CHAPTER 6

GENERAL DISCUSSION

Our studies in the pig complement and expand previous works in rodents and humans. We have conducted an immunohistochemical study of porcine retinal histogenesis and characterized antibodies to retina-specific markers commonly used in rodents. We conclude that retinal development in pigs parallels retinal development in humans (Chapter 2). We show that a small proportion of cells from the newborn pig CE can proliferate and give rise to sphere cultures with limited self-renewal potential *in vitro* under controlled culture conditions (Chapter 3). Our data reveal that CE-derived cells can generate not only retinal, but also RPE phenotypes *in vivo* and *in vitro*, and suggest the hypothesis that these cells posses a broad developmental potential (Chapter 5). Similar results were recently reported in other mouse studies *in vitro* (Aruta et al, 2011) and *in vivo* (Ballios et al, 2010), further supporting our work. The dual lineage potential of CE-derived cells might be advantageous in the context of cell replacement. On the one hand, CE-derived RPE-like cells could contribute a protective effect by promoting photoreceptor survival (Carr et al, 2009b; Lin et al, 1996) while, at the same time, newly differentiated photoreceptors could replace lost ones. Finally, our studies confirmed that the pig is a viable animal model in alternative to non-human primates for pre-clinical testing of cell-based therapies in the retina. We have also shown that allotransplantation in the large porcine eye is successful and does not appear to cause any obvious immune response, providing pre-clinical data and support for translation to the human eye (Chapter 5).

Some aspects of our work require further investigation: the age of CE-derived cells; the demonstration of functional differentiation; and the use of disease models.

Optimal age of CE-derived cells - We chose to collect CE-derived cells from newborn piglets (1-2 week old) because they were more readily available and cost-effective. These cells may possess greater plasticity and enhanced capacity to generate retinal and RPE phenotypes compared to cells isolated from adult CE that would be used in human autologous transplantation procedures. Although earlier reports indicate that CE-derived cells from neonatal, young and old human donors possess at least

comparable *in vitro* proliferation and clonogenic properties (Coles et al, 2004), the true potential for auto-transplantation of porcine CE-derived cells should be confirmed with cells from adult donors.

Demonstration of functional differentiation of CE-derived cells - The use of reporter genes upregulated coincidentally with differentiation of a specific cell type (i.e. rod photoreceptors) has provided convincing evidence of the capacity of RPC and ES cells to generate the correct cell type for replacement (Decembrini et al, 2011; Lamba et al, 2009; Lamba et al, 2006; Lamba et al, 2010; MacLaren et al, 2006; Zhou et al, 2011). In our studies, we base our assessment of the transplanted cells on morphology and co-localization of cell-specific markers with a labeling dye. The use of viral constructs to express reporter genes under control of photoreceptor-specific promoters as shown in the cited papers could strengthen our results. Furthermore, the unequivocal direct proof that cells expressing photoreceptors genes possess photoreceptor function would validate their identity. The vital dye we used in this studies should be sufficient to localize the transplanted cells and record neuronal function by Ca^{++} imaging and patch clamping in retinal slice preparations (Homma K. et al., unpublished data).

Transplantation in porcine models of retinal degeneration - We have completed a full round of cell isolation and transplantation in the normal pig with relative success in terms of cell integration, expression of phenotypic markers of differentiated retinal and RPE cells, and no immune rejection. The next step would be transplantation in a pig model of retinal degeneration. Work in rodents suggests that the partially degenerated retina might be more permissive to cell integration and differentiation, depending on the degree of degeneration at the time of transplantation (Chacko et al, 2003) and supports the working hypothesis that transplanting pig models with retinal degeneration might yield visible improvements of retinal histology and function.

The debate on Retinal Stem Cells

The nature and developmental potential of cells derived from the adult CE have been the object of controversy in recent literature. Mike Dyer and his group, based on

morphological and molecular criteria, conclude that CE-derived spheres do not consist of stem cells, but rather of proliferating pigmented CE cells with epithelial morphology that fail to differentiate into retinal neurons *in vitro* and *in vivo* (Cicero et al, 2009). Furthermore, Robin Ali's and Jane Sowden's groups provide evidence that CE-derived cells expanded in monolayer culture lose their pigmentation, and despite lacking neuronal morphology, co-express markers of eye field, retinal progenitor, and differentiated CE. Upon stimulation, these cells fail to activate the Nrl-regulated rod photoreceptor differentiation program (Gualdoni et al, 2010).

Three earlier publications had also reported detailed data on the epithelial properties of CE-derived cells. However, all concur that CE-derived spheres contain a mixed population of proliferative cells with epithelial and neural properties (Kohno et al, 2006; Moe et al, 2009; Xu et al, 2007a). The epithelial phenotype is manifested in some of the cells by the presence of pigmentation, of junctional complexes (desmosomes, gap, adherent, and tight junctions), expression of epithelial markers, and flattened/polarized appearance. Neural properties are evidenced by the expression, among others, of the immature neuroepithelial marker nestin, the retinal field markers PAX6 and SOX2, and the retinal progenitor marker CHX10. The balance between epithelial and neural properties is regulated by central signaling pathways (Moe et al, 2009). The epithelial phenotype is sustained by activation of the canonical Wnt pathway while the neureoepithelial phenotype depends upon increased expression of TGF-β receptor. These data can reconcile the terms of the controversy in that slight changes in culture conditions (e.g., concentration of growth factors and culture density) might tilt the balance towards one phenotype or the other. Furthermore, they explain the limited self-renewal capacity of CE-derived cells that has been consistently reported in the published literature (Ahmad et al, 2000; Coles et al, 2004; De Marzo et al, 2010; Inoue et al, 2006; Inoue et al, 2005; Mayer et al, 2005; Tropepe et al, 2000). Thus, although the nature of RSCs remains controversial, the partial self-renewal capacity of CE-derived cells is suggestive of the undifferentiated and mitotic state.

In our studies by IHC and RT-PCR, we focused on the neuronal properties of CE-derived cells. Although we did not specifically look for epithelial properties, we reported that a fraction of the cells in the CE-derived spheres were pigmented. However, in our experimental conditions, the proportion of pigmented cells diminished with passages in culture. Furthermore, we anecdotally observed that CE-derived spheres were more difficult to dissociate than RPC or neural stem cell (NSC) spheres. This characteristic did not change over passages in culture and could provide indirect evidence of the presence of epithelial junctions in CE-derived cells. Our data are therefore compatible with the existence of mixed epithelial and neural phenotypes in CE-derived cells that can be manipulated by changing culture conditions. Notably, while some reports indicate that pigmented CE-derived cells constitute the proliferating RSC population (Tropepe et al, 2000), others suggest that non-pigmented CE cells are proliferating RSCs (Bhatia et al, 2009).

Work from several laboratories has shown differentiation *in vitro* and *in vivo* of CE-derived cells into neuronal- and photoreceptor-like phenotypes (Coles et al, 2004; De Marzo et al, 2010; Inoue et al, 2006; Inoue et al, 2005; Jomary & Jones, 2008; Jomary et al, 2010). Our differentiation cultures are different from Dyer's in that we plated dissociated cells and not intact spheres. By disrupting cell-cell interactions, we might have biased the cells towards neural differentiation. Furthermore, we differentiated our cultures in the presence of high serum or low serum and growth factors; this too might have contributed to driving the cells along the neural lineage. Most importantly, we used cells from newborn pig, which corresponds to <1 month old mouse, whereas Dyer used 6-8 week old mice. As already discussed, newborn cells might maintain neuronal progenitor properties that are lost in cells from later ages.

Similarly, our experiments are not comparable to those from Ali's and Sowden's groups since they differentiate cells maintained in monolayer cultures and we used cells cultured as neurospheres. When we used monolayer culture conditions in our initial studies, we could only detect differentiated cells positive for the glial marker GFAP and none of the other retinal neuronal markers. Since Ali's and Sowden's groups were

specifically looking for rod photoreceptor differentiation in their experiments, they might have missed glial differentiation that probably occurred in their cultures.

Concluding remarks

Based on the literature and on our data, a small proportion of porcine CE-derived cells can proliferate and give rise to characteristic spheres under controlled culture conditions *in vitro*. Cells in the spheres have limited self-renewal potential and display mixed epithelial and neural properties. Under permissive differentiating conditions *in vitro*, cells from dissociated spheres can express markers of mature retinal cells. Similarly, *in vivo*, CE-derived cells migrate from the site of injection and express markers of mature retinal cells, including photoreceptors and RPE. Further investigations are required to improve the expandability and differentiation potential of CE-derived cells if they are to be considered for translational applications. In addition, stronger functional data should be generated to provide evidence of their regenerative capacity in the diseased retina. Finally, recent concerns on the rapid accumulation of severe chromosomal aberrations during cultivation remain to be addressed (Djojosubroto et al, 2009).

The exact nature of CE-derived cells in culture remains unclear. The literature strongly suggests that cellular environment and paracrine factors play an important role in determining their phenotype and fate *in vitro* and *in vivo*. Thus, cell amplification followed by genetic (re)programming and pre-differentiation may be required to facilitate the generation of the desired cell phenotypes and to restore retinal function. Recent advances in differentiating retinal neurons from other pluripotent stem cell sources such as ESCs and iPSCs have overshadowed studies on CE-derived cells. Retinal neurons have been successfully differentiated from human ESCs *in vitro* under culture conditions that recapitulate retinal development, using known developmental cues (Hirano et al, 2003; Ikeda et al, 2005; Lamba et al, 2006; Osakada et al, 2008; Osakada et al, 2009a; Osakada et al, 2009b; Reh et al, 2010; Zhao et al, 2002), small-molecule induction protocols (Osakada et al, 2009b), and guiding morphogenesis using

biomaterial scaffolds (McUsic et al, 2012). Once transplanted in animal models of retinal degeneration, human ESC-derived retinal cells populate the ONL, express markers of rod and cone photoreceptors (Hambright et al, 2012), and partially restore function (Lamba et al, 2009). Similarly, photoreceptor progenitors have been differentiated from human iPSCs (Hirami et al, 2009), and successfully integrated in the mouse retina (Lamba et al, 2010). Finally, iPSC-derived photoreceptors allotransplanted in the swine retina have been shown to integrate and express markers of differentiation (Zhou et al, 2011). In retinal degenerative diseases such as LCA and AMD, although vision impairment is consequent to photoreceptor death, the latter fail because of a dysfunctional RPE. Thus, at early stages of disease, when the ONL is still preserved, RPE replacement could prevent photoreceptor loss. RPE derived from human and other non-human primate ESCs and iPSCs is functional and can phagocytose shed photoreceptor membranes *in vitro* and *in vivo* after transplantation in rodent models of retinal degeneration (Carr et al, 2009a; Carr et al, 2009b; Idelson et al, 2009; Okamoto & Takahashi, 2011; Wang et al, 2010). Furthermore, the first clinical trial for safety and tolerability of transplanted human ESC-derived RPE was initiated in 2011 in patients with Stargardt's macular dystrophy and dry AMD (Schwartz et al, 2012). The authors suggest that it is premature to conclusively interpret data on efficacy. However, they show that RPE integrates in the host as a mature, quiescent monolayer and they observe no signs of hyperproliferation, abnormal growth, or immune rejection four months after transplantation, suggesting that the cells are safe and well tolerated and supporting the promise of this cell-based approach.

Although other cell types are currently under more intense investigation, there are still questions worth asking and answering about CE-derived cells before discarding them as a possible source for retinal transplantation and repair. This is true especially in consideration of their amenability to autologous transplantation and the relatively limited manipulation they require before injection in the degenerative retina. Finally, all data concur in showing the remarkable plasticity of CE-derived cells in neurosphere cultures. Cell plasticity is a phenomenon that is attracting increasing research interest

and CE-derived cells may provide a useful model for studies on its regulation, ultimately providing insights and methods to manipulate stem cell sources for repair in the retina.