APPENDICES

SUMMARY

This thesis reports the isolation, characterization and allotransplantation in porcine retina of ciliary epithelium (CE)-derived cells, also known as retinal stem cells (RSCs). The self-renewal capacity and differentiation potential of these cells *in vitro* and *in vivo* makes them candidate donors in cell replacement approaches for treating retinal degenerative diseases. The use of pig as the animal model should facilitate translation of these findings to applications for human conditions.

Chapter 2 presents data on retinal histogenesis in the pig, generated upon validation of antibodies commonly used in rodents. The porcine retina displays similar marker distribution and histogenesis as the human retina, supporting the pig as a preclinical model to study retinal degeneration and repair.

Chapter 3 describes the isolation and characterization of newborn porcine CE-derived RSCs in comparison with retinal progenitor cells (RPCs) derived from the developing retina and neural stem cells (NSCs) from the developing brain. Porcine CE-derived RSCs share similar characteristics with their human counterpart: their self-renewal potential is limited and they cannot be maintained indefinitely in culture, suggesting that they are not bona fide stem cells like NSCs; however, under conditions that favor differentiation, they can express markers of mature retinal cells and rudimentary morphological features of neurons.

In Chapter 4, we undertook a novel approach to predict microRNA that could have gene regulatory functions in the retina and in RSCs based on gene expression profiling data obtained by microarray hybridization. A limited number of microRNA was predicted to affect gene expression in RSCs compared to postnatal and adult retina, consistent with their undifferentiated nature. Furthermore, among the microRNAs that ranked highest in our prediction and were validated by RT-PCR, miR24 and miR122, had previously been shown to be involved in development.

Chapter 5 completes the work presented in Chapters 2 and 3 by testing the fate of newborn pig CE-derived cells after transplantation in the young porcine retina. CE-derived cells migrated both radially and tangentially from the subretinal site of injection. The majority of transplanted cells appeared integrated in multilayered RPE-like structures and expressed RPE65, while a smaller proportion of cells integrated in the neuroretina and expressed markers of mature retinal cells. Differences in cell fate observed *in vitro*, where RPE marker expression was low and retinal phenotypes comparably higher, suggest that the environment and extrinsic cues are critical in guiding the differentiation of CE-derived cells.

In summary, this thesis shows that cultures of self-renewing cells can be obtained *in vitro* from the newborn porcine CE and that these cells can differentiate into retinal and RPE phenotypes both *in vitro* and *in vivo* in allorecipients. However, the nature and physiological role of these cells in potential endogenous repair mechanisms still remain to be explored. Notably, their remarkable plasticity *in vitro* makes them an interesting cell model to study this phenomenon of recent identification.

NEDERLANDSE SAMENVATTING

Dit proefschrift beschrijft de isolatie en karakterisering van cellen afkomstig van het ciliair epitheel (CE) van het netvlies van het varken. Deze cellen hebben eigenschappen van netvlies stamcellen (retinal stem cells, RSC's). Tevens wordt het lot van deze cellen beschreven na (allo)transplantatie in het varkensoog.

De vooronderstelling bij de beschreven experimenten was tweeledig, nl. i. dat het zelf-vernieuwingsvermogen en het differentiatie potentieel van RSC's hen tot goede kandidaten maakt voor de behandeling -door middel van celvervanging- van oogziekten, waarbij het netvlies degenereert en ii. dat het varken een goed modeldier is om de vertaalslag naar toepassing bij de mens te maken van celtherapeutisch onderzoek naar netvliesherstel (Hoofdstuk 1).

Hoofdstuk 2 beschrijft de ontwikkeling van het netvlies van het varken. Bij dit onderzoek is gebruik gemaakt van immuunhistochemie met antilichamen tegen celmerker-eiwitten, die goed gekarakteriseerd zijn bij de muis. De resultaten laten zien dat de verdeling van de celmerker-eiwitten in het varkensnetvlies grote overeenkomst vertoont met die in het menselijke netvlies. Dit gegeven ondersteunt de vooronderstelling over het gebruik van het varken als modeldier bij preklinisch onderzoek naar netvlies degeneratie en herstel.

Hoofdstuk 3 gaat in op de isolatie en karakterisering van de CE-afgeleide RSC's van pasgeboren varkens. Deze RSC's worden vergeleken met retinale voorloper (progenitor) cellen (RPC's) in het zich ontwikkelende netvlies en neurale stamcellen (NSC) van het zich ontwikkelende brein. Uit dit onderzoek bleek dat varken-CE afgeleide RSC's veel overeenkomst vertonen met hun menselijke tegenhangers in de zin dat hun zelfvernieuwend vermogen beperkt is en dat ze niet onbeperkt in kweek kunnen worden gehouden. Dit laatste geeft overigens aan dat RSC's geen bona fide stamcellen zijn, zoals NSC's. Wel is echter duidelijk geworden dat ze celmerkers tot expressie

kunnen brengen, die ook in gedifferentieerde netvliescellen worden aangetroffen en dat ze morfologische kenmerken vertonen die aan neuronen doen denken.

In Hoofdstuk 4 is een nieuwe methode ontwikkeld en toegepast die voorspelt welke microRNA's een gen-regulatoire functie zouden kunnen hebben in het netvlies en RSC's. Hierbij is gebruik gemaakt van door microarray-hybridisatie verkregen genexpressieprofielen van RSC's en netvliezen van pasgeboren en volwassen varkens. Vergeleken met de netvliezen werd er voor RSC's een klein aantal microRNA's met effect op genexpressie voorspeld. Het geringe aantal strookt met het ongedifferentieerde karakter van RSC's. Onder de microRNA's, die het hoogst op de ranglijst van effectvoorspelling stonden, bevonden zich miR24 en miR122, waarvan al eerder aangetoond dat ze betrokken zijn bij de ontwikkeling. Het voorspelde effect is vervolgens kwantitatief bevestigd met behulp van RT-PCR.

In Hoofdstuk 5 wordt het werk beschreven in de Hoofdstukken 2 en 3 aangevuld met onderzoek naar het lot van CE-afgeleide cellen van pasgeboren varkens na allotransplantatie. Hiertoe is hun distributie en fenotype bestudeerd na subretinale injectie in het oog van jonge varkens. Gebleken is dat de cellen zowel radiaal als tangentieel vanaf de plek van injectie migreren, dat de meerderheid van de getransplanteerde cellen in het retina pigment epitheel (RPE) terecht komen en de RPE-merker RPE65 tot expressie brengen. Een kleiner deel van de cellen komt terecht in de neuroretina en brengt eiwitmerkers tot expressie, die karakteristiek zijn voor volwassen netvliescellen. Deze fenotypische in vivo bevindingen zijn niet helemaal congruent met de in vitro resultaten. In vitro was het percentage cellen met de RPE-merkerexpressie laag en die van cellen met netvliesfenotypes relatief hoog, hetgeen sterk suggereert dat de directe omgeving en extrinsieke signalen een kritische rol spelen bij de differentiatie van de CE-afgeleide cellen.

Samenvattend, de resultaten beschreven in dit proefschrift laten zien dat uit het ciliair epitheel van pasgeboren varkens cellen met een zelf-vernieuwend vermogen in kweek gebracht kunnen worden en dat deze cellen zowel in vitro als in vivo (na allotransplantie) kunnen differentiëren tot cellen met fenotypes die karakteristiek zijn

voor cellen van het netvlies en het retinale pigmentepitheel. Voordat ze kunnen worden toegepast zal echter hun fysiologische rol in netvliesherstelmechanismen verder onderzocht moeten worden. Vooral de nieuw ontdekte en opmerkelijke plasticiteit in vitro maakt deze cellen tot een waardevol onderwerp van verder preklinisch onderzoek.

RIASSUNTO IN ITALIANO

Questa tesi descrive l'isolamento, la caratterizzazione e il trapianto allogenico nella retina suina di cellule derivate dall'epitelio ciliare (EC), anche chiamate cellule staminali retiniche (CSR). La capacita' di autoreplicarsi e il potenziale di generare cellule differenziate *in vitro* e *in vivo* ne suggeriscono l'uso in terapie cellulari per il trattamento delle malattie degenerative della retina. La scelta del maiale come modello animale faciliterebbe l'applicazione all'uomo dei risultati di questa ricerca.

Nel Capitolo 2 e' illustrata l'istogenesi della retina nel maiale. I dati sono stati ottenuti dopo avere validato anticorpi comunemente usati in roditori. La retina suina presenta una distribuzione di marcatori cellulari e un'istogenesi simili a quelli della retina umana. Questo incoraggia l'uso del maiale come modello preclinico per studi sulla degenerazione e rigenerazione della retina.

Il Capitolo 3 descrive l'isolamento e la caratterizzazione di CSR derivate dall'EC di maiale neonato. Le loro caratteristiche vengono confrontate con quelle di cellule progenitrici retiniche (CPR) ottenute dalla retina e cellule staminali nervose (CSN) ottenute dal cervello di embrioni di maiale. The CSR derivate dall'EC suino posseggono cartteristiche simili a quelle delle corrispondenti cellule umane: il loro potenziale di autoreplicazione e' limitato e non possono essere mantenute in coltura a lungo. Queste caratteristiche suggeriscono che queste cellule non siano *bona fide* cellule staminali come the CSN. Tuttavia, in condizioni che favoriscono il differenziamento, le CSR possono esprimere marcatori cellulari tipici delle cellule retiniche mature e caratteristiche morfologiche rudimentali che suggeriscono la loro natura di neuroni.

Nel Capitolo 4 abbiamo applicato un nuovo metodo per prevedere quali microRNA potessero avere funzione regolatoria nella retina e nelle CSR basandosi sul profilo di espressione ottenuto con l'ibridazione su micro arrays. Un numero ridotto di microRNA e' risultato essere coinvolto nell'espressione genica nelle CSR rispetto alla retina postnatale e adulta, come ci si aspetterebbe sulla base della loro natura

indifferenziata. Inoltre, tra i microRNA che sono risultati in alto alla lista e che sono stati validati con la RT-PCR, miR24 e miR22 erano gia' noti per il loro ruolo nello sviluppo.

Il Capitolo 5 illustra il destino di cellule derivate dall'EC di maiale neonato dopo trapianto nella retina di giovani maiali. Questo Capitolo conclude la ricerca presentata nei Capitoli 2 e 3. Le cellule derivate dall'EC migrano in direzione radiale e tangenziale dal punto di iniezione nello spazio subretinale. La maggioranza delle cellule trapiantate sembrano integrarsi in una struttura a piu' strati simile all'epitelio retinico pigmentato (ERP) ed esprimono il marcatore cellulare RPE65. Una piccola parte delle cellule risulta integrata nella neuroretina ed esprime marcatori cellulari caratteristici delle cellule retiniche mature. Le differenze osservate rispetto al differenziamento *in vitro*, dove a confronto l'espressione di RPE65 e' minima e le cellule che esprimono marcatori retinici numerose, suggeriscono che stimoli ambientali e segnali estrinsici sono fondamentali nel guidare il differenziamento delle cellule derivate dall'EC.

In conclusione, questa tesi dimostra che colture di cellule con capacita' di autoreplicazione possono essere ottenute *in vitro* dall'EC di maiali neonati. Queste cellule possono essere differenziate per generare fenotipi retinici e ERP *in vitro* e *in vivo* dopo allotrapianto. Tuttavia, la natura e il ruolo fisiologico di queste cellule in potenziali meccanismi autologhi di rigenerazione rimangono ignoti. La plasticita' cellulare *in vitro* aumenta l'interesse per queste cellule che potrebbero essere usate come modello per studiare questo fenomeno di recente identificazione.

ABBREVIATIONS

AGE	Advanced Glycosylation End-products
AMD	Age-related Macular Degeneration
α-AA	Alpha-aminoadipate
BLBP	Brain Lipid Binding Protein
BMP	Bone Morphogenic Protein
BrdU	Bromodeoxyuridine
CE	Ciliary Epithelium
Cdh2	N-cadherin
Chx10	Visual System Homeobox 2
CMZ	Ciliary Marginal Zone
CNTF	Ciliary Neurotrophic Factor
CRALBP	Cellular Retinaldehyde-Binding Protein
CZG	Circumferential Germinal Zone
DME	Diabetic Macular Edema
DR	Diabetic Retinopathy
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ES	Embryonic Stem (cells)
FGF	Fibroblast Growth Factor
FLP	Focal Laser Photocoagulation
GCL	Ganglion Cell Layer
GFAP	Glial Fibrillary Acidic Protein
GDNF	Glial cell-Derived Neurotrophic Factor
GH	Growth Hormone
GS	Glutamine Synthetase
IGF	Insulin-like Growth Factor

IHC	Immunohistochemistry
IL	Interleukin
INL	Inner Nuclear Layer
IPL	Inner Plexiform Layer
iPS	Induced Pluripotent Stem (cell)
LCA	Leber Congenital Amaurosis
LIF	Leukemia Inhibitory Factor
LPC	Laser Photocoagulation
NFL	Nerve Fiber Layer
NMDA	N-Methyl-D-aspartic acid
NMU	N-methyl N-nItrosourea
NSC	Neural Stem Cell
ONL	Outer Nuclear Layer
OPL	Outer Plexiform Layer
OS	Outer Segments
Pax6a	Paired Box gene 6a
PCNA	Proliferating Cell Nuclear Antigen
PDGF	Platelet-Derived Growth Factor
PDT	Photodynamic Therapy
PEDF	Pigment Epithelium Derived Factor
PNA	Peanut Agglutinin
PRL	Photoreceptor Layer
β-6PDE	β-6 Phosphodiesterase
RA	Retinoic Acid
RCS	Royal College of Surgeon's
RGC	Retinal Ganglion Cell
Rho	Rhodopsin
RP	Retinitis Pigmentosa
RPC	Retinal Progenitor Cell

RPE	Retinal Pigment Epithelium
RSC	Retinal Stem Cell
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
Rx1	Retinal Homeobox 1
SHH	Sonic Hedgehog
Smo	Smoothened
TGF	Transforming growth factor
TTT	Transpupillary thermotherapy
VEGF	Vascular Endothelial Growth Factor
Vsx2/Chx10	Visual System Homeobox 2

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