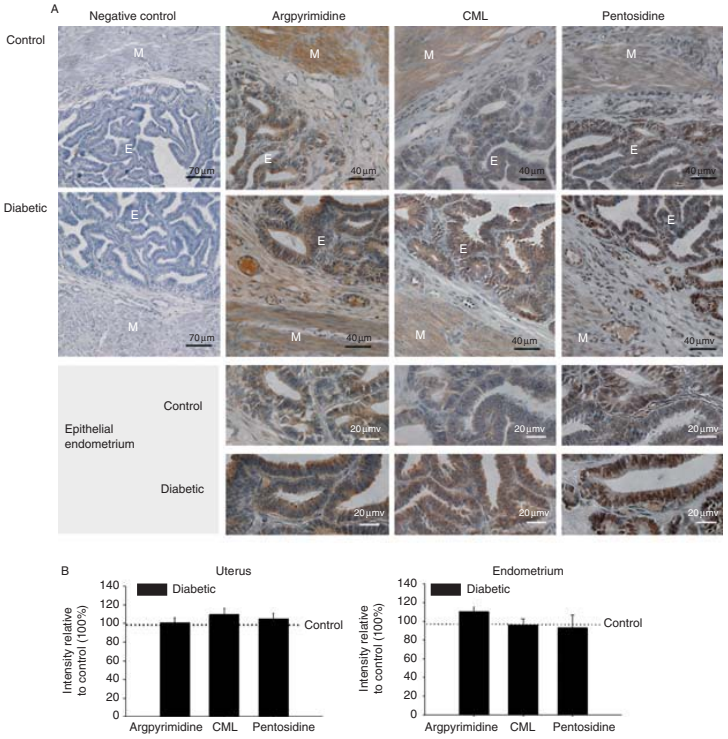

Figure 3 (A) Immunohistochemical analysis of argpyrimidine, N^{ε}-carboxymethyllysine (CML), and pentosidine in the pregnant uterus of rabbits with type 1 diabetes mellitus and in healthy controls. The AGE-modified proteins were visualized by the peroxidase–diaminobenzidine reaction (brown color). The nucleus was counterstained with hematoxylin (blue colour). The negative control is the control reaction of the HRP-conjugated secondary goat anti-mouse IgG without a primary antibody. CML and argpyrimidine show an exclusive cytosolic staining, whereas pentosidine is localized to both the cytosol and the nucleus. The epithelial endometrium (E) of diabetic rabbits shows a stronger staining, whereas the staining of the myometrium (M) seems to be unchanged. (B) Relative amounts of protein-bound argpyrimidine, CML, and pentosidine in the entire uterus and the separated endometrium from diabetic and healthy rabbits measured by slot blot analysis (mean$\pm$S.E.M.; $N=3$, $n=9$). The amounts of AGE are related to the protein load (Ponceau S staining).

CML, and pentosidine are present in the endometrium and myometrium. AGEs are localized to smooth muscle cells, not only in the myometrium but also in the endothelium of vessels. In the endometrium, CML and pentosidine are exclusively present in the epithelium; argpyrimidine is also slightly present in the stroma. CML and argpyrimidine were mainly localized to the cytoplasm. Pentosidine showed a cytosolic staining and stained nuclei. CML, pentosidine, and argpyrimidine showed a strongly stained endometrial epithelium in diabetic rabbits (Fig. 3A).

Slot blot analyses revealed no differences in protein-bound CML, pentosidine, and argpyrimidine between diabetic and normoglycemic rabbits for the entire uterus and for the endometrium in particular (Fig. 3B).

Immunohistochemical staining of the ovary revealed no differences between normoglycemic and diabetic females. Figure 4 shows the AGE distribution in the ovary from normoglycemic controls. All determined AGEs were detectable in the ovary. CML and argpyrimidine showed an exclusive cytosolic staining, whereas pentosidine was localized to both the cytoplasm and nuclei. The oocyte showed positive staining for CML, pentosidine, and argpyrimidine in the cytoplasm, but not in the nucleus in the investigated follicle stages (primary, secondary, and tertiary follicle).

Determination of AGE modifications in 6-day-old rabbit blastocysts developed under diabetic conditions

Immunohistochemical detection of pentosidine, CML, and argpyrimidine showed strong staining in the embryoblast (EB) and well-stained trophoblast (TB) cells (Fig. 5A). All detected AGEs were exclusively present in the cytoplasm. Slot blot analysis revealed a significantly higher level of protein-bound CML in blastocysts from diabetic mothers (Fig. 5B). Similarly, argpyrimidine level was tendentially increased. The detection of the reactive α-dicarbonyls MGO showed no differences between blastocysts from diabetic and normoglycemic rabbits (Fig. 5C). GO was under the detection limit.

The BCF is known to be an important reservoir for nutrients during preimplantation. We used the fluorescent properties of AGEs to determine AGE accumulation in the BCF. Specific peaks for the known AGE fluorescence with excitation and emission at wavelengths of 330/405 and 360/440 nm, respectively, were detectable. Both were significantly increased in the BCF of blastocysts developed under diabetic conditions (Fig. 6A). The protein content of the BCF was equal in both groups with 0.43 ± 0.012 µg/µl. Besides fluorescent AGEs, the non-fluorescent CML as a free adduct was identified by HPLC/MS in the BCF. The quantification of

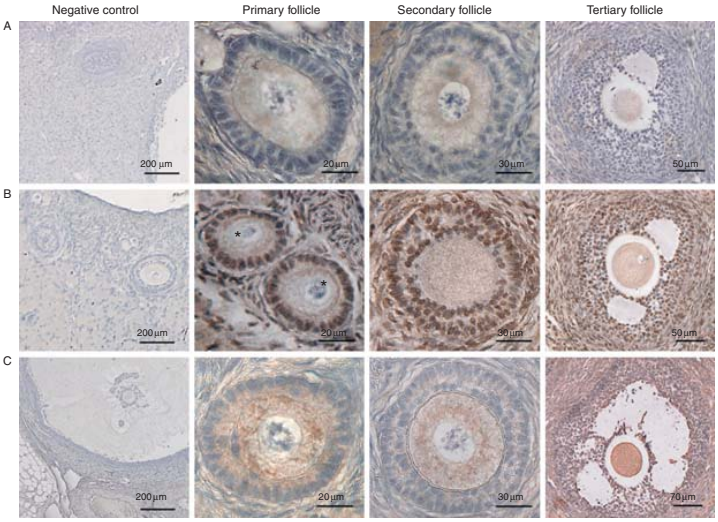

Figure 4 Immunohistochemical analysis of N^{ε}-carboxymethyllysine (A), pentosidine (B), and argpyrimidine (C) in the ovary of healthy rabbits. Follicle growth was stimulated by s.c. injection of pregnant mare's serum gonadotropin. The AGE-modified proteins were visualized by the peroxidase–diaminobenzidine reaction (brown color). The nuclei were counterstained with hematoxylin (blue color). The negative control is the control reaction of the HRP-conjugated secondary goat anti-mouse IgG without a primary antibody. CML and argpyrimidine show an exclusive cytosolic staining, whereas pentosidine was localized to both the cytoplasm and the nuclei, except for nuclei of the oocytes*.

CML showed a higher amount of soluble CML in the BCF of blastocysts from diabetic mothers with 0.26 ± 0.05 µmol/l compared with controls with 0.18 ± 0.02 µmol/l (Fig. 6B).

AGER *mRNA amount in rabbit blastocysts*

AGER mRNA was detectable from the early blastocyst stage (day 4 p.c.) onwards (Ott *et al.* 2014). At day 6 p.c., the amount of *AGER* mRNA was significantly increased under diabetic conditions (Fig. 7).

Discussion

An intrauterine exposure to hyperglycemia has been shown to cause alterations in pre- and postnatal growth patterns. The underlying metabolic disorder may predispose offspring to develop metabolic diseases in later life (Silveira *et al.* 2007). Several animal studies demonstrated a developmental delay for embryos recovered from diabetic mothers (Giavini *et al.* 1986, Moley *et al.* 1991, Ramin *et al.* 2010). Our study provides new insights into the effects of a maternal DM during the preimplantation period by analyzing the AGE formation in both, the mother and the developing embryo. Thus far, AGE formation is mostly associated with aging and diseases. By contrast, Ling *et al.* (2001) had already reported AGE modifications in fetal rats from day 10 p.c. onwards. A recently published study has revealed a high rate of glycated and oxidized proteins in undifferentiated mouse embryonic stem cells (ESCs) and in mouse blastocysts at day 3.5 p.c. HSPA8 (HSC70) was identified as the major protein modified by CML in undifferentiated mouse ESCs (Hernebring *et al.* 2006). Similar to the findings of our study on day 6 rabbit blastocysts, AGE-modified proteins were mainly present in the EB, and strongly stained cells were also found in the TB (Fig. 5A). The protein modifications by pentosidine, CML, and argpyrimidine were almost exclusively observed in the cytoplasm.

It is known that AGEs accumulate intracellularly (Goldin *et al.* 2006). Besides the physiological appearance of AGEs in preimplantation embryos, we observed pathological AGE accumulations in blastocysts from diabetic mothers. Blastocysts are obviously susceptible for AGE formation while growing up in a diabetic uterine milieu. A 6-day-old rabbit blastocyst developed in a diabetic uterine milieu showed tendentially more AGEs intracellularly and in the BCF compared with age- and stage-matched control blastocysts (Figs 5 and 6). Protein-bound CML and argpyrimidine levels were elevated, whereas protein-bound pentosidine was unchanged. CML and argpyrimidine are AGEs resulting from reactions of α-dicarbonyls (GO and MGO) and amino groups that are known to be highly reactive. Although glucose and fructose are present in significant concentrations in uterine secretions, the fact that they react slowly with proteins to form AGEs must be taken into consideration. Pentosidine is sourced mostly from ribose but also from glucose (Dyer *et al.* 1991, Grandhee & Monnier 1991). Previous investigations had demonstrated an unchanged glucose uptake in rabbit blastocysts developed under normoglycemic and diabetic conditions (Schindler *et al.* 2013). These findings might explain the unchanged protein-bound pentosidine concentration. The intracellular glucose concentration has not been determined so far. We found measurable, but unaltered, concentrations of MGO in blastocysts

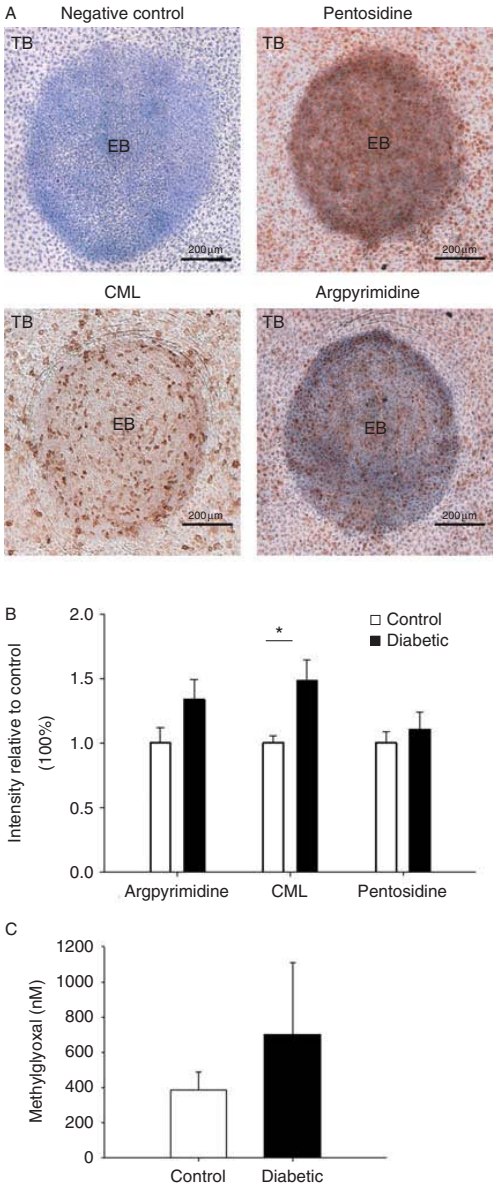

Figure 5 (A) Immunohistochemical analysis of argpyrimidine, N^{ε}-carboxymethyllysine (CML), and pentosidine in embryoblast (EB) and trophoblast (TB) cells of 6-day-old rabbit blastocysts. The AGE-modified proteins were visualized by the peroxidase–diaminobenzidine reaction (brown color). The used antibodies show an exclusive cytosolic staining. The EB has tendentially more AGE modifications than the TB. The nucleus was counterstained with hematoxylin (blue colour). The negative control is the control reaction of the HRP-conjugated secondary goat anti-mouse IgG without a primary antibody. (B) Relative amounts of protein-bound argpyrimidine, CML, and pentosidine in 6-day-old rabbit blastocysts from diabetic and healthy (control) rabbits analyzed by slot blot analysis (mean±S.E.M.; $N=6$, $n=8$–10; $^{*}P<0.05$). The amount of AGE is related to the protein load (Ponceau S staining). (C) Methylglyoxal quantification in 6-day-old rabbit blastocyst by HPLC (mean±S.E.M.; $N=3$, $n=8$–10).

from diabetic and normoglycemic rabbits (Fig. 5C). The quantification of GO in blastocysts failed at the detection limit of 300 nM. Recent estimates of the cellular concentrations are 1–5 µM for MGO and 0.1–1 µM for GO (Dobler *et al.* 2006). Formation of MGO arises from a decreased activity of the reductive pentose phosphate pathway and formation of GO arises from lipid peroxidation (Fu *et al.* 1996, Januszewski *et al.* 2003, Thornalley & Rabbani 2009). We have new evidence that the lipid metabolism is altered in the rabbit blastocyst in case of a maternal DM. Besides noticeable lipid accumulations, the transport protein for fatty acids and other lipophilic substances, fatty acid-binding protein, is upregulated (Schindler *et al.* 2014). The two pathways – altered fatty acid metabolism and AGE formation – may interact and contribute to specific aspects of maternal subfertility in DM. In the rabbit model, maternal DM is associated with a reduced ovulation rate and lower number of blastocysts. Blastocyst development is retarded (Ramin *et al.* 2010). A recently published study has demonstrated that in ART patients an increased AGE concentration in human follicular fluid is associated with diminished follicle growth, a lower fertilization rate, and delayed embryo development (Jinno *et al.* 2011).

A major reason for AGE-mediated damage is the activation of RAGEs (Bierhaus *et al.* 2005, Nass *et al.* 2007, Berbaum *et al.* 2008, Rouhiainen *et al.* 2013). RAGE–ligand interaction leads to an increase in the expression of AGER. This type of positive feedback loop results in prolonged NFκB activation (Bierhaus & Nawroth 2009, Fritz 2011). In our study, we found a similar positive correlation between AGE accumulation and an increased *AGER* mRNA expression in blastocysts from diabetic mothers, confirming the view that AGE–RAGE interaction leads to an upregulation of RAGEs. This finding could be a proof for an active AGE–RAGE system in the blastocyst, which may negatively affect the embryo quality in diabetic mothers.

From which source the observed AGEs in the early embryo originate is uncertain. As AGE formation, especially due to glucose reactions, is an exceedingly

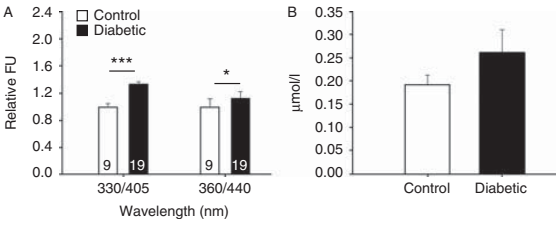

Figure 6 (A) Relative AGE fluorescence in the blastocyst cavity fluid (BCF) of blastocysts from diabetic and control rabbits (mean±S.E.M., $N=3$, $n=$as indicated; $^{*}P<0.05$ and $^{***}P<0.001$). (B) The free AGE adduct N^{ε}-carboxymethyllysine (CML) in the BCF was quantified by HPLC/mass spectrometry ($N=4$, $n=15$–18).

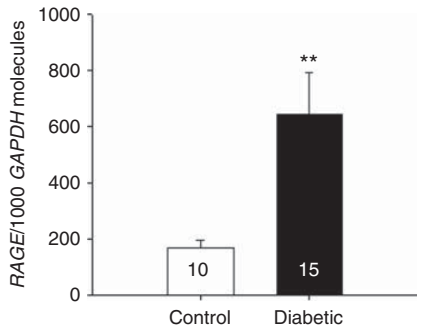

Figure 7 *RAGE* mRNA amount in 6-day-old blastocysts from diabetic and control rabbits. *RAGE* mRNA was related to the amount of *GAPDH* mRNA molecules per blastocyst (mean±S.E.M.; $N=6$, n=as indicated, $^{**}P<0.01$).

slow process and the degradation of AGEs *in vivo* is negligible, it can be assumed that AGEs may descent from germ cells. Matsumine *et al.* (2008) have demonstrated increased levels of pentosidine in the primordial, primary, and atretic follicles in premenopausal women. The reduced fertility and reduction in the follicle quality during aging are supposed to be, besides other reasons, due to AGE accumulation (Tatone & Amicarelli 2013). Our rabbit model confirms the presence of AGEs in oocytes (Fig. 4). As the experimentally induced type 1 DM had no effect on the AGE concentration in oocytes, it is unlikely that the accumulated AGEs in diabetic blastocysts are of oocyte origin in our model. However, it is likely that AGEs found in the oocyte might be still present in the developing embryo. This view may be relevant to women of older age, as AGE accumulation takes place for a longer term in this case, and to women with a long-term poorly controlled DM.

The increased CML concentration in the diabetic blastocyst (Fig. 5B) was additionally reflected by increased CML concentrations in the BCF (Fig. 6B). Furthermore, diabetic mothers showed increased amounts of CML in the plasma (Fig. 2B) and, through immunohistochemical detection, in the endometrium (Fig. 3A). The disparity between the results of immunohistochemistry and slot blot analysis in uterine tissue (Fig. 3) may be caused by the broad spectrum of AGE modification in tissues. The used antibodies are able to detect, besides protein-bound modifications, free AGEs. It is known that the non-enzymatic reaction between reducing sugars and amino groups also affects lipids and nucleic acids. The immunohistochemical method might capture more AGE modifications than slot blot analysis that detects only protein-bound AGEs. As CML level is increases in both, mother and blastocyst, it is possible that the maternally formed CML are transferred to the embryo. Little is known about the transport mechanism of AGEs. Experiments with Caco-2 cells showed a low transepithelial flux of CML. However, there was no measurable active transport for CML across the epithelial monolayer, neither via PEPT1 (SLC15A1) nor by carriers for neutral amino acids. The observation led to the conclusion that the transport is based on simple diffusion (Grunwald *et al.* 2006). Further studies are necessary to clarify this hypothesis.

For the first time, we demonstrate that one consequence of maternal DM is AGE formation in preimplantation embryos. AGEs do accumulate in blastocysts if the maternal DM is poorly controlled. Although our results do not provide a causative mechanism between embryo toxicity and DM, it is likely that AGEs play a role as stimuli for activating intracellular stress pathways and, additionally, do affect the molecular function of intracellular proteins. It is known that even moderate changes in the preimplantation environment can adversely affect the pre- and postnatal phenotypes (Fleming *et al.* 2004, Sinclair & Singh 2007). A clear consequence of these findings is the necessity for a strict control of maternal blood glucose levels during pregnancy from the day of conception onwards.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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4. Ergebnisse und Diskussion

Das Wachstum des Embryos ist abhängig von der Versorgung mit Nährstoffen über den mütterlichen Organismus. Um sich kurzfristig an veränderte Wachstumsbedingungen anzupassen, besitzt der Embryo vermittelt über auto- und parakrine Regulationsmechanismen eine große metabolische Plastizität. Die Adaptation ist die Fähigkeit von Präimplantationsembryonen, Wachstums- und Nahrungsfaktoren zu produzieren und dadurch seinen Stoffwechsel auf das veränderte Nährstoffangebot einzustellen. Eine Anpassung des embryonalen Stoffwechsels an suboptimale Bedingungen könnte sich jedoch negativ auf die weitere Entwicklung auswirken und zu einer nachhaltigen Prägung der embryonalen Zellen führen. Im Verlauf der weiteren Entwicklung und Differenzierung könnte es dann zu Abweichungen vom eigentlichen „metabolischen" Entwicklungsprogramm kommen, wodurch der Phänotyp des Organismus auf Dauer beeinflusst wird. Welche Rolle der Transkriptionsfaktor CREB bei der Anpassung an veränderte Umgebungsbedingungen einnimmt, wird im Folgenden dargestellt und diskutiert. Zuerst wird die Rolle für das IIRS betrachtet.

4.1. Kompensatorische Erhöhung der IGFs und verminderte Insulin- und IGF-Sensitivität

Der Einfluss eines mütterlichen Diabetes mellitus auf die Expression der IIRS-Familie während der Präimplantationphase des Kaninchens wurde in den zwei Publikationen „*Maternal diabetes impairs gastrulation and insulin and IGF-I receptor expression in rabbit blastocysts*" und „*Insulin growth factor adjustment in preimplantation rabbit blastocysts and uterine tissues in response to maternal type 1 diabetes*" beschrieben (Ramin et al. 2010, Thieme et al. 2012a) und soll im Folgenden diskutiert werden.

Vor Einführung der Insulintherapie Anfang des 20igsten Jahrhunderts war Diabetes mellitus die Hauptursache für Infertilität bei Frauen im gebärfähigen Alter. Falls sich doch eine Schwangerschaft einstellte, war die fetale und neonatale Mortalität mit 60% extrem hoch (Vargas et al. 2010). Mit Beginn der Insulinbehandlung konnten die Erfolgschancen, überhaupt schwanger zu werden und ein gesundes Kind zur Welt zu bringen, wesentlich verbessert werden. Jedoch zeigt sich, dass trotz einer engmaschigen Blutzuckerkontrolle und verbesserter Insulintherapie das Auftreten von Komplikationen, wie zum Beispiel fetale Makrosomie, weitgehend unverändert blieb (Zhao and Reece 2013). Im Jahre 2008 wurde die größte Studie, die HAPO-Study, mit 23316 Probandinnen veröffentlicht (HAPO Study Cooperative Research Group et al. 2008). Diese belegt eindeutig die

negativen Einflüsse einer Hyperglykämie während der Schwangerschaft. Eine übergeordnete Rolle bei den Ursachen spielt aber auch der Insulinmangel der Mutter während der Frühschwangerschaft. Erst ab der 11. Entwicklungswoche ist der humane Fötus selbst in der Lage, Insulin zu produzieren (Reiher et al. 1983). Um den Verlust des mütterlichen Insulin zu kompensieren, müssen somit endokrine und lokale (parakrine und autokrine) Kompensationsmechanismen aktiviert werden.

Wichtige Mediatoren in diesem Zusammenspiel sind IGF1 und IGF2. Sie wirken synergistisch mit Insulin und gewährleisten so im Verlauf einer Schwangerschaft die Kopplung des embryonalen Metabolismus an das mütterliche Nährstoffangebot. In Folge eines mütterlichen Diabetes Typ 1 wird sowohl die uterine IGF-Produktion als auch die embryonale IGF-Synthese gesteigert und somit die lokale IGF-Menge deutlich erhöht (Thieme et al. 2012a). Diese kompensatorische Regulation wird direkt über den Insulinmangel ausgelöst, da Insulin die IGF-Synthese inhibiert (Thieme et al. 2012a). Auch bei schwangeren Diabetikerinnen findet man eine Erhöhung der fetalen IGF1- und IGF2-Spiegel (Bhaumick 1986, Roth et al. 1996, Hiden et al. 2009).

Inwieweit diese kompensatorische Erhöhung ausreichend ist, um den Insulinmangel vollständig zu kompensieren, ist fraglich. Zum einen ist in Blastozysten nur eine 1,6-fache Steigerung von IGF1 auf Proteinebene nachzuweisen (Thieme et al. 2012b). Zum anderen beeinflusst ein mütterlicher Diabetes nicht nur die Liganden des IIRS, sondern auch dessen Rezeptoren. Sowohl die Transkription des IGF1R als auch des IR sind deutlich unterdrückt (Ramin et al. 2010). Verantwortlich dafür könnten zum einen die erhöhten IGF-Spiegel sein. Supraphysiologische Konzentrationen an IGF1 und IGF2 führen zu einer Inhibierung der IGF1R-Expression (Papa et al. 1991, Chi et al. 2000, Prelle et al. 2001). Kaninchenblastozysten, die mit IGF1 stimuliert wurden, zeigen deutlich geringe IGF1R- und IR-Transkriptmengen im Vergleich zur korrespondierenden Behandlungskontrolle (Thieme et al. 2012a). Auch hohe Glukosekonzentrationen*pinpvitro* führen zu einer Unterdrückung der IGF1R- und IR-Transkription in Kaninchenblastozysten (Ramin et al. 2010). Aber auch Adiponektin ist in der Lage, die Transkription des IGF1R und IR zu inhibieren (Schindler, unveröffentlicht). Die geringere Rezeptivität gegenüber den Liganden führt zu einer eingeschränkten Aktivierbarkeit der Signalkaskade (mitogen-activated protein kinase (MAPK) und Proteinkinase B (Akt)) und einer reduzierten Expression zentraler Schlüsselenzyme im Glukosemetabolismus, der PEPCK und Hexokinase (Ramin et al. 2010). Somit legt unser derzeitiger Wissensstand nahe, dass aufgrund des Rezeptormangels der Verlust des Insulin durch die höhere lokale Verfügbarkeit an IGF nur partiell kompensiert werden kann.

Diese ersten Analysen umfassten die gesamte Blastozyste am Tag 6 *p.c*. (Ramin et al. 2010, Thieme et al. 2012a). Bedingt durch die zelltypabhängige Expression der Rezeptoren (Einleitung Kapitel 1.4.) sowie die hohe Produktion der Liganden im Embryoblasten (Thieme et al. 2012a) würde die Wirkung

erhöhter IGF-Spiegel und damit die para- und autokrine Wirkung insbesondere die Embryoblastzellen betreffen. Dies wird durch die Untersuchungen der CREB-vermittelten Insulin/IGF-abhängigen Adiponektin-Regulation unterstrichen (siehe Kapitel 4.3). Im Trophoblasten muss somit ein anderes Sicherheitssystem greifen, um den mütterlichen Insulinmangel und damit die fehlende Regulation des Stoffwechsels zu kompensieren. Ein potentieller Kandidat ist das Hormon Adiponektin, das in Präimplantationsembryonen den Glukose- und Lipidmetabolismus regulieren kann (Fischer et al. 2010, Kim et al. 2011).

4.2. Adiponektin und die gegenseitige Wechselwirkung mit dem IIRS

Adiponektin wirkt als embryogenes Hormon fördernd auf die Glukoseaufnahme und ersetzt die metabolischen Wirkungen von Insulin bei Insulinmangel des Embryos. Eine gegenseitige Interaktion des IIRS und ARS ist seit längerem im adulten Organismus bekannt. Humane Studien zeigen, dass Adiponektin bei einem Diabetes mellitus Typ 1 in höheren Konzentrationen im Blut zu finden ist (Imagawa et al. 2002, Maahs et al. 2007, Leth et al. 2008). Eine kompensatorische Übernahme der Insulinfunktion durch Adiponektin wird diskutiert. Adiponektin kann die Insulinwirkung über verschiedene Vermittler der Insulinsignalkaskade beeinflussen. Die Adiponektin-abhängige Aktivierung der AMPK führt zu einer Inhibierung der S6-Kinase (S6K) und zu einer Aktivierung des TSC1/2-Komplexes und damit zu einer Aktivierung des PI3K/Akt-Signalweges (Wang et al. 2007). Dieser Signalweg reguliert sowohl die verstärkte Glukoseaufnahme über die GLUT4-Translokation in die Zelle, als auch die Glukoneogenese über die Reduzierung der PEPCK-Transkription (Übersichtsartikel Yamauchi et al. 2014). Umgekehrt ist Insulin auch ein potenter Regulator des ARS. Patienten mit einem genetisch-bedingten IR-Defekt weisen erhöhte Adiponektinspiegel im Blut auf (Imagawa et al. 2002, Semple et al. 2006). Bei KO-Mäusen, die keinen funktionellen IR im Fettgewebe exprimieren, ist ebenfalls der Adiponektinspiegel erhöht (Blüher et al. 2004). In *in vitro*-Zellversuchen mit 3T3-L1-Präadipozyten führt eine Insulinstimulation zu einer geringeren Adiponektinexpression (Fasshauer et al. 2002).

In Blastozysten des Kaninchens konnte eine Insulin/IGF-abhängige Regulation von Adiponektin sowohl *in vivo* als auch *in vitro* gezeigt werden. Die Ergebnisse sind in der Publikation „*Cyclic AMP responsive element binding protein - a vital link in embryonic hormonal adaptation*“ veröffentlicht (Schindler et al. 2013). Die Reaktionen des Embryoblasten und Trophoblasten auf den Insulinmangel sind unterschiedlich. Im Embryoblasten diabetischer Kaninchen war Adiponektin nicht mehr nachweisbar, im Trophoblasten hingegen deutlich erhöht (Schindler et al. 2013). Eine Analyse der Blastozystenflüssigkeit zeigte deutlich erhöhte Adiponektinspiegel bei diabetischen Tieren (Schindler et al. 2013). Dies kann zum einen durch die erhöhte Produktion im Trophoblasten erklärt werden. Aber auch eine erhöhte Aufnahme aus dem uterinen Sekret ist möglich, da auch die diabetischen

Muttertiere eine erhöhte Adiponektinkonzentration im Serum (endokrin) und Endometrium (parakrin) aufweisen (Schindler et al. 2013). Dies würde einen gerichteten Transport des mütterlichen Adiponektins voraussetzen. Ob und wie der trans- und parazelluläre Transport in embryonalen Zellen erfolgt ist unklar. Bisher wurde ein gerichteter Adiponektintransport nur in Endothelzellen untersucht (Rutkowski et al. 2014).

Auch der AdipoR1 wird in Kaninchenblastozysten transkriptionell über Insulin und IGF1 *in vitro* und *in vivo* reguliert, im Gegensatz zum AdipoR2, der keine Insulin- oder IGF1-abhängige Transkriptionsregulation zeigte (Schindler et al. 2013)). Dieser Befund weist auf eine potentiell unterschiedliche Funktion der Rezeptoren in der Adiponektin-Insulin-Interaktion hin. Dies wurde auch in anderen tierexperimentellen Versuchen belegt. In Muskelgewebe führt Insulin zu einer verringerten AdipoR1-Transkription (Inukai et al. 2005, Cui et al. 2011, Sattar and Sattar 2012). Der molekulare Mechanismus dieser Regulation ist wahrscheinlich über den Transkriptionsfaktor *forkhead box protein O*1 (FOXO1) zu erklären (Tsuchida et al. 2004). Eine Interaktion der zwei Signalwege, IIRS und ARS, konnte somit in der vorliegenden Arbeit zum ersten Mal auch in embryonalen Zellen belegt werden.

Zusammenfassend kann man feststellen, dass Blastozysten diabetischer Mütter zum einen mit mehr Adiponektin durch das uterine Sekret versorgt werden, zum anderen aber auch selbst mehr Adiponektin in Trophoblastzellen produzieren. Zudem weisen die embryonalen Zellen eine höhere Rezeptivität gegenüber Adiponektin durch eine gesteigerte AdipoR1-Bildung auf. Ein Grund dieser kompensatorischen Regulation könnte in der „metabolischen *switch*-Funktion“ des Adiponektins in Präimplatationsembryonen (Einleitung Kapitel 1.5.) liegen. Unter diabetischen Stoffwechsel-bedingungen könnte somit das ARS die Aufgabe, den Glukose- und Lipidstoffwechsel zu koordinieren, übernehmen, eine Aufgabe, die durch den Insulinmangel frei wird. Diese Hypothese wird durch die gleich hohe Glukoseaufnahme in die embryonalen Zellen gesunder und diabetischer Kaninchen gestützt (Schindler et al. 2013).

4.3. CREB - der zentrale Schalter zwischen IIRS und ARS

Damit stellt sich die Frage nach dem zentralen Vermittler zwischen den beiden hormonellen Signalsystemen IIRS und ARS. Ein potentieller Kandidat ist der Transkriptionsfaktor CREB.

In der vorliegenden Arbeit konnte erstmals gezeigt werden, dass nicht nur CREB, sondern auch die anderen Familienmitglieder ATF1, ATF2, ATF3 und ATF4 in Präimplantationsembryonen des Kaninchens exprimiert werden. In der Publikation „*Cyclic AMP responsive element binding protein - a vital link in embryonic hormonal adaptation*“ sind die Ergebnisse für CREB, ATF1, ATF3, ATF4 zusammengefasst (Schindler et al. 2013). Die ATF2-Expression konnte von Tag 3 p.c. an und sowohl

im Embryoblasten und Trophoblasten bei Tag 6 alten Blastozysten nachgewiesen werden. Transkripte von CREM, ATF5 oder ATF6 waren in den untersuchten Stadien nicht nachweisbar oder lagen unterhalb der methodischen Nachweisgrenze (Schindler, unveröffentlicht). Da auch in Maus präimplantationsembryonen bisher keine Expression von CREM gezeigt werden konnte (Bleckmann et al. 2002), ist nicht von einer biologischen Bedeutung der drei Faktoren in der Präimplantationsphase auszugehen.

In Kaninchenblastozysten konnte eine Aktivierung von CREB *in vitro* nach Insulin- oder IGF-Stimulation innerhalb von 10min nachgewiesen werden (Schindler et al. 2013)). Zur Stimulation wurde 17nM Insulin, 1,3nM IGF1 oder 13nM IGF2 in physiologischen Konzentrationen eingesetzt, um eine spezifische Ligand-Rezeptor-Bindung und die Aktivierung biologischer Prozesse zu gewährleisten (Einleitung Kapitel 1.4.) (Frasca et al. 1999, Chi et al. 2000, Gicquel and Le Bouc 2006). Auch andere Arbeitsgruppen konnten eine Insulin- oder IGF-abhängige CREB-Phosphorylierung nach 10min zeigen (Reusch et al. 1994, Reusch et al. 1995, Klemm et al. 1998, Zheng and Quirion 2006). Diese Aktivierung kann über die MAP-Kinase (MAPK) oder p38 MAPK erfolgen. In Kaninchenblastozysten wurde die Phosphorylierung der MAPK durch Insulin- und IGF1 nachgewiesen (Navarrete Santos et al. 2008). Diese Aktivierung ist potentiell der Weg für die Aktivierung von CREB. Bemerkenswert ist, dass eine Stimulation über einen längeren Zeitraum von 12 Stunden zu einer reduzierten CREB-Expression führte (Schindler et al. 2013). Dies weist auf einen negativ-gekoppelten Regulationsmechanismus in Kaninchenblastozysten hin. Dieser Rückkopplungsmechanismus ist vermutlich die Erklärung für die zwar deutlich geringere Aktivierung von CREB, jedoch höhere CREB-Expression in den Blastozysten diabetischer Muttertiere (Schindler et al. 2013)).

Durch die CREB-vermittelte Insulin- und IGF-Wirkung kann auch die unterschiedliche Adiponektinexpression in Embryo- und Trophoblasten erklärt werden (Abb. 7): Unter diabetischen Entwicklungsbedingungen wird in Blastozysten eine gesteigerte parakrine und autokrine IGF-Verfügbarkeit nachgewiesen (Thieme et al. 2012a), die über den IGF1R im Embryoblasten wirken kann (Navarrete Santos et al. 2008). Dies wiederum führt zu einer gesteigerten Phosphorylierung von CREB und dessen Lokalisation in den Zellkern. Somit wird die Expression von Adiponektin im Embryoblasten unterdrückt (Abb. 7 A) (Schindler et al. 2013).

Im Trophoblasten sind IGF1 und IGF2 nicht in der Lage, das Fehlen des Insulin zu kompensieren, da der IGF1R nicht exprimiert wird und eine Aktivierung von CREB als *downstream*-Molekül ausbleibt. CREB verbleibt im Zytoplasma und die transkriptionelle Unterdrückung der Adiponektinexpression unterbleibt, was wiederum zur erhöhten Adiponektinexpression in Trophoblastzellen diabetischer Muttertiere führt (Abb. 7 B) (Schindler et al. 2013).

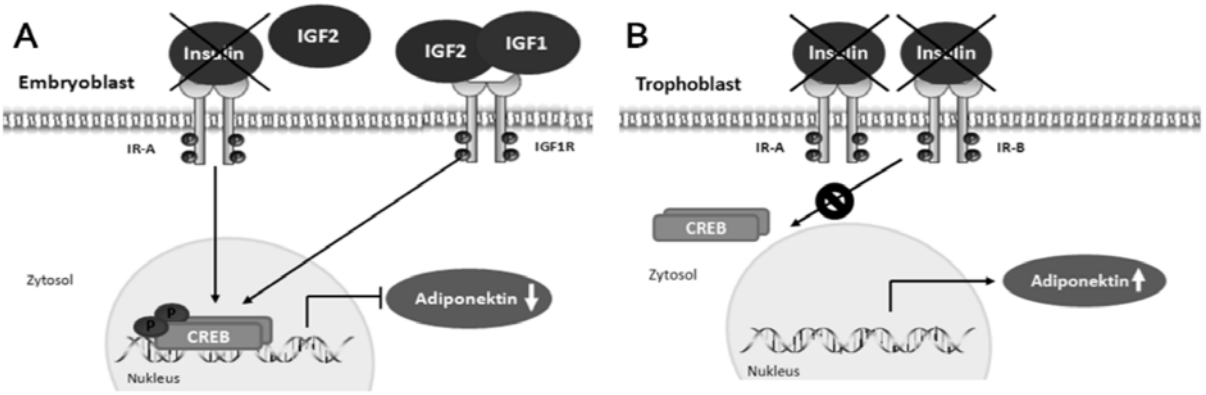

Abb. 7 **Schematische Darstellung der CREB-vermittelten Regulation von Adiponektin im Embryoblasten und Trophoblasten (aus Schindler et al. 2013)**

A) Im Embryoblasten werden neben dem IR-A auch der *Insulin-like growth factors* 1 receptor (IGF1R) exprimiert. Die Insulinähnlichen Wachstumsfaktoren (*Insulin-like growth factors,* IGF) 1 und 2 binden sowohl an den IGF1R und IR-A. Durch die erhöhte endogene und lokale Produktion an IGF1 und IGF2 wird CREB verstärkt phoshoryliert und wandert in den Zellkern. Dort inhibiert CREB die Adiponektintranskription.
B) Im Trophoblasten werden beide Insulinrezeptor-Isoformen (IR-A und IR-B) exprimiert. Unter diabetischen Entwicklungsbedingungen führt der Mangel an Insulin zu einer fehlenden Phosphorylierung des *cyclic AMP (cAMP) resposive element binding protein* (CREB) und dessen Verbleib im Zytosol. Die Adiponektintranskription wird forciert.
(• Phosphorylierung)

Als Transkriptionsfaktor ist CREB in der Lage, die Transkription seiner Zielgene sowohl zu stimulieren als auch zu inhibieren. Die Inhibierung der Zielgen-Transkription wird dabei indirekt, über eine Interaktion mit Co-Faktoren, organisiert (Hai and Hartman 2001, Johannessen et al. 2004). Dies wirft die Frage auf, wer an der CREB-vermittelten Adiponektininhibierung beteiligt sein könnte? Ein potentieller Kandidat für eine inhibitorische Regulation wäre ATF3 (Kim et al. 2006, Qi et al. 2009), jedoch wird ATF3 genauso wie CREB durch Insulin und IGF1 inhibiert (Schindler et al. 2013). Auch in Blastozysten diabetischer Kaninchen war kein Unterschied in der ATF3-Expression messbar (Schindler et al. 2013). Die Transkriptmenge von ATF4 hingegen, welcher auch zu einer Unterdrückung der CRE-Zielgentranskription führen kann, wird durch Insulin und IGF1 erhöht (Schindler et al. 2013). In Blastozysten, besonders im Embryoblasten diabetischer Kaninchen, waren deutlich erhöhte ATF4-mRNA-Mengen nachweisbar (Schindler et al. 2013). Dies weist darauf hin, dass ATF4 das inhibitorische Stellglied in der Insulin-CREB-Adiponektin-Signalkaskade sein könnte. Jedoch zeigen Mäuse, die ATF4 überexprimieren oder kein funktionelles ATF4 aufweisen, keinen Unterschied im Adiponektinspiegel (Yoshizawa et al. 2009). Daher wird die Bedeutung anderer Cofaktoren in dieser Signalkaskade nicht ausgeschlossen.

CREB reguliert nicht nur die Expression von Adiponektin, sondern ist in eine Vielzahl metabolischer Prozesse involviert (Conkright et al. 2003, Zhang et al. 2005). Bereits vor Beginn der hier diskutierten Untersuchungen war bekannt, dass klassische CREB-Zielgene wie die PEPCK und Hexokinase 2, die eine zentrale Bedeutung im Glukosemetabolismus einnehmen, in Blastozysten diabetischer Kaninchen verändert exprimiert werden (Ramin 2010). CREB beeinflusst im adulten Organismus die Transkription von verschiedenen Genen des Lipidmetabolismus. Dazu zählen unter anderem PPARγ (Reusch et al. 2000, Herzig et al. 2003, Fox et al. 2006), FABP4 (Reusch et al. 2000), FATP4 (Zhang et al. 2005), CPT1B (Louet et al., 2002) und *sterol regulatory element-binding protein* 1 (SREBP1) (Dooley et al. 1999, Zhou et al. 2007) (Übersichtsartikel: Altarejos and Montminy 2011, Datenbank: http://natural.salk.deu/CREB).

Außerdem ist bekannt, dass CREB die intrazelluläre Akkumulation von Triglyceriden infolge einer mitochondrialen Dysfunktion reguliert ((Vankoningsloo et al. 2006)). Eine Veränderung des embryonalen Lipidstoffwechsels infolge der veränderten CREB-Aktivierung in Embryonen diabetischer Kaninchen ist somit wahrscheinlich.

4.4. Ein mütterlicher Diabetes mellitus und die Auswirkung auf den embryonalen Lipidmetabolismus

Erste gewonnene Ergebnisse zum embryonalen Lipidmetabolismus unter diabetischen Entwicklungsbedingungen sind in der Publikation „*Maternal diabetes leads to unphysiological high lipid accumulation in rabbit preimplantation embryos*“ veröffentlicht (Schindler et al. 2014). Weitere bisher nicht publizierte Daten werden in diesem Kapitel als Ergebnisse dargestellt und diskutiert.

4.4.1. Intrazelluläre Lipidspeicherung und Expression lipogener Schlüsselmoleküle

Das wohl deutlichste Zeichen, dass die Umstellung des embryonalen Metabolismus auf die diabetischen Entwicklungsbedingungen zu einer Störung im Lipidstoffwechsel führt, ist die massive intrazelluläre Akkumulation von Lipiden im EB und TB (Schindler et al. 2014). Dabei wurde der Nachweis von Lipidtropfen mittels Oil Red O-Färbung geführt. Um zusätzlich eine quantitative Aussage treffen zu können, wurde eine neue Methode basierend auf Nile Red-Färbung und Fluoreszenz-aktivierte Zellsortierung (FACS, *flow cytometry*) entwickelt. Zur Durchführung dieser Methode mussten unter Verwendung eines kommerziellen Kits (*neural tissue*, Miltony Biotec) die embryonalen Zellen der Blastozysten vereinzelt werden. Beide Versuchsansätze belegten eine deutlich höhere Akkumulation von Lipidvesikeln in Blastozysten diabetischer Kaninchen (Schindler et al. 2014). Es ist bekannt, dass Präimplantationsembryonen in der Lage sind, intrazellulär Lipide in Form von Lipidvesikeln zu speichern ((Tsujii et al. 2001)). Die gespeicherten Lipide dienen zum einen als Energiequelle, beeinflussen aber auch die Zellproliferation, Zell-Zellinteraktion, die Funktion von

Membranen und damit den inter- und intrazellulären Transport (Übersichtsartikel (Stubbs and Smith 1984). Die Größe und Anzahl der intrazellulären Lipidvesikel ist durch das umgebende Milieu beeinflussbar (Abe et al. 1999, Hoshi 2003) und ein wichtiger Marker für die Vitalität der Embryonen. Eine erhöhte Fettvesikelmenge weist auf eine schlechte Embryonenqualität, ein geringeres Entwicklungspotential und eine geringe Kryotoleranz hin (Dobrinsky et al. 1999, Nagano et al. 2006, Jeong et al. 2009). Somit unterstützt der Befund einer massiven Akkumulation von intrazellulären Lipidvesikeln in Blastozysten diabetischer Kaninchen die bereits bekannte schlechtere Embryonenqualität und Entwicklungsverzögerung (Ramin et al. 2010).

Zudem sind wichtige Schlüsselmoleküle des Lipidmetabolismus durch den mütterlichen Diabetes verändert. Abgesehen von Adipophilin, konnten diese in der vorliegenden Arbeit zum ersten Mal im Präimplantationsembryo des Kaninchens nachgewiesen werden (Schindler et al. 2014). Adipophilin und Perilipin, auch als PLIN1 und PLIN2 bekannt, sind Lipidvesikel-assoziierte Proteine und regulieren Aufbau und Zusammensetzung von Lipidvesikeln, sowie die Lipolyse ((Heid et al. 1998), (Miura et al. 2002, Brasaemle 2007). Picone und Koautoren konnten Adipophilin-Transkripte bereits ab Tag 2 *p.c.* im Kaninchenembryo nachweisen (Picone et al. 2011). Erst kürzlich wurde die Lokalisation von Adipophilin um Lipidvesikel gezeigt (Al Darwich et al. 2014). Diese wird durch verschiedene Kulturbedingungen beeinflusst. Blastozysten diabetischer Kaninchen wiesen eine erhöhte Expression von Adipophilin und Perilipin auf (Abb. 8) (Schindler et al. 2014).

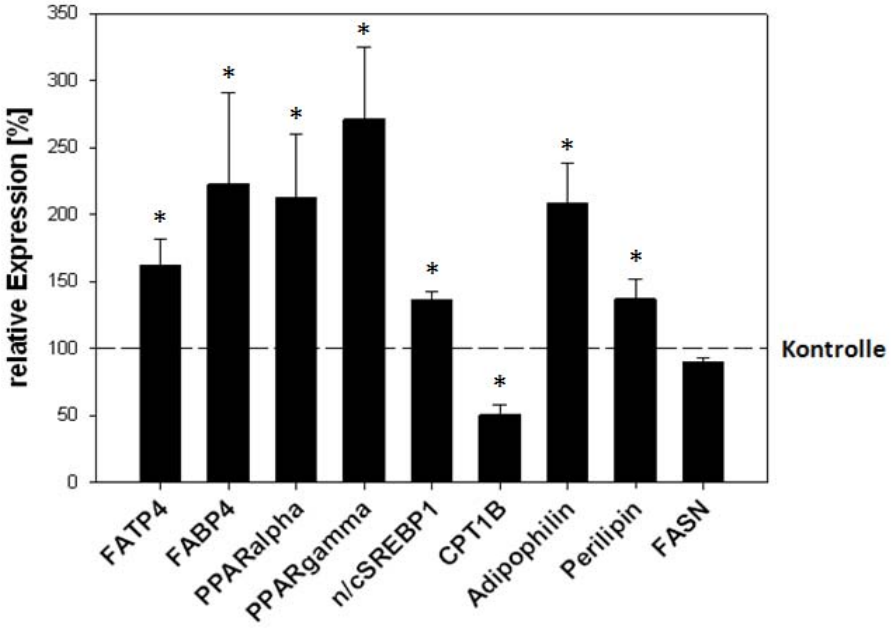

Abb. 8 **Relative Menge lipogener Signalmoleküle in Tag 6 Blastozysten diabetischer Kaninchen**

Die Expression der lipogenen Signalmoleküle wurde mittels qRT-PCR oder Western Blot ermittelt. Dafür wurden am Tag 6 p.c. Blastozysten normoglykämischer und diabetischer Kaninchen aus dem Uterus gespült, aufgearbeitet und bei -80°C gelagert. Nach mRNA-Isolation mittels Dynabeads-Verfahren und cDNA-Synthese erfolgte die qRT-PCR-Messung folgender Gene: Adipophilin, *fatty acid transport protein 4* (FATP4), *fatty acid binding protein 4* (FABP4), Carnitin-Palmitoyltransferase 1 B (CPT1B). Die Proteinisolation erfolgte unter Verwendung des RIPA-Puffers. 25µg Gesamtprotein pro Probe wurden für eine Analyse mittels SDS-PAGE und Western Blot verwendet. Die Expression von Perilipin, Peroxisom-Proliferator-aktivierter Rezeptor α und γ (PPARα, PPARγ), *sterol regulatory element-binding protein* 1 (SREBP1) und Fettsäure-Synthase (FASN) wurden auf Proteinebene bestimmt. Die Ergebnisse sind relativ zur Expression in Blastozysten aus gesunden Kaninchen (Kontrolle =100%) berechnet [Mittelwert in % ± Standardfehler].

Aber auch Gene, die entscheidend für die intrazelluläre Aufnahme, für Transport und Speicherung von Lipiden, FATP4 und FABP4, sind (Heid et al. 1998, Storch and Thumser 2000, Stahl 2004, Storch and McDermott 2009), sind höher in Blastozysten diabetischer Kaninchen exprimiert (Schindler et al. 2014). In der Blastozystenhöhlenflüssigkeit diabetischer Kaninchen konnte zudem eine höhere Konzentration an FABP4 nachgewiesen werden (Schindler et al. 2014), was im Zusammenhang mit einem vermehrten Transport von Fettsäuren in embryonale Zellen stehen könnte. Humane Studien belegen, dass sowohl die Expression von FATP4 und FABP4, als auch die Menge an FABP4 im Serum ein Indikator für Übergewicht, Insulinresistenz und Dyslipidämien ist (Gertow et al. 2004, Xu et al. 2006, Karakas et al. 2009). Zusätzlich sind sowohl Embryoblast- als auch Trophoblastzellen diabetischer Kaninchen durch eine deutlich stärkere Lokalisation von FABP4 im Zellkern

gekennzeichnet (Schindler et al. 2014). FABP4 transportiert Fettsäuren in den Zellkern und kann dort durch die Interaktion mit den Trankriptionsfaktoren PPARγ und PPARα die Transkription von Zielgenen regulieren (Storch and McDermott 2009)). Der Befund einer deutlich prominenteren Lokalisation im Zellkern unterstützt die Sicht einer verstärkten regulatorischen Interaktion mit den PPARs unter diabetischen Bedingungen. Die PPARs gehören zu den Liganden-aktivierten Transkriptionsfaktoren ((Dreyer et al. 1993, Desvergne and Wahli 1999). Es können drei verschiedene Isoformen, PPARα, PPARβ/δ und PPARγ, unterschieden werden. Die Expression von PPARα und PPARγ ist in Blastozysten diabetischer Kaninchen erhöht (Abb. 8) (Schindler, unveröffentlicht). PPARγ, von dem 2 verschiedene Proteinisoformen bekannt sind (PPARγ1 und PPARγ2), wurde als wichtiger Regulator der Adipozytendifferenzierung und des Glukosemetabolismus identifiziert (Tontonoz et al. 1994, Desvergne et al. 2006, Übersichtsartikel: Kumari, 2014). PPARα kontrolliert den intrazellulären Lipid– und Glukosemetabolismus über die direkte transkriptionelle Regulation von Genen, die entscheidend für die β-Oxidation, Fettsäureaufnahme und den Triglyceridkatabolismus sind (Lefebvre et al. 2006). Dazu gehört auch CPT1B, das den initialen Schritt der β-Oxidation, den Transport von Fettsäuren in die Mitochondrien, steuert. Die Expression von CPT1B ist in den Blastozysten diabetischer Kaninchen reduziert (Abb. 8) (Schindler, unveröffentlicht) und spricht damit für eine geringere β-Oxidation. Kritisch muss man anmerken, dass die höhere PPARα-Expression und die Verfügbarkeit an Lipiden aufgrund der mütterlichen Hyperlipidämie eher eine Steigerung der β-Oxidation vermuten ließen. Ob die verringerte CPT1B-Transkriptmenge auch zu einer Absenkung der β-Oxidation führt, muss durch weitere Untersuchungen im Bereich des Fettsäurekatabolismus von Embryonen geklärt werden. Bekannt ist bereits, dass eine Hemmung der β-Oxidation während der Präimplantationsentwicklung zu einer geringeren Proliferationsrate und Blastozystenentwicklung führt (Hewitson et al. 1996, Ferguson and Leese 2006) und damit zu der beobachteten Entwicklungsverzögerung bei diabetischen Kaninchenembryonen beitragen kann (Ramin et al. 2010).

Das zentrale Schlüsselenzym der Lipogenese, SREBP1, wird als inaktive Vorstufe (*Precursor*) gebildet, das als integrales Membranprotein im Endoplasmatischen Retikulum vorliegt. Durch zwei proteolytische Spaltungen entsteht das aktive SREBP1-Protein, das auch als nukleäres SREBP1 (nSREBP1) bezeichnet wird (Kim et al. 1995, Edwards et al. 2000). Bei der Analyse von SREBP1 zeigte sich eine höhere Akkumulation des aktiven nuklearen SREBP1 (nSREBP1) in Blastozysten der diabetischen Muttertiere (Schindler et al. 2014). Dies spricht für eine höhere transkriptionelle Aktivität in diabetischen Blastozysten. Eine transkriptionelle Regulation des wichtigen Zielgens, die Fettsäure-Synthase (FASN), erfolgt jedoch nicht (Abb. 8) (Schindler et al. 2014). Die FASN, das Schlüsselenzym der *de novo* Lipogenese, katalysiert die Produktion langkettiger Fettsäuren aus Malonyl-CoA ((Wakil 1989, Semenkovich 1997)). Der Mangel an funktionellem FASN-Protein führt zur

embryonalen Letalität noch während der Präimplantationsphase (Chirala et al. 2003) und unterstreicht damit die essentielle Bedeutung des Lipidmetabolismus während der ersten Tage der Entwicklung.

Die Veränderungen im Lipidmetabolismus können auch mit der von uns gesteigerten Bildung von *advanced glycation end products* (AGEs) in Zusmannenhang gebracht werden (Haucke et al. 2014). Es ist bekannt, dass die Peroxidation von Lipiden zur Bildung von reaktiven Dicarbonylverbindungen, wie zum Beispiel Methylglyoxal und Glyoxal, führt (Uribarri 2006 AGEs and nephortoxity of high protein diets). Die Bildung und Akkumulation von AGEs induziert einen anhaltenden oxidativen Stress (Sell 1991, Lee & Cerami 1992, Brownlee 1995). AGEs akkumulieren im Blut und Endometrium diabetischer Muttertiere (Haucke 2014). Zudem weisen Blastozysten eine deutlich erhöhte Menge Glyoxal im Embryo selbst und anderer AGEs in der Blastozystenflüssigkeit auf (Haucke et al. 2014). Dieser Befund korreliert mit einer erhöhten Expression des AGE-Rezeptors, RAGE (Haucke et al. 2014). Eine RAGE-Aktivierung führt über den Transkriptionsfaktor *nuclear factor of kappa-light-chain polypeptide gene enhancer in B-cells* (NFκB) zu einer Ausschüttung proinflammatorischer Zytokine und freier Radikale (Berbaum et al. 2008, Bierhaus & Nawroth 2009, Fritz et al. 2011). Zusammen mit der bereits beschriebenen erhöhten „Lipidbelastung“ kann die erhöhte AGE-Bildung und Akkumulation für die beobachtete schlechtere Embryonenqualität ursächlich sein (Ramin et al. 2010).

Erste Untersuchungen einer breiten Palette von Genen des Lipidmetabolismus weisen auf eine unterschiedliche Regulation im Embryoblasten und Trophoblasten hin. In 6 Tage alten Blastozysten gesunder Kaninchen wird FABP4 und FASN hauptsächlich im Embryoblasten exprimiert (Schindler et al. 2014). FATP4- und Adipophilin-Transkripte können im Embryoblasten und Trophoblasten mit gleicher Intensität nachgewiesen werden (Schindler et al. 2014). Der Vergleich von Blastozysten gesunder und diabetischer Kaninchen zeigt, dass die erhöhte Transkription von FABP4 und Adipophilin auf den Embryoblasten beschränkt ist (Schindler et al. 2014). FATP4 ist sowohl im Embryoblasten und Trophoblasten diabetischer Kaninchen höher exprimiert (Schindler et al. 2014). Diese Befunde werden durch eine signifikant erhöhte Nile Red-Absorption unterstützt, die nur im Embryoblasten zu finden ist (Schindler et al. 2014). Unser derzeitiger Wissensstand und diese ersten Ergebnisse weisen darauf hin, dass im Embryoblasten und Trophoblasten der zelluläre Metabolismus über unterschiedliche Adaptationsmechanismen gesteuert wird. Potentielle Mechanismen sollen im Folgenden diskutiert werden.

4.4.2. Mögliche Regulatoren des embryonalen Lipidstoffwechsels

Welche Faktoren für die beobachten Veränderungen im Lipidmetabolismus verantwortlich sind, ist noch nicht geklärt. Eine zentrale Vermittlerrolle von CREB ist aufgrund der gezeigten und bereits

erörterten regulatorischen Funktion für lipogene Gene wahrscheinlich (siehe Kapitel Diskussion 4.3.). In Leberzellen gesunder Mäuse führt der Mangel an funktionellem CREB zu einer erhöhten Akkumulation von Lipiden und somit zu einer Fettleber. Diese Wirkung wird über PPARγ vermittelt (Herzig et al. 2003). In verschiedenen Insulin-defizienten Mausmodellen scheint der Verlust an CREB eine gegenteilige Wirkung zu vermitteln. Diese Mäuse weisen eine reduzierte hepatische Lipogenese und geringere Cholesterol- und Triglyceridspiegel im Plasma auf (Erion et al. 2009). Das IIRS scheint also bei der Art der Wirkung von CREB eine wesentliche Rolle zu spielen.

Neben CREB kann auch die Regulation der anderen CREB/ATF-Familienmitglieder ausschlaggebend sein. Dabei rückt ATF4 verstärkt in den Fokus. ATF4-defiziente Mäuse sind dünn und weisen eine gesteigerte Lipolyse und inhibierte Lipogenese auf (Yoshizawa et al. 2009, Seo et al. 2009, Wang et al., 2010). Die Serumspiegel an Triglyceriden und Fettsäuren sind deutlich geringer als in Wildtypmäusen (Wang et al. 2010). Ursächlich dafür ist unter anderem die Steuerung der zentralen Schlüsselmoleküle im Lipidmetabolismus, FASN und SREBP1c, sowie eine induzierte β-Oxidation über die erhöhte Transkription von CPT1B und UCP1 (Wang et al. 2010). Die erhöhte Transkription von ATF4 in Blastozysten diabetischer Kaninchen kann somit auch für die Regulation von SREBP1 und CPT1B verantwortlich sein.

Es gibt aber auch CREB/ATF-unabhängige Wege, auf denen das IIRS den embryonalen Lipidstoffwechsel regulieren kann. Sowohl Insulin als auch IGF gelten als anabole Faktoren und steuern die Expression und Enzymaktivität der FASN (Moustaïd et al. 1996, Claycombe et al. 1998, Wang et al. 2004), der FABP4-Expression (Smith et al. 1988, Blake and Clarke 1990) sowie der SREBP1-Transkription und Prozessierung (Foretz et al. 1999, Fleischmann and Iynedjian 2000, Han et al. 2009). Eine Auswirkung des veränderten IIRS auf die Blastozysten der diabetischen Muttertiere ist somit wahrscheinlich. Zusätzlich können auch die erhöhten Adiponektinkonzentrationen, die sowohl im Uterus als auch in der Blastocoelflüssigkeit nachgewiesen wurden, den Lipidmetabolismus im Embryo- und Trophoblasten beeinflussen. Wie bereits erwähnt, reguliert Adiponektin neben dem Glukosestoffwechsel auch den Lipidmetabolismus. Über verschiedene Signalmoleküle, wie AMPK, Acetyl-CoA Carboxylase (ACC), PPARs, FATPs und CPT1B, werden die intrazelluläre Aufnahme von Fettsäuren sowie Lipogenese und Lipolyse gesteuert (Einleitung Kapitel 1.4 Abb. 5). Bereits vor Beginn der hier diskutierten Ergebnisse war bekannt, dass die FATP1, ACC und AMPK in Präimplantationsembryonen der Maus und Kaninchen exprimiert werden (Fischer et al. 2010, Kim et al. 2011). Inwieweit eine Steuerung über Adiponektin für die beobachteten Effekte in Blastozysten diabetischer Kaninchen verantwortlich ist, muss in kommenden *in vitro*-Versuchen abgeklärt werden.

Der Einfluss einer Hyperglykämie auf den embryonalen Lipidmetabolismus wurde bereits untersucht und in dem Artikel „*Maternal diabetes leads to unphysiological high lipid accumulation in rabbit*

preimplantation embryos“ publiziert (Schindler et al. 2014). Deshalb soll an dieser Stelle nur eine kurze Zusammenfassung folgen. Hohe Glukosekonzentrationen führen zu einer erhöhten Akkumulation von intrazellulären Lipiden. Dies ist bedingt durch die erhöhte Expression der FASN, von FABP4 sowie von Perilipin und Adipophilin. Im Vergleich zu dem Expressionsmuster *in vivo* fällt auf, dass *in vitro* die FASN über Glukose reguliert wird, FATP4 jedoch nicht (Abb. 9). Somit führen hohe Glukosekonzentrationen zu einer vermehrten Lipidspeicherung durch eine gesteigerte Neubildung von Lipiden (erhöhte FASN-Expression, siehe Abb. 9). *In vivo* allerdings ist eine veränderte Transkription der FASN nicht nachweisbar. Da die FASN-Aktivität auf transkriptioneller Ebene reguliert wird (Paulauskis and Sul 1989, Latasa et al. 2003, Griffin and Sul 2004), weist dieser Befund auf eine erhöhte Versorgung mit Lipiden über die Aufnahme von Fettsäuren hin. (keine veränderte FASN-Expression, erhöhte FATP4- und FABP4-Transkription, siehe Abb. 9) (Schindler et al. 2014). Diese Ergebnisse belegen, dass die erhöhte Glukosekonzentration nicht alleine ausreichend ist, um die beobachte verstärkte Lipidakkumulation zu erklären.

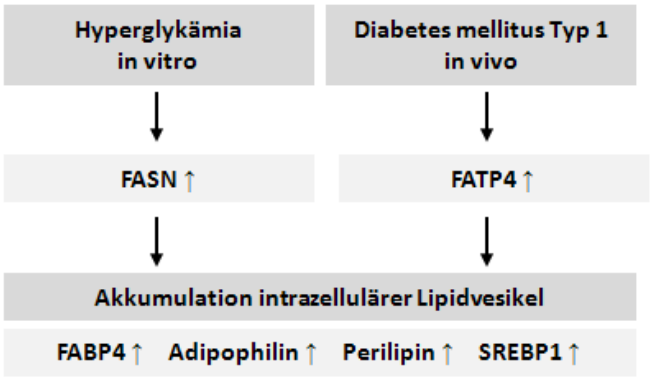

Abb. 9 **Schematische Darstellung der intrazellulären Lipidakkumulation in Blastozysten durch eine Hyperglykämie *in vitro* und eines Diabetes mellitus *in vivo* (aus Schindler et al. 2014)**

Hohe Glukosespiegel *in vivo* (Diabetes mellitus) und *in vitro* führen zu einer deutlich erhöhten Anzahl an Lipidvesikeln mit einer erhöhten Expression der lipogenen Schlüsselmoleküle Adipophilin, Perilipin, *fatty acid binding protein 4* (FABP4) und *sterol regulatory element-binding protein* 1 (SREBP1). Ein unterschiedliches Expressionsmuster fand sich bei der Transkription der Fettsäure-Synthase (FASN) und *fatty acid transport protein 4* (FATP4). Eine Hyperglykämie *in vitro* erhöhte die FASN-mRNA-Menge. *In vivo* hingegen ist die FATP4-Transkription gesteigert.

Unter Berücksichtigung der von Krisher und Prather aufgestellten Hypothese (Einleitung Kapitel 1.3.) kann man jedoch auch vermuten, dass die deutlich erhöhten Glukosespiegel unter diabetischen Bedingungen eine Umstellung des embryonalen Metabolismus verursachen. Unter normalen Entwicklungsbedingungen, so die Autoren, wird Glukose nur zu einem geringen Anteil für die Energiegewinnung verwendet und die Deckung des Energiebedarfs über Fettsäuren abgesichert (Krisher and Prather 2012). Das Mehr an Glukose führt nun dazu, dass nicht Fettsäuren, sondern

Glukose zur Energiebereitstellung verwendet wird. Dies würde auch erklären, warum die β-Oxidation in Blastozysten diabetischer Kaninchen inhibiert ist. Somit ist diese Umstellung des intrazellulären Stoffwechsels eine mögliche weitere Ursache für die geringere Vitalität diabetischer Blastozysten.

Neben erhöhten Glukosespiegeln ist ein Diabetes mellitus weiterhin durch sekundäre Stoffwechselstörungen charakterisiert. Diabetiker weisen häufig erhöhte freie Fettsäure- und Triglyceridspiegel sowie höhere *low-density* Lipoprotein- (LDL-) und erniedrigte *high-density* Lipoprotein- (HDL-) Cholesterolkonzentrationen im Serum auf (Kissebah et al. 1976, Kilby et al. 1998, Merzouk et al. 2000, Mooradian 2009, Scifres et al. 2011). Diabetische Kaninchen weisen ebenfalls höhere Triglyceridspiegel auf (Schindler et al. 2014). Zudem ist die Verteilung von Cholesterol und Triglyceriden in den Lipoproteinen deutlich verändert (Schindler, unveröffentlicht). Im Serum diabetischer Kaninchen findet sich eine deutlich erhöhte Konzentration an Triglyceriden in der *very-low-density* Lipoprotein (VLDL) und LDL-Fraktion. Für Cholesterol war eine höhere Konzentration nur in der VLDL zu beobachten. Ein Beweis, dass sich die mütterliche Hyperlipidämie zudem auf das Umgebungsmilieu der Blastozysten auswirkt ist, dass die Cholesterolkonzentration im Uterussekret um das Doppelte erhöht ist (Schindler, unveröffentlicht).

Eine Hyperlipidämie führt zu einer erhöhten Akkumulation intrazellulärer Lipidvesikel, einer höheren Expression von Adipophilin, FATP4 und FABP4 (Amri et al. 1991, Elchalal et al. 2005, Pathmaperuma et al. 2010, Scifres et al. 2011), also von Schlüsselmolekülen, die auch in den Blastozysten diabetischer Kaninchen erhöht exprimiert werden. Diese Effekte können langfristige Auswirkungen haben. Die Arbeitsgruppe um Dr. Pascale Chavatte-Palmer vom INRA Jouy-en-Josas analysierte die Plazenta der Nachkommen, die sich in den ersten 4 Tagen unter diabetischen Umgebungsbedingungen entwickelt hatten. Die Blastozysten wurden im Anschluss in gesunde Empfängerkaninchen transferiert. Trotz der weiteren normalen Entwicklungsbedingungen wies die Plazenta eine erhöhte Akkumulation intrazellulärer Lipide auf (Tarrade/Chavatte-Palmer, unveröffentlicht). Diese Ergebnisse basieren bis jetzt nur auf einer Versuchswiederholung und müssen aus diesem Grund dringend wiederholt werden. Bereits bekannt ist jedoch, dass eine mütterliche Hypercholesterinämie neben einer veränderten Lipidzusammensetzung der Plazenta auch die endogene Cholesterolsynthese erhöht (Montoudis et al. 1999, Tarrade et al. 2013). Neue Studien zeigen, dass diese Effekte schon im Blastozystenstadium ihren Ursprung haben. Schlüsselmoleküle, die ursächlich für die erhöhte Anzahl der Lipidtropfen sein können, sind dabei Adipophilin, Leber X-Rezeptoren α (LXRα) und der *low density lipoprotein receptor* (LDLR) (Picone et al. 2011, Tarrade et al. 2013). Welche weiteren Auswirkungen eine mütterliche Hyperlipidämie auf den Lipidmetabolismus in Embryonen und Feten hat, muss Gegenstand weiterer Untersuchungen

sein. Für Effekte unabhängig erhöhter Glukosespiegel spricht auch, dass trotz eines gut eingestellten Diabetes die Schwangerschaften höhere Komplikationsraten aufweisen (Evers et al. 2004).

Ausgangspunkte für den veränderten Metabolismus in diabetischen Embryonen wurden an dieser Stelle genannt. Wahrscheinlich ist, dass nicht nur ein Faktor der ausschlaggebende Punkt ist, sondern dass die (Fehl-)Adaptation das Resultat eines komplexen Zusammenspiels ist, bei dem CREB eine zentrale Vermittlerrolle einnimmt (Abb. 10).

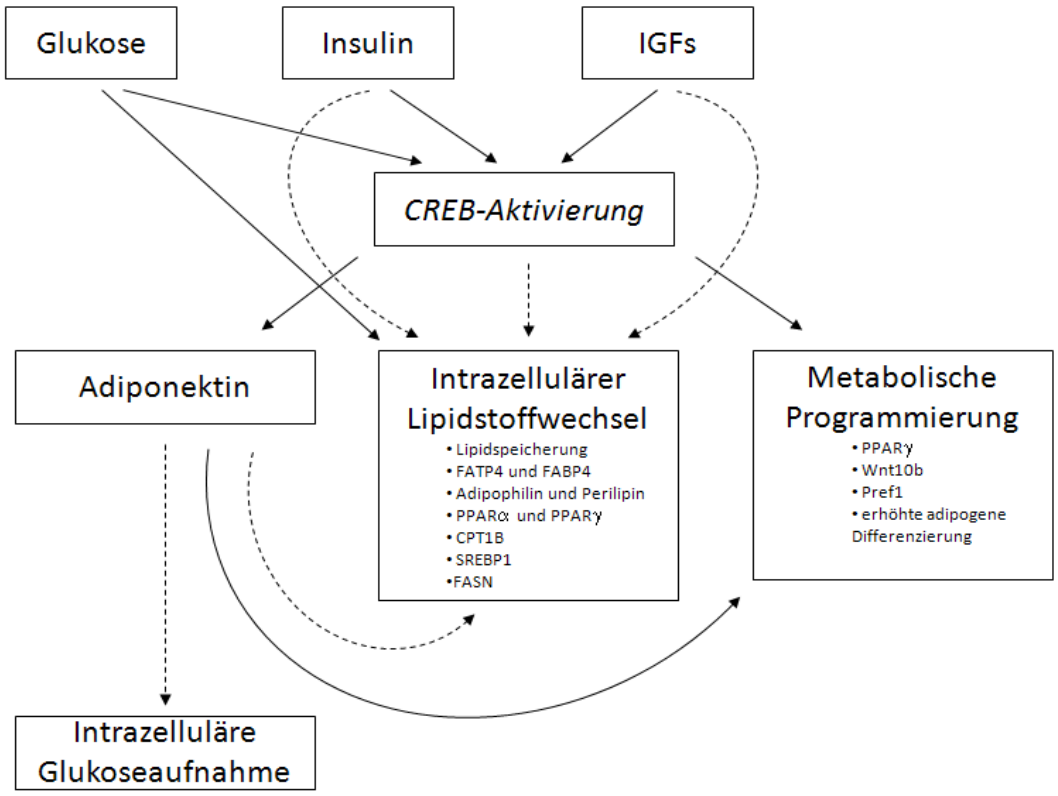

Abb. 10 **Schematische Darstellung der zentralen Rolle von CREB im Embryo**

⟶ *in der vorliegenden Arbeit gezeigte Ergebnisse,* ⇢ *aus der Literatur bekannte Zusammenhänge*
(IGFs; insulinähnliche Wachstumsfaktoren, CREB; *cyclic AMP (cAMP) resposive element binding protein*, FATP4; *fatty acid transport protein 4*, FABP4; *fatty acid binding protein 4*, PPARα; Peroxisom-Proliferator-aktivierter Rezeptor α, PPARγ; Peroxisom-Proliferator-aktivierter Rezeptor γ, CPT1B; Carnitin-Palmitoyltransferase 1 B, SREBP1; *sterol regulatory element-binding protein* 1, FASN; Fettsäure-Synthase, Wnt10b; *wingless-type MMTV Integration site family member* 10b, Pref1; *Preadipocyte factor* 1)

4.5. Die Rolle von CREB für die langfristige Prägung embryonaler Zellen

Es wird deutlich, dass diabetische Entwicklungsbedingungen während der Frühschwangerschaft zu komplexen Veränderungen im Stoffwechsel und in der molekularen Regulation zellulärer Prozesse beim Embryo führen (Abb.10). Welche Konsequenzen die zellulären Anpassungsreaktionen auf das

Differenzierungspotential embryonaler Zellen haben, wurde *in vitro* mit embryonalen Stammzellen untersucht. Die Daten dieser Untersuchungen wurden bisher noch nicht publiziert und werden deshalb in diesem Kapitel als Ergebnisse und Diskussion ausführlich dargestellt.

Ausgehend von der *in vivo*-Beobachtung einer erhöhten Adiponektinmenge *in utero* unter diabetischen Entwicklungsbedingungen wurde die Wirkung von Adiponektin auf pluripotente Embryonale Stammzellen (ES) und deren adipogene Differenzierung untersucht. Für diese Experimente wurde die embryonale Stammzelllinie CGR8 ausgewählt. Dabei handelt es sich um ein etabliertes Zellmodell, das 1994 von Prof. Austin Smith aus Embryonen des Mausstammes 129 etabliert wurde (Mountford et al. 1994). CGR8-Zellen sind durch folgende Eigenschaften gekennzeichnet:

Durch die routinemäßige Zugabe des Zytokins *leukaemia inhibitory factor* (LIF), das alle stammzellspezifischen Charakteristika aufrechterhält (Silva-Cote and Cardier 2011), können die Zellen ohne Kokultur mit embryonalen Fibroblasten kultiviert werden. Eine Entfernung von LIF führt zur sofortigen Differenzierung der Zellen (Hirai et al. 2011). Die Anzahl zelltypspezifisch terminal differenzierter Zellen ist bei einer spontanen Differenzierung sehr gering (hohe Heterogenität terminaler Zelltypen) und setzt somit die Zugabe von Differenzierungsfaktoren für eine gerichtete Zelldifferenzierung voraus.

Die gerichtete adipogene Differenzierung erfolgt unter Einhaltung des schematisch in Abb. 11 dargestellten Protokolls, bei dem am Ende 70-80% der *embryoid bodies* (EBs) große Adipozytenansammlungen aufweisen (Dani et al. 1997).

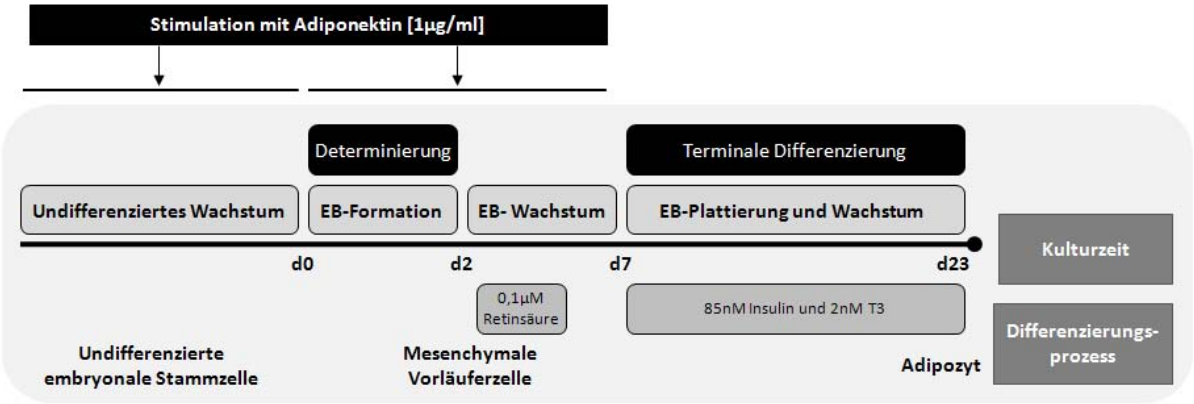

Abb. 11 **Schematische Darstellung des adipogenen Differenzierungsprotokolls der embryonalen Stammzelllinie CGR8**

Die Pluripotenz der CGR8-Zellen wird durch die Kultur mit 10%igen fetalen Kälberserum (FCS) und der Zugabe des Zytokin *leukemia inihibtory factor* (LIF) aufrechterhalten. Um die adipogene Differenzierung zu initiieren, erfolgt die Kultivierung als dreidimensionale Aggregate (*embryoid bodies*, EBs) mit 20%igem FCS und ohne differenzierungshemmende Faktoren. Im Anschluss erfolgt die kurzzeitige Behandlung der EBs mit Retinsäure (RA). Nach anschließender Ausplattierung auf Gelatine-beschichtete Schalen und Kultur in Anwesenheit von Insulin und Trijodthyronin (T_3) bilden sich reife Adipozyten innerhalb der nächsten 23 Tage im EB *outgrowth*. Die CGR8-Zellen wurden entweder für 5 Tage während des undifferenzierten Wachstums (adi und) oder während der Determinierungsphase bis Tag 7 (adi d0-7) mit 1µg/ml Adiponektin kultiviert. (d = Tage)

Allgemein gilt: aus pluripotenten embryonalen Stammzellen differenzieren sich mesenchymale Stammzellen, die sich in weiteren Schritten in Osteoblasten, Chondroblasten, Muskelzellen und Adipozyten differenzieren können (Poissonnet et al. 1984, Poissonnet et al. 1988, Rosen and MacDougald 2006). Die Entstehung von Fettzellen *in vitro* bietet die Möglichkeit, die einzelnen Schritte detailliert zu analysieren und zeigt, dass der Prozess der Zelldeterminierung über verschiedene Signalmoleküle reguliert wird. Eine wichtige Rolle in diesem Netzwerk spielt CREB (Reusch et al. 2000, Rosen and MacDougald 2006, Fox et al. 2006, Fox et al. 2008, Qi et al. 2009, Tang et al. 2012).

Dies wird auch durch Ergebnisse in der vorliegenden Arbeit bestätigt. Die Kultivierung mit Adiponektin während des undifferenzierten Wachstums (CGR8 adi und) und während der Determinierungsphase (CGR8 adi d0-7) der embryonalen Stammzelllinie CGR8 führte zu einer erhöhten CREB-Aktivität (Abb. 12 B). Dafür wurde Adiponektin in einer Konzentration von 1µg/ml verwendet, was einer physiologischen Serumkonzentration entspricht. Die erhöhte transkriptionelle Aktivität von CREB führt zu einer gesteigerten Adipozytendifferenzierung (Abb. 12A) und Expression adipogener Signalmoleküle. Dazu zählen *preadipocyte factor* 1 (Pref1; auch als *delta-like 1 homolog*

(DLK1) bekannt), *wingless-type MMTV integration site family member* 10b (Wnt10b) und PPARγ2 (Abb.12C).

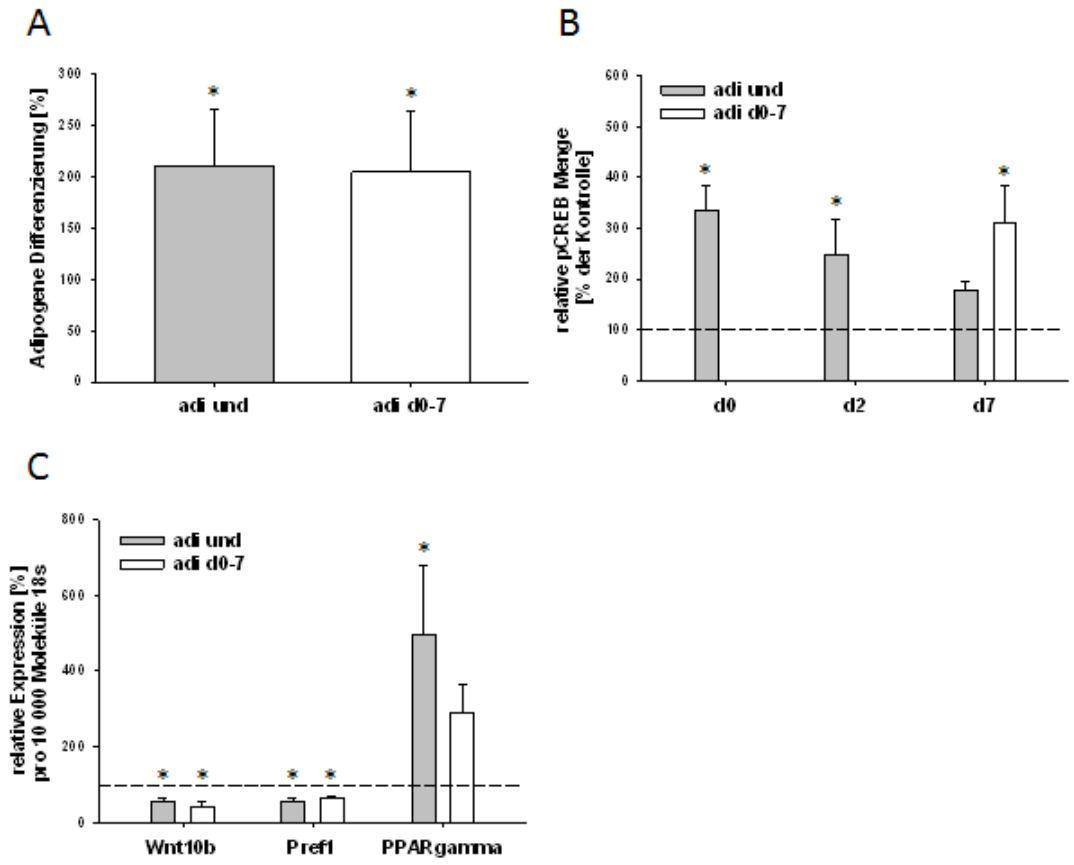

Abb. 12 **Relative Menge reifer Adipozyten (A), Aktivierung von CREB (B) und die Regulation von CREB-Zielgenen (C) nach Adiponektin-Stimulation in CGR8-Zellen**

CGR8-Zellen wurden in zwei verschieden Intervallen mit 1µg/ml Adiponektin stimuliert. Intervall I: für 5 Tage während des undifferenzierten Wachstums (adi und) und Intervall II: während der 7 Tage Determinierungsphase (adi d0-7). **A)** Bestimmung der relativen Menge von Adipozyten nach 23 Tagen Kultur der CGR8-Zellen. In den Versuchsgruppen wurde nach 23 Kulturtagen die Anzahl reifer Adipozyten mittels fluoreszenz-aktivierter Zellsortierung (FACS) bestimmt wie in Schädlich et al. (2010) beschrieben. Die Ergebnisse sind als Mittelwert in % ± Standardfehler relativ zur Behandlungskontrolle (gestrichelte Linie, 100% gesetzt) dargestellt. **B)** Aktivierung von CREB in undifferenzierten Zellen (d0), an Tag 2 (d2) und Tag 7 (d7) der Differenzierung. Die Analyse wurde mittels SDS-PAGE und Western Blot durchgeführt. Die Phosphorylierung nach Adiponektin-Stimulation wird relativ zur Behandlungskontrolle dargestellt, die 100% gesetzt ist und im Diagramm als gestrichelte Linie dargestellt wird [Mittelwert in % ± Standardfehler]. **C)** Expression von Wnt10b, Pref1 (beides an Tag 7) und PPARγ (an Tag 14) in CGR8-Zellen nach Adiponektin-Stimulation. Die mRNA-Menge wurde nach RNA-Isolation und cDNA-Synthese mittels qRT-PCR bestimmt. Die Ergebnisse sind relativ zur Expression in der Behandlungskontrolle angegeben (dargestellt als gestrichelte Linie), die 100% gesetzt wurden [Mittelwert in % ± Standardfehler].

PPARγ ist ein früher Marker der Adipogenese und induziert die Expression von Signalmolekülen, die wichtig für die Induktion und Aufrechterhaltung der adipogenen Differenzierung sind (Rosen & Spiegelmann 2000). Wie bereits erwähnt, gibt es von PPARγ zwei verschiedene Isoformen, wobei PPARγ2 nur in Adipozyten exprimiert wird und daher ein idealer adipogener Marker ist. Die erhöhte Expression von PPARγ2 am Tag 14 der Differenzierung (Abb. 12C) unterstützt somit den Befund einer erhöhten adipogenen Differenzierung (Abb. 12A) nach Adiponektin-Stimulation. Erste Studien zu Wnt10b zeigen eine abnehmende Expression mit Beginn der initialen Phase der Adipogenese *in vivo* und *in vitro* (Ross et al. 2000, Bennett et al. 2002) und belegen, dass eine Inhibierung von Wnt10b erst die Differenzierung zu reifen Adipozyten zulässt. Eine ektope Expression von Wnt10b hingegen hemmt die adipogene Differenzierung mesenchymaler Stammzellen (Ross et al. 2000). Schlüsselmoleküle für diesen Mechanismus sind PPARγ und CCAAT/*enhancer binding protein* α (C/EBPα) (Ross et al. 2000). Neben Wnt10b wirkt auch Pref1 inhibitorisch auf die Adipogenese (Smas and Sul 1993). Über das Pref1-Zielgen *sex determing region* (SRY)-box 9 (Sox9) wird, wie bei Wnt10b, die Expression von PPARγ und C/EBPα unterdrückt (Wang and Sul 2009). Zusätzlich wird die Transkription von „späten" adipogenen Markern, wie FASN und FABP4, inhibiert und die Lipideinlagerung gehemmt (Wang et al. 2006). CGR8-Zellen, die nach Adiponektin-Stimulation eine höhere CREB-Phosphorylierung zeigen, weisen eine geringere mRNA-Menge an Wnt10b und Pref1 auf, zwei wichtige Inhibitoren der Adipogenese. Diese Beobachtung ist vermutlich für die gesteigerte adipogene Differenzierung nach Adiponektin-Stimulation ausschlaggebend.

Die hier vorgestellten Ergebnisse stehen im Einklang mit bisherigen Studien, die die Bedeutung von CREB in 3T3-L1-Präadipozytenzelllinien untersucht haben. (Reusch et al. 2000, Klemm et al. 2001, Zhang et al. 2004, Fox et al. 2006, Fox et al. 2008). Die Expression eines konstitutiv aktiven CREBs alleine ist ausreichend, um die Adipogenese in diesen Zellen zu induzieren (Reusch et al. 2000, Klemm et al. 2001). Zentrale Schlüsselmoleküle sind dabei die klassischen adipogenen Faktoren PPARγ2 und C/EBP α und β (Reusch et al. 2000, Klemm et al. 2001). Weitere Zielgene der CREB-induzierten Adipogenese sind Cyclin D1 und Wnt10b (Fox et al. 2008). Fox und Koautoren postulieren, dass die CREB-abhängige Regulation der Wnt10b-Transkription über die Methylierung der Promotorregion gesteuert wird (Fox et al. 2008). Die in der Literatur bisher verwendeten 3T3-L1-Zellen sind Präadipozyten, die sich zu Adipozyten differenzieren können. Daher war bisher nur eine Analyse der terminalen Differenzierung von Präadipozyten zu Adipozyten möglich. Die in der vorliegenden Arbeit gezeigten Ergebnisse belegen erstmals die Bedeutung von CREB während der frühen Phase der Zelldeterminierung. Zielgen ist dabei neben den bereits bekannten Wnt10b und PPARγ2 auch Pref1.

Ein eindeutiger Beweis, dass CREB an den Promotor von Pref1, Wnt10b und PPARγ2 in CGR8-Zellen bindet, ist bisher noch nicht erfolgt und muss daher Gegenstand weiterer Untersuchungen sein. Versuche mittels siRNA-Technik zeigen, dass der *knock down* von CREB zu einer reduzierten adipogenen Differenzierungseffizienz (Abb. 13A) führt, die durch die reduzierte CREB-Transkriptmenge (Abb. 13B) und erhöhte Wnt10b und Pref1-Transkription (Abb. 13C) verursacht wird. Somit ist von einer direkten oder indirekten Interaktion von CREB mit Wnt 10b und Pref1 auszugehen. Dies beeinflusst die Adipogenese langfristig.

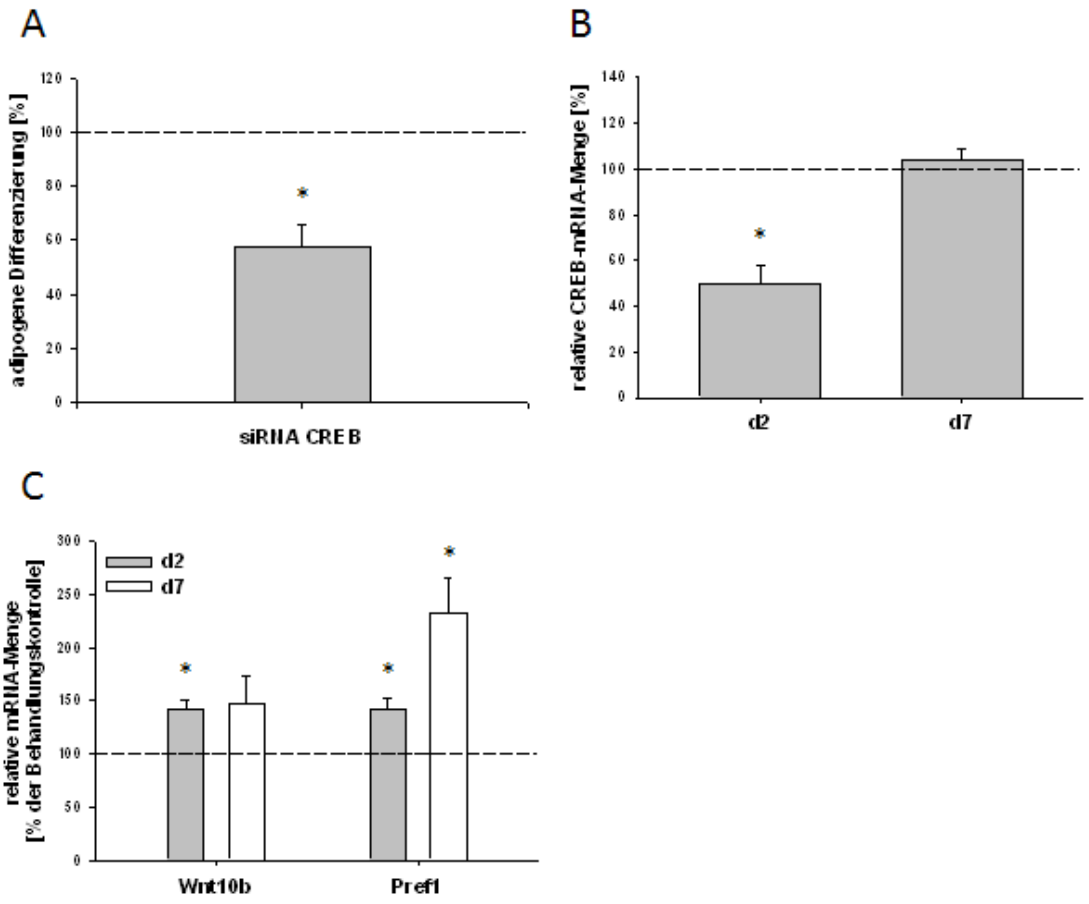

Abb. 13 **Adipogene Differenzierung (A), Aktivierung von CREB (B) und die Regulation von CREB-Zielgenen (C) nach siRNA-vermittelter Herunterregulation von CREB in CGR8-Zellen**

CGR8-Zellen wurden mit dem Einleiten der Determinierung (d0) einmalig mit siRNA gegen CREB behandelt. Ein Einfluss durch die siRNA-Behandlung im Vergleich zur nicht-transfizierten Kontrolle zeigte sich nicht. Aus diesem Grund sind die nachfolgenden Ergebnisse immer relativ zur siRNA-Behandlungskontrolle (*mock control*) dargestellt. **A)** Bestimmung der relativen Menge von Adipozyten nach 23 Tagen Kultur der CGR8-Zellen. In den Versuchsgruppen wurde nach 23 Kulturtagen die Anzahl reifer Adipozyten mittels fluoreszenz-aktivierter Zellsortierung (FACS), wie in Schädlich et al. (2010) beschrieben wurde, bestimmt. Das Ergebnis ist als Mittelwert in % ± Standardfehler relativ zur siRNA-Behandlungskontrolle (gestrichelte Linie, 100% gesetzt) dargestellt. **B)** CREB-mRNA-Menge an Tag 2 (d2)

und Tag 7 (d7) der Differenzierung. Die mRNA-Menge wurde nach RNA-Isolation und cDNA-Synthese mittels qRT-PCR bestimmt. Die Ergebnisse sind relativ zur Expression in der siRNA-Behandlungskontrolle ausgedrückt (dargestellt als gestrichelte Linie), die 100% gesetzt wurden [Mittelwert in % ± Standardfehler]. **C)** Expression von Wnt10b und Pref1 (an Tag 2 und 7) in CGR8-Zellen nach siRNA-Behandlung. Die mRNA-Menge wurde nach RNA-Isolation und cDNA-Synthese mittels qRT-PCR bestimmt. Die Ergebnisse sind relativ zur Expression in der Behandlungskontrolle ausgedrückt (dargestellt als gestrichelte Linie), die 100% gesetzt wurden [Mittelwert in % ± Standardfehler].

Inzwischen gibt es auch experimentell untermauerte Hinweise, dass neben Adiponektin auch Glukose metabolische Differenzierungseffekte auf die adipogene Differenzierung der CGR8-Zellen vermittelt. Hypoglykämische Entwicklungsbedingungen während der Determinierungsphase führen zu einer signifikant geringeren Adipozytenanzahl am Ende der adipogenen Differenzierung (Dissertation Knelangen 2012). Kritisch muss man jedoch anmerken, dass dieser Effekt nicht auftrat, wenn man die gleichen Glukosekonzentrationen während des undifferenzierten Wachstums verwendete (Dissertation Knelangen 2012). Eine mögliche Rolle von CREB kann nicht ausgeschlossen werde, da auch Glukose zu einer Beeinflussung der CREB-Aktivität in adulten Zellen führt (Jansson et al. 2008).

4.6. Ausblick

Im Hinblick auf eine langfristige Auswirkung der veränderten intrauterinen Entwicklungsbedingungen belegen die Ergebnisse, dass bereits in den ersten Tagen eines jeden Individuums wesentliche Grundlagen und Voraussetzungen für einen gestörten Stoffwechsel und damit für eine potentielle zukünftige Anfälligkeit gegenüber metabolischen Krankheiten wie Übergewicht im weiteren Leben determiniert werden. Dies stellt nicht nur eine große persönliche und klinische Herausforderung für bereits vor der Schwangerschaft bekannte mütterliche Stoffwechselstörungen dar, sondern auch für die assistierten Reproduktionstechniken (ART). Bei einer Befruchtung außerhalb des Mutterleibes werden die Embryonen für eine kurze Zeit in einem (derzeit unzureichend) definierten Nährmedium kultiviert. Trotz der zunehmenden Erfolge der letzten Jahre im Bereich der *in vitro*-Embryokultur (*baby take home*-Raten) fehlen Langzeitbeobachtungen. Noch ist nicht geklärt, was der Embryo während seiner ersten Tage für eine optimale und gesunde Entwicklung braucht. Dass die Nährmedien nicht dem natürlichen Eileiter und Uterusmilieu entsprechen, beweisen verschiedene experimentelle und epidemiologische Studien bei Mensch und Tier (Bavister 1995, Young et al. 1998, Sinclair et al. 2000, McEvoy 2003, Dumoulin et al. 2010, Mäkinen et al. 2013, Kleijkers et al. 2014, Hemkemeyer et al. 2014, Hoelker et al. 2014). In der Veterinärmedizin ist das höhere Risiko des *large offspring syndrom* (LOS) wohlbekannt, das durch eine erhöhte Abortrate und perinatale Sterblichkeit gekennzeichnet ist (Walker et al. 1996, Young et al. 1998, Niemann and Wrenzycki 2000). Inwieweit

eine *in vitro*-Kultur das Geburtsgewicht beim Menschen beeinflusst, wird aktuell kontrovers diskutiert (Lin et al. 2013, Carrasco et al. 2013, Kleijkers et al. 2014). Eine wichtige Schlussfolgerung aus der vorliegenden Arbeit ist, dass im Hinblick auf potentielle negative Konsequenzen die optimalen Kulturbedingungen herausgefunden werden müssen, um eine Beeinträchtigung und gegebenenfalls persistierende Veränderungen des embryonalen Metabolismus und somit langfristig Gesundheitschäden zu verhindern.

5. Zusammenfassung

Der Embryo besitzt eine hohe metabolische Plastizität, um sich an veränderte Entwicklungsbedingungen anzupassen. Durch einen mütterlichen Diabetes mellitus werden die lokalen und endokrinen Regulationsmechanismen, die das „optimale“ intrauterine Umgebungsmilieu des Embryos garantieren, gestört. Dies führt bereits während der Präimplantationsphase zu komplexen Veränderungen des embryonalen Stoffwechsels. Die Adaptation des embryonalen Stoffwechsels an ein verändertes Nährstoffangebot kann sich negativ auf die weitere Entwicklung embryonaler Zellen auswirken. Ein mütterlicher Diabetes mellitus, insbesondere bei mangelnder Compliance der Patientinnen, ist eine Ursache für eine solche adaptive Fehlprogrammierung und steht im direkten Zusammenhang mit einem späteren Übergewicht oder erhöhtem Diabetes-Risiko der Nachkommen.

Die vorliegende Arbeit zeigt, welche Auswirkungen eine diabetische Stoffwechsellage der Mutter auf den embryonalen Metabolismus während der Präimplantationsentwicklung hat. Als ein zentrales Stellglied des molekularen Anpassungsmechanismus wurde der Transkriptionsfaktor CREB untersucht. Dafür wurden Blastozysten diabetischer Kaninchen (Diabetisches Tiermodell) analysiert. Um den Einfluss der hormonellen Faktoren, Insulin, IGFs und Adiponektin zu diskriminieren, wurden Blastozysten gesunder Kaninchen *in vitro hyperglykämischen und hypoinsulinämischen* Kulturbedingungen ausgesetzt und molekulare Adaptationsmechanismen analysiert.

In beiden Versuchsmodellen wurde bewiesen, dass sich der Präimplantationsembryo an ein hyperglykämisches Entwicklungsmilieu hormonell und metabolisch anpasst. Die Ergebnisse dieser Arbeit lassen sich wie folgt zusammenfassen:

- Eine diabetische Stoffwechsellage der Mutter führt zu einer kompensatorischen Erhöhung der IGF-Produktion und verminderten Insulin- und IGF-Sensitivität im uterinen und embryonalen Gewebe am Tag 6 der Gravidität beim Kaninchen.
- Aufgrund der Interaktion zwischen dem IIRS und Adiponektin wird unter diabetischen Entwicklungsbedingungen in 6 Tage alten Blastozysten die metabolische Funktion des Insulins durch Adiponektin weitgehend kompensiert.
- CREB fungiert als zentraler Vermittler zwischen Insulin/IGF und Adiponektin in Kaninchenblastozysten (Tag 6 *p.c.*) diabetischer Tiere.

- Aufgrund der unterschiedlichen Rezeptorausstattung des IIRS im Embryo- und Trophoblasten ist eine gegenläufige Adiponektinregulation in den beiden Zellkompartimenten in Blastozysten diabetischer Kaninchen nachweisbar.

Als Folge dieser Anpassung an das diabetische Umgebungsmilieu treten im Lipidmetabolismus folgende Veränderungen auf:

- Blastozysten diabetischer Kaninchen weisen eine massive Akkumulation intrazellulärer Lipidvesikel am Tag 6 der Gravidität auf.
- Die Expression von Schlüsselmolekülen für den Lipidmetabolismus (PPARγ und SREBP1c), die intrazelluläre Lipidspeicherung (Perilipin und Adipophilin), den Fettsäuretransport und Metabolismus (FABP4 und FATP4) und die adipogene Differenzierung (PPARα) sind in Blastozysten diabetischer Kaninchen erhöht.
- In Blastozysten (Tag 6 *p.c.*) diabetischer Kaninchen weist die reduzierte Transkription von CPT1B auf eine reduzierte β-Oxidation hin.

Die beobachteten Effekte sprechen für eine inhibierte Energiegewinnung aus Fettsäuren und eine forcierte intrazelluläre Lipidspeicherung. Die genaue Ursache bedarf weiterer Untersuchungen. Erhöhte Glukosespiegel im Uterus können jedoch nur bedingt dafür verantwortlich gemacht werden.

Langfristige Konsequenzen einer Anpassung an veränderte Entwicklungsbedingungen wurden mit Hilfe der embryonalen Mausstammzelllinie CGR8 untersucht. In dieser konnten Adiponektin-vermittelte Effekte auf die adipogene Differenzierung belegt werden.

- Eine frühe Adiponektinexposition führt zu einer gesteigerten Anzahl reifer Adipozyten am Ende der adipogenen Differenzierung muriner embryonaler Stammzellen (CGR8).
- Für murine embryonale Stammzellen sind das undifferenzierte Wachstum und die Determinierungsphase die sensiblen Fenster für diese metabolische Programmierung.
- In die metabolische Programmierung von embryonalen Zellen ist die CREB-abhängige Regulation von Pref1, Wnt10b und PPARαinvolviert.

Die erzielten Ergebnisse bieten erste Ansatzpunkte für ein besseres Verständnis dafür, wie der embryonale Metabolismus die weitere Entwicklungsfähigkeit von Embryonen und die spätere Anfälligkeit gegenüber metabolischen Erkrankungen beeinflusst. Dabei ist eine Anpassung an das intrauterine diabetische Umgebungsmilieu, die die Weiterentwicklung sichert, der wichtigste Auslöser für die gezeigten embryonalen Effekte. Neben Glukose und Insulin scheinen auch andere Faktoren wie Lipide und das Adipozytokin Adiponektin eine zentrale Bedeutung zu haben.

6. Summary

Prior to implantation embryo development is regulated by maternal and embryonic factors, interacting at the cellular level within the uterine lumen. Embryonic cells are sensitive to nutritional and hormonal changes with a high adaptive plasticity to secure differentiation and survival. A maternal type 1 diabetes mellitus (T1DM) impairs endocrine regulatory mechanisms normally ensuring an optimal intrauterine milieu. This leads to complex changes in embryonic metabolism during the preimplantation period with consequences for further development.

The purpose of the current study was to examine the relationship between maternal diabetes mellitus and embryonal metabolism in effort to better understood molecular adaptation mechanisms to a diabetic uterine milieu. As a central mediator the transcription factor cyclic AMP-responsive element-binding protein (CREB) and its transcription factor family (ATFs and CREM) were characterised in rabbit blastocysts in normal development *in vivt* and in a diabetic pregnancy model in the rabbit (diabetic rabbit animal model). To better discriminate the influence of individual factors such as insulin, IGFs and adiponectin blastocysts from healthy rabbits were cultured *in virot* with various medium supplements.

Both experimental approaches showed that CREB takes part in the embryo-maternal crosstalk connecting the maternal insulin signalling and embryonic adiponectin synthesis. The main results can be summarised as followed:

- A maternal diabetes mellitus leads to a compensatory increased production of IGFs and decreased insulin/IGF sensitivity in blastocysts and uteri on day 6 of pregnancy in the rabbit.
- The lack of insulin is compensated by adiponectin as part of a metabolic adaptation process to secure embryonic glucose metabolism.
- In 6 day old rabbit blastocysts CREB acts as an arbiter in the embryo-maternal crosstalk connecting maternal insulin/IGF signalling and embryonic adiponectin receptor system (ARS).
- Due to the different insulin and IGF signaling in embryoblast and trophoblast cells in diabetic blastocysts, the embryo has a cell lineage-specific CREB regulation and adiponectin expression on day 6 of gravidity.

The molecular adaptation to a diabetic uterine environment leads to alterations in embryonic lipid metabolism and triglyceride accumulation:

- Blastocysts developed in diabetic environment accumulate intracellular lipids far above the normal level.

- Expression of key genes for lipid metabolism (PPARγ), lipid storage (perilipin and adipophilin), fatty acid transport and metabolism (FATP4 and FABP4), lipogenesis (SREBP1) and adipogenic differentiation (PPARα) are increased in blastocysts from diabetic rabbits at day 6 pregnancy.
- Transcription von CPT1B is decreased in blastocysts (day 6 *pini*) from diabetic rabbits, pointing to a reduced β-oxidation.

The underlying mechanism for the enforced intracellular lipid accumulation in blastocysts from diabetic rabbits needs further analysis and can only be partly explained by hyperglycemia.

Long-lasting consequences of embryonic adaptation to a diabetic environment (metabolic programming) were studied in pluripotent murine embryonic stem cells (CGR8). CGR8 cells encompass the full process of adipogenesis, thus offering a suitable model to study adipogenesis *in vitro*. The developmental impact of adiponectin during undifferentiated growth and determination of embryonic stem cells were elucidated by investigating gene expression and function of terminally differentiated cells. The observed effects can be summarised as followed:

- Adiponectin exposure during early phases of development leads to an increased number of functional terminally differentiated adipocytes, demonstrating a direct influence of adiponectin on adipogenesis in CGR8 cells.
- The undifferentiated growth and determination are two sensible phases for adiponectin-mediated metabolic programming in CGR8 cells.
- The CREB-mediated regulation of Pref1, Wnt10b and PPARα is crucial for the observed effects in CGR8 cells.

A maternal diabetes influences embryo development in myriad ways. Besides both “traditional” factors (insulin and glucose) other nutrients, particularly lipids, and adipokines, like adiponectin, need more attention in respect to their capacity to regulate embryonic metabolism. The embryo itself has an astonishing capacity to adapt to metabolic changes. The current study clearly shows the pivotal role of CREB in the embryonic adaptation process. This molecular adaptation may lay the foundation for susceptibility to diseases throughout later life. The results from this study together with current knowledge demand an intensive care already during early pregnancy in order to prevent long-lasting effects, like obesity in later life.

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8. Abbildungsverzeichnis

9. Abkürzungsverzeichnis

(c)AMP	(zyklisches) Adenosinmonophosphat
(c)DNA	(komplementäre) Desoxyribonukleinsäure
(m)RNA	(*messanger*) Ribonukleinsäure
(q)RT-PCR	(quantitative) Reverse Transkriptase-Polymerase-Kettenreaktion
3T3-L1	Murine unipotente Präadipozyten der Linie C3H10T1/2
AAPL1	*Adaptor Protein Containg Pleckstrin Homology Domain* 1
Abb.	Abbildung
ACC	Acetyl-CoA Carboxylase
AdipoQ/Acrp30	Adiponectin
AdipoR	Adiponectin-Rezeptor
Akt	*Proteinkinase B*
AMPK	AMP-aktivierte Proteinkinase
ARS	*Adiponektin-Rezeptor-System*
ART	assistierten Reproduktionsmedizin
ATF	*activating transcription factor*
BSA	*Bovines Serum Albumin*
BSM	Basales Synthetisches Medium
bZIP	Leucin-Zipper Domäne
C/EBP	*CCAAT/enhancer-binding protein*
ca.	circa
CD36	*Cluster of Differentiation* 36
CGR8	Murine pluripotente embryonale Stammzelllinie
CPT1B	Carnitin-Palmitoyltransferase 1 B
CRE	*cAMP responsive elment*
CREB	*cAMP responsive element binding protein*
CREM	*CREB modulatory protein*
CREM-ICER	*repressor CREB modulatory protein*
d	Tag
DLK1	*Delta-like 1 homolog* alias *Preadipocyte factor 1*
EB	Embryoblast
EBs	e*mbryoid bodies*

ESC	pluripotente embryonale Stammzelle
FABP4	*Fatty acid binding protein 4*
FACS	*Fluorescence-activated cell sorting, flow cytometry*
FASN	Fettsäure-Synthase
FATP	*fatty acid transporter protein*
FCS	*Fetal calf serum*
FOXO1	*forkhead box protein O*1
G6P	Glukose-6-Phosphatase
GDM	Gestationsdiabetes
GLUT2	Glukosetransporter Typ 2
GLUT4	Glukosetransporter Typ 4
h	Stunde
HAPO	Hyperglycemia and Adverse Pregnancy Outcomes
hCG	humanes Choriongonadotropin
HDL	*high-density* Lipoprotein
ICM	*inner cell mass*
ICSI	Intrazytoplasmatische Spermieninjektion
IGF	*Insulin-like growth factors*
IGF1R	IGF1-Rezeptor
IGF2R	IGF2-Rezeptor
IIRS	Insulin-IGF-Rezeptor-System
IR	Insulin-Rezeptor
IVF	*In vitro-Fertilisation*
KID	Kinase-induzierbare Domäne
LDL	*low-density* Lipoprotein
LDLR	*low density lipoprotein receptor*
LGA	*large for gastational age*
LIF	*Leukaemia inhibitory factor*
LOS	*large offspring syndrom*
LXRα	Leber X-Rezeptoren α
min	Minute
MAPK	*Mitogen-activated protein kinase*
MSC	Multipotente mesenchymale Stammzellen
p.c.	*post coitum*
PCOS	polyzystisches Ovarsydrom

PEPCK	Phosphoenolpyruvatcarboxykinase
PGE	posteriore Gastrulationsextension
PI3K	Phosphoinositid-3-Kinase
PLIN1	Perilipin
PLIN2	Adipophilin
PMSG	*Pregnant Mare Serum Gonadotropin*
PPAR	Peroxisomen-Proliferator-aktivierter Rezeptor
PREF1	*Preadipocyte factor* 1
RAB5	*RAS-related protein 5*
RIPA	*Radio-Immunoprecipitation Assay*
ROS	Reaktive Sauerstoffspezies
S6K	S6-Kinase
SDS -PAGE	Natriumdodecylsulfat-Polyacrylamidgelelektrophorese
SGA	*small for gastational age*
siRNA	s*mall interfering* RNA
SREBP1	*sterol regulatory element-binding protein* 1
T1DM	Typ 1 Diabetes mellitus
T2DM	Typ 2 Diabetes mellitus
T_3	Trijodthyronin
TB	Trophoblast
TSC1/2	*tuberous sclerosis* 1/2
UCP	*Uncoupling protein*
VRB	Vordere Randbogen
Wnt	*Wingless-type MMTV integration site family*
Wnt10b	*wingless-type MMTV Integration site family member* 10b

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Declaration under Oath

Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegeben Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich und inhaltlich entnommen Stellen als solche kenntlich gemacht habe.
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I herby declare that I have no criminal record and that no preliminary investigations are pending against me.

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