Mesodermal Differentiation of Skin-derived Precursor cells

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Abstract

Neural crest stem cells (NCSCs) are embryonic multipotent cells that give rise to a wide range of cell types that include those forming the peripheral neural cells and the mesodermal cells of the face including the facial bones. In neonatal and adult skin, skin-derived precursor cells (SKPs) are multipotent dermal precursors that share similarities with NCSCs and can differentiate into peripheral neural and mesodermal cells, such as adipocytes. Based on the similarities between SKPs and NCSCs, I asked, in this thesis, whether rodent or human SKPs can differentiate into skeletal mesodermal cell types by determining their ability to differentiate into osteoblasts and chondrocytes. In culture, rodent and human SKPs differentiated into alkaline phosphatase-, osteopontin- and type-I collagen-positive osteoblasts that produced mineral deposits and into type-II collagen expressing chondrocytes. Clonal analysis showed that SKPs are multipotent for the osteogenic and chondrogenic lineages. To ask whether SKPs can generate these cells in vivo, genetically-tagged naïve rat SKPs were transplanted into a tibia bone fracture model. Six weeks post-transplantation, SKP-derived osteoblasts and osteocytes were present in the newly formed bone, showing their osteogenic differentiation in vivo. At three weeks post-transplantation, some of the injected cells differentiated into hypertrophic chondrocytes in the callus and others into perivascular cells in areas just outside the callus. To test whether it is the local environment that dictates the phenotype of transplanted SKPs, GFP-tagged undifferentiated rat SKPs were injected into the hypodermis of the skin, an adipogenic environment. Four weeks post-transplantation, SKPs differentiated into adipocytes, but not in inappropriate cell types. These results further the known differentiation potential of SKPs, show that local environment of a bone fracture or the hypodermis of the skin is sufficient to induce the differentiation of undifferentiated SKPs into appropriate cell types and suggest the use of SKPs as source of mesodermal precursor cells for cell therapy.
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Chapter 1
General Introduction

1 Introduction

Stem cells are a source of promise and controversy. For some, they represent a potential cure for degenerative diseases such as Alzheimer’s, Parkinson’s and diabetes or for the healing of various tissues that fail to regenerate properly including the spinal cord, cardiac muscle, knee cartilage, as well as a variety of bone defects such as bone malformation or disease, non-union healing, and osteoporosis. The main controversy with the use of stem cells is mainly associated with their source, particularly the use of embryos to isolate embryonic stem cells and their tumorigenic potential. Over the course of the last few decades, the finding that many adult organs contain precursor or stem cells brings the possibility for cell therapy without the controversial use of embryos. The identification and characterization of these adult stem cells have also resulted in a progression from the traditional view that cells derived from a given embryonic lineage cannot differentiate into cell types of other embryonic lineages.

Therefore the premise of stem cell research is not limited to therapeutic purposes but also includes the expansion of our understanding of the mechanisms governing cell differentiation. This chapter introduces and discusses three types of precursor/stem cells: neural crest stem cells (NCSCs), mesenchymal stem cells (MSCs) and skin-derived precursor cells (SKPs) with an emphasis on the latter two. While NCSCs are uniquely found during a short period of embryogenesis, both MSCs and SKPs can be isolated from juvenile and adult mammals. MSCs can be differentiated into a variety of cell types with therapeutic applications. Multiple lines of evidence demonstrate that SKPs, recently identified in the laboratory of Dr. Miller, resemble neural crest cells and therefore may also have great potential for therapeutic applications. In this thesis I hypothesized that SKPs can differentiate into skeletal mesodermal cell types. This was tested by differentiating SKPs into osteoblasts and chondrocytes in vitro using conditions developed for the differentiation of MSCs. The differentiation potential was also tested in vivo by transplanting SKPs into a bone fracture. The results, showing the mesodermal differentiation of SKPs into osteoblasts and chondrocytes are presented in Chapter 2. The mesodermal differentiation potential of SKPs was not limited to these two cell types, and I hypothesized that it is the local environment that dictates the cell types into which they differentiate. The
description of the other mesoderm-derived cell types into which SKPs differentiated, such as perivascular cells, adipocytes, and possibly periosteum cells, depending on their tissue location is presented in Chapter 3. These findings prompt a consideration of the exciting possibility of using SKPs to treat certain skeletal defects. The discussion of these results and their implications is then presented in Chapter 5.

1.1 Neural crest cells

Neural crest cells are multipotent embryonic cells that migrate extensively in the embryo to contribute to the formation of a number of organs and structures. Early during embryogenesis, at the gastrulation stage, three cell layers form: ectoderm, endoderm and mesoderm. The ectoderm gives rise to the nervous system and the skin, the endoderm generates the organs associated with the digestive and respiratory systems, and the mesoderm forms most of the skeleton, the circulatory system and the conjunctive tissues. A strip of ectoderm along the anterior-posterior axis located just above the notochord forms the neural plate. The neural plate folds over itself to generate the neural tube. During this process the ectoderm on each side of the neural plate gives rise to the neural folds, also referred to here as the neural crests, that eventually fuse, resulting in the formation of a neural tube overlaid by a continuous ectoderm that gives rise to skin (Knecht and Bronner-Fraser, 2002). A subset of the dorsal neuroepithelial cells of the neural tube, that are in close proximity to the ectoderm, leave the neural tube and migrate into a variety of regions throughout the embryo. These migratory cells are termed neural crest precursor cells or neural crest stem cells and have the capacity to differentiate into a large array of cell types.

Early work mapping the fate of neural crest cells was initially performed in the chick embryo either by tagging the cells with a dye or by using the quail/chick chimera model. In this model, neural crest cells from a quail embryo are transplanted into a chicken host, or vice versa, at the time that the host neural crest cells normally migrate. The host and transplanted cells are distinguished in vivo based on their nuclear morphology. This technique demonstrated that neural crest cells migrate to various tissues and differentiate into the different cell types that contribute to the formation of the organ or structure appropriate to the migration site. Their migration route and the cell types they will generate are determined by their original position along the rostrocaudal axis. For example, the cephalic neural crest cells give rise to most mesodermal derivatives of the face, facial bone and cartilage, smooth muscles/pericytes of the
head blood vessels, and the dermis including the adipocytes (Creuzet et al., 2005; Etchevers et al., 2001); the vagal neural crest cells (somite 1-7) which are located posterior to the cephalic neural crest cells give rise to the neurons and glia of the enteric nervous system (Crane and Trainor, 2006) and also contain the cardiac neural crest cells, which contribute to the cardiac ganglia, the aorticopulmonary septum, and aortic smooth muscles (Kirby et al., 1983; Kirby and Stewart, 1983); the dorsal neural crest cells (from somite 8 to 28) give rise to the neurons and glia of the peripheral nervous system, melanocytes and adrenal medulla (Le Douarin et al., 2004); and the sacral neural crest cells (rostral to somite 28) contribute to the enteric nervous system (Crane and Trainor, 2006). Based on the work showing that they have the capacity to self-renew and differentiate into a variety of cell types (Fraser and Bronner-Fraser, 1991; Stemple and Anderson, 1992), they have been named neural crest stem cells, NCSCs (See section 1.1.3).

1.1.1 Neural crest cell markers

A series of markers have been identified and used to describe neural crest cells. It is important to note that most of them are not exclusive to neural crest cells. One of the first markers shown to immunostain neural crest cells was the yet to be characterized glycoprotein epitope recognised by the HNK1 and NC1 antibodies (Tucker et al., 1984). One limitation of this marker is its temporal expression; for example, it is only transiently expressed by some cephalic neural crest cells, those giving rise to the ectomesoderm. The second limitation of this marker is its specificity. HNK1 immunoreactivity has been observed in other tissues such as the CNS, periosteum, perichondrium, kidney tubules and digestive tract (Le Douarin and Smith, 1988). Nonetheless, this marker is used to identify the neural crest cells at the early stages of migration, at which time the other immunoreactive tissues have not yet formed or do not express the epitope (Le Douarin and Smith, 1988).

Multipotent self-renewing neural crest cells were shown to express the p75 neurotrophin receptor (p75NTR), in vitro (Stemple and Anderson, 1992). These cells also expressed nestin (Stemple and Anderson, 1992), a marker for neural precursor cells. The expression of p75NTR is not limited to neural crest cells as other cell types, such as neuroblasts and neurons, express this receptor. A more specific marker for neural crest cells is Wnt1, a secreted morphogenetic
ligand that exerts multiple roles during development and in pathologies (Hausmann et al., 2007; Katoh, 2007). Expression of Wnt1 was found in neural crest cells in vivo using the reporter gene LacZ under the control of the Wnt1 promoter (Echelard et al., 1994). The expression of this marker was used in a genetic recombination strategy to map the neural crest derivatives in the mouse embryo (see below). Sox1 was reported as another specific marker for neural crest cells (Takashima et al., 2007) and to mark neuroepithelial cells that give rise to neural crest cells (Aubert et al., 2003; Pevny et al., 1998). It remains to be shown whether Sox1 and Wnt1 mark the same population of neural crest cells. The expression of a number of genes can also be used as markers for at least a subpopulation of neural crest cells. These include Pax3, Twist, snail, slug, Hoxa-3, Crabp1, FoxD3, Prx1 and Prx2. Some of these genes are also expressed in another type of precursor cell, SKPs, discussed in this introduction (see Section 1.6). The next section focuses on those markers with relevance to SKPs.

**Pax3** is a member of the paired box gene family that is expressed, at the mRNA level, in the dorsal portion of the neural tube, the cephalic neural crest, the cranio-facial neural crest derivatives, the developing dorsal root ganglia, the undifferentiated mesenchyme of the forelimbs and hindlimbs and transiently expressed in the dermomyotome (Goulding et al., 1991). *Pax3* was shown to be expressed by cranial neural crest cells that also express the markers Hoxa-3, Crabp1, Prx1, Prx2, and c-met (Conway et al., 1997).

**Twist** mRNA is expressed in a wide range of embryonic tissues including the neural tube, pre-somitic mesoderm, epithelial somites and anterior mesoderm. However, Twist protein expression is more restricted and is present in cranial neural crest cells, branchial arches, limb-bud mesenchyme, somatic lateral plate, and the sclerotome and dermatome of the somites (Gitelman, 1997). *Twist* expression is required for the proper patterning of cranial neural crest cells, and the migration and differentiation of both cranial and cardiac neural crest cells (Soo et al., 2002; Vincentz et al., 2008). In addition, Twist, which is a member of the basic-helix-loop-helix (bHLH) transcription factor family, can activate the expression of another neural crest cell associated marker, *snail*.

**Snail** is a DNA binding protein containing zinc finger domains. In the mouse it is first expressed at the gastrulation stage in both the mesoderm and the primitive ectoderm (Nieto et al., 1992; Smith et al., 1992). Later, *snail* expression is found in the mesodermal structures, the
sclerotome and myotome, and also in premigratory and migratory neural crest cells (Nieto et al., 1992; Smith et al., 1992). It is interesting to note that this expression of *snail* is species specific as in the chicken, premigratory and migratory neural crest cell do not express *snail*, with the exception of a subpopulation of migratory hindbrain neural crest (Sefton et al., 1998). In the mouse, *snail* is also expressed in mesenchymal condensates originating from either the mesoderm or the neural crest that will give rise to cartilage or bone, and in the developing whisker follicle (Smith et al., 1992).

**Slug** is a zinc finger protein of the Snail family, also known as Snail2. In the chick embryo, *Slug* is first expressed in the cells flanking the primitive streak (one of the first structures formed during gastrulation) and then continues to be expressed in the more lateral mesoderm (Nieto et al., 1994). At a later stage of development, when the neural crests begin to form, *slug* expression is observed in the ridges at the edges of the neural plate and marks the premigratory and migratory neural crest cells (Nieto et al., 1994). These neural crest cells expressing *slug* are also immunoreactive for HNK1 (Nieto et al., 1994). *Slug* expression is required for the proper closure of the hindbrain and cervical neural tube, the epithelial-mesenchymal transition and the migration of neural crest cells (Nieto et al., 1994). In the mouse, *slug* expression marks migrating neural crest cells and some mesodermal structures found in the eye, the ventral part of the somites and in the limb buds (Sefton et al., 1998). It is important to note that the expression patterns of *slug* and *snail* differ in the mouse and chicken. In the mouse, the expression pattern of *snail* corresponds to that of *slug* in the chicken and *vice versa* (Sefton et al., 1998). One exception to this is the expression of *slug* in the developing eye, which is seen in both mouse and chicken (Sefton et al., 1998).

**Group E Sox** genes are expressed in the chicken, mouse and human neural crest (Hong and Saint-Jeannet, 2005). This group is composed of *Sox8*, *Sox9*, and *Sox10*. While these *Sox* genes play an important role in neural crest cells, it must be noted that their expression is not restricted to the neural crest. For example, Sox9 mRNA is expressed in chondrocytes (Wright et al., 1995) and in gonads (Zhao et al., 1997). In the chicken, Sox8 is expressed in many structures of the embryo and its expression in neural crest cells overlaps that of Sox10 (McKeown et al., 2005). The specific role of Sox8 remains to be determined as the mouse null for Sox8 does not display any developmental abnormalities other than a reduced weight (Sock et al., 2001). This
apparent lack of severe phenotype has been proposed to be due to a functional redundancy between the various members of the group E Sox genes (Sock et al., 2001).

In mouse and chicken, Sox9 is expressed in premigratory neural crest cells and is down-regulated during their migration (Cheung and Briscoe, 2003; Cheung et al., 2005; McKeown et al., 2005; Wright et al., 1995). Sox9 is then observed in prechondrogenic tissue of the somatic sclerotome and in the notochord (McKeown et al., 2005; Zhao et al., 1997). The role of Sox9 in the neural crest was further elucidated by experiments in which Sox9 was over-expressed or down-regulated. Ectopic expression of Sox9 in the neural plate of both chicken and mouse induced the differentiation of neuroepithelial cells into neural crest-like cells, as assessed by staining for HNK1, but was not sufficient to induce the migration of these cells (Cheung and Briscoe, 2003; Cheung et al., 2005). Sox9 expression colocalises with the neural crest cell markers FoxD3, Slug (in chicken) and Wnt1. Interestingly, forced expression of the combination of Sox9, FoxD3 and Slug in chicken neuroepithelial cells resulted in the generation of neural crest-like cells and the migration of these cells, whereas none of these markers alone could generate this phenotype (Cheung et al., 2005). The importance of Sox9 in neural crest cells is further demonstrated by the severe phenotype of the Sox9 loss-of-function transgenic mouse. The dorsal neural crest cells of this animal fail to migrate to certain sites such as the sympathetic ganglia, in part due to an increase in apoptosis among the premigratory neural crest cells (Cheung et al., 2005). This phenotype was not as pronounced in the cephalic neural crest cells but apoptotic cells were also observed along the migratory route of these anterior neural crest cells (Cheung et al., 2005). This suggests that Sox9 plays an important role in either the differentiation of neuroepithelial cells into neural crest cells or in their survival depending on their position along the anterior-posterior axis.

Sox10 is expressed in premigratory neural crest cells just before they start to migrate (McKeown et al., 2005). Sox10 expression is mainly confined to the trunk neural crest cells with the exception of transient expression in the cranial neural crest cells populating the pharyngeal arches (Hong and Saint-Jeannet, 2005). Consistent with this expression pattern, humans, mice and zebrafish expressing a defective Sox10 suffer from aberrations of vagual and trunk neural crest derivatives such as the absence of adrenal chromaffin cells, and a decrease in the size of the enteric and paravertebral sympathetic ganglia (Hong and Saint-Jeannet, 2005; Kapur, 1999). However, Sox10 is dispensable for the generation of the neural crest-derived craniofacial
structures and the heart (Kapur, 1999). In mice lacking Sox10 expression, the neural crest cells generated undergo apoptosis as they leave the neural tube (Kapur, 1999). This suggests a pro-survival role for Sox10 in these cells. Forced expression of Sox10 demonstrated the important role of this Sox protein in the maintenance of the glial and neural differentiation potential of neural crest cells \textit{in vivo} and in the prevention of premature cell differentiation (Kim et al., 2003; McKeown et al., 2005).

1.1.2 Neural crest derivatives in mouse

The use of neural crest cell-specific markers such as Wnt1 and Sox1 in combination with the Cre-lox technology, allowed the mapping of the fate of neural crest cells in the mouse. In these experiments, Cre recombinase was expressed under the control of the Wnt1 or Sox1 promoter in knock-in animals. These animals were crossed with ROSA26R (R26R) animals in which the reporter gene LacZ is normally silenced by an element flanked by loxP sites. Following the Cre-mediated excision of the silencer, the cells and their progeny express LacZ in a constitutive manner. In the Wnt1-Cre:R26R compound mouse, all neural crest-derived cells are labelled by their expression of LacZ. Analysis of these animals revealed that mouse neural crest cells contribute to the same embryonic structures as chick neural crest cells. In particular, they contribute to the facial skeleton, the facial dermis and portions of the whisker follicles, the dorsal route ganglia, the peripheral nervous system (neurons and glial cells), melanocytes, the adrenal medulla, and the smooth muscles of the aorta (Chai et al., 2000; Jiang et al., 2002; Jiang et al., 2000; Sieber-Blum and Grim, 2004). Using the Sox1-Cre mouse, neural crest cells were also shown to contribute to the first wave of bone-derived mesenchymal stem cells (Takashima et al., 2007) (See section 1.5.4).

1.1.3 Neural crest cells are multipotent

Neural crest cells located at discrete positions along the rostrocaudal axis differentiate into distinct cell types. This raises the question of how cell fate determination occurs. Either a cell autonomous mechanism or signals present in the local environment may dictate a cell’s fate. Experiments in which neural crest cells from a more rostral location were transplanted to a more caudal position, and \textit{vice versa}, were performed using the quail-chick chimera model to address this question. Results of these experiments combined with those of co-culture explants showed that the fate adopted by neural crest cells is in part dependent on their environment, including the
location they migrate from, the target tissue to which they migrate and the path of migration (Le Douarin, 1980; Le Douarin and Smith, 1988). One of the exceptions to this rule are the cranial neural crest cells that, when transplanted at more caudal locations, retain their capacity to generate cartilage in the dermis and kidney of host embryos (Couly et al., 1998; Le Douarin and Smith, 1988). Another example of an exception is that neural crest cells derived from locations posterior to rumbomere 3 cannot form facial skeleton regardless of transplant location. The set of *hox* genes expressed in these more posterior neural crest cells prevents them from generating the facial skeleton and membranous bone (Couly et al., 1998; Creuzet et al., 2005; Kanzler et al., 1998). The conclusion of these *in vivo* experiments was also supported by a series of experiments in which clonal cultures of neural crest cells were differentiated *in vitro*. These data suggested that neural crest cells are, at least in culture, a heterogeneous mixture of cells with different degrees of multipotentiality (Le Douarin and Smith, 1988). Supporting this conclusion, Stemple and Anderson, showed that clonal cultures of rat neural crest cells can give rise to clones that self-renew, that these secondary clones can in turn generate clones that yield cells with either multipotent or restricted differentiation capacity, and that it is the substratum on which these cells grow that will influence their fate (Stemple and Anderson, 1992). This work and the *in vivo* clonal analysis by Fraser and Bronner-Fraser, show that at least a fraction of the neural crest cells that migrate from the neural crest are, at least initially, multipotent and that their subsequent environment can instruct their differentiation potential (Fraser and Bronner-Fraser, 1991).

### 1.1.4 Cell therapy potential

The potential of neural crest cells to self-renew and to generate a multitude of different cell types suggest that they would provide a good source of cells for cell-based therapies. However, neural crest cells are only transiently present in the embryo. This makes their efficient isolation a major impediment to their use as a source of therapeutic cells. However, in recent decades, other adult stem cells have been identified that have similar or more limited potential and may be more readily isolated from their respective niches (see sections 1.5 and 1.6).

### 1.2 Skeletogenesis

Cranial neural crest cells generate mesodermal derivatives such as bone and cartilage in the ventral part of the head. Elsewhere in the body these mesodermal structures are derived from the mesoderm, which also gives rise to adipose and conjunctive tissues, and most of the vascular
system. The following sections will introduce the developmental formation of the bone and cartilage that make up the skeleton. Bone and cartilage formation can be separated into four steps. First the mesenchymal cells migrate to the site where skeletogenesis will take place. Second, the newly arrived mesenchymal cells interact with the overlaying epithelial cells, a process which induces mesenchymal condensation. Third, the process of mesenchymal condensation results in the initiation of the formation of skeletal elements. Fourth, the condensed cells overtly differentiate into skeletal cells, the chondrocytes and osteoblasts. Since the purpose of this discussion is to give an overview of the mechanisms involved in chondrogenic and osteogenic differentiation, it will focus on the last two steps, mesodermal condensation and overt differentiation.

1.2.1 Mesodermal condensation

Mesodermal condensation occurs once mesodermal and cranial neural crest cells have migrated to the sites where they will form bone or cartilage. This process, not unique to skeletogenesis, is also observed during organogenesis, and is very important for the reprogramming or fate determination of the cells present in these condensates. In skeletogenesis, it results in the generation of chondrocytes and osteoblasts. Mesodermal condensation is characterized by an increase in cell density as cells draw close together, without an increase in cell division (Hall and Miyake, 1995). The size of the condensate is important, if it is too small no bone will form, but if it is too large extraskeletal structures will form (Smith and Schneider, 1998). This increase in cell density is associated with an increase in cell-cell adhesion and the formation of gap junctions. In the context of skeletogenesis, the end result of this condensation is the chondrogenic or osteogenic differentiation of the cells in the condensate. It is not yet clear which signals dictate the differentiation pathway that the mesenchymal cells will follow, but osteogenic commitment has been suggested to occur prior to condensation, during intramembranous ossification (Hall and Miyake, 1995) (Section 1.3.1).

Recent advances in the elucidation of the molecular mechanisms involved in mesenchymal condensation have revealed an important interplay of a number of growth factors such as TGF-β, and BMPs, extracellular matrix proteins such as fibronectin, versican, tenascin, adhesion molecules like N-CAM and N-Cadherin and transcription factors that include Pax-1, Pax-9, Hoxa-2, Prx-1, Prx-2, (Hall and Miyake, 2000). This discussion focuses on the roles of
TGF-β and BMPs, and some of their downstream targets in this process. TGFβ is important in the initial stage of condensation as it induces the expression of the extracellular matrix proteins fibronectin and tenascin and the cell adhesion proteins N-CAM and N-Cadherin (Chimal-Monroy and Diaz de Leon, 1999).

N-Cadherin has been reported to be expressed first and then followed by N-CAM in embryonic chicken mesenchymal cells differentiating into chondrocytes (Tavella et al., 1994). The functional importance of these adhesion molecules was demonstrated by the results of loss-of-function experiments. Interference with N-CAM binding, using a Fab fragment of an antibody generated against N-CAM, reduced chondrogenesis severely but had a less pronounced effect on pre-chondrogenic condensation (Widelitz et al., 1993). In contrast, over-expression of N-CAM resulted in a larger condensate size (Widelitz et al., 1993). Similarly, experiments using a function-blocking antibody showed that N-Cadherin binding is important for the first 24 hours of condensation while the presence of this antibody had a minimal effect on chondrogenesis (Oberlender and Tuan, 1994). These observations suggest that N-cadherin is associated with the initiation of cell-cell adhesion while N-CAM serves in the maintenance of the adhesion (Hall and Miyake, 2000). However, its role in condensation remains controversial as mice null for N-CAM develop a normal skeleton (Cremer et al., 1994). These apparently conflicting results may be due to a compensation mechanism, possibly involving N-cadherin which is also expressed at that time.

In addition to its function in the process of mesenchymal condensation, TGF-β plays another important role in the initiation of chondrogenesis: it can induce the expression of the chondrogenic gene Sox-9 (Chimal-Monroy et al., 2003). Experimental evidence suggests that TGF-β and BMP2, factors also implicated in chondrogenesis, exert their regulatory effects at complementary but distinct levels in the process of chondrogenic differentiation. In support of this idea is the observation that insertion of a TGF-β soaked bead in the embryonic chicken leg bud resulted in the formation of ectopic cartilage and in the stimulation of the expression of the BMP receptor 1B (BMPR1B) in the differentiating cells (Chimal-Monroy et al., 2003). This is consistent with a model where TGF-β can induce the ability of these pre-chondrocytes to response to BMP signalling. A second piece of evidence comes from the characterisation of the conditional knock-out mouse for BMPR1A and BMPR1B in collagen type-II expressing cells.
that are chondroprogenitor cells. These cells showed delayed and reduced differentiation of chondrocytes as well as a decrease in their proliferation and survival (Yoon et al., 2005). This phenotype shows that BMP signalling is important, at least in part, for the survival and maturation of these chondroprogenitors into pre-chondrocytes and more mature chondrocytes. The molecular analysis of the condensates in this mouse suggests that BMP signalling is required for the induction or the maintenance of the expression of the chondrogenic genes Sox9, Sox5 and Sox6 in pre-chondrocytes (Yoon et al., 2005) (See section 1.2.2.1). To test whether TGF-β is important in the initiation of cartilage formation, Ito and colleagues looked at the requirement of TGF signalling in the formation of cranial cartilage (Ito et al., 2003b). They analysed the phenotype of the mouse with conditional knock-out for TGF-β signalling in cranial neural crest cells (Wnt1-cre/TGFR-II<sup>fl<sup>ox/flox</sup></sup>). This mutant mouse has mandible and cranial bone defects (Ito et al., 2003b); cell proliferation was decreased and cartilage was not induced in the mandible anlagen, though bone matrix was present (Ito et al., 2003b; Oka et al., 2008). These results are consistent with the role of TGF-β signalling in the initiation of the formation of cartilage. Analysis of the type-II collagen-cre/TGFR-II<sup>fl<sup>ox/flox</sup></sup> mouse, in which all TGF-β signalling is blocked in chondroprogenitor cells, showed mild defects in long bone formation but severe defects in two skull bones and in the vertebrae (Baffi et al., 2004). This phenotype is consistent with the conclusion that the loss of TGF-β signalling in cells already committed toward the chondrogenic fate, chondroprogenitor cells, does not block their maturation into more mature chondrocytes, which are required to form long bones. This phenotype also suggests that TGF-β may play distinct roles in the formation of different bone and cartilage structures. It is important to note that TGF-β is also implicated in joint morphogenesis (Spagnoli et al., 2007), and thus critically involved in multiple steps of skeletal development. Taken together the results of these studies suggest that TGF-β signalling is more important at the initiation stage of chondrogenesis, in pre-condensation or condensing cells, and that BMP-2 signalling is more potent in chondroprogenitors or pre-chondrocytes; and that signalling from both TGF-β and BMP2 are important for the proper differentiation of these mesenchymal cells into chondrocytes.

1.2.2 Skeletal cell types

The net result of mesenchymal condensation is the differentiation of mesenchymal cells into chondrocytes and osteoblasts. These two cell types are implicated in the formation of bones, a
process which may occur by two different mechanisms. One mechanism is *intramembranous ossification*, which is characterised by the direct formation of bone matrix by osteoblasts derived from the mesenchymal condensates. The second mechanism is *endochondral ossification*, which is characterised by the formation of a cartilage mould that is then replaced with bone matrix. Three bone cell types are involved in this process, chondrocytes, osteoblasts and osteoclasts, also referred as chondroclasts. Below is a short review of the roles, markers and differentiation typical of these three cell types.

### 1.2.2.1 Chondrocyte differentiation

Chondrocytes are the cartilage forming cells. They are derived from mesenchymal cells originating from either the mesoderm or the neural crest cells that underwent mesenchymal condensation. Two fates can be adopted by chondrocytes: their maturation program may arrest and they then generate joint cartilage or they may differentiate into hypertrophic chondrocytes that play a critical role in endochondral ossification. One very important gene regulating chondrogenesis is *Sox9*. Its expression, essential for the induction of other chondrogenic genes, can be modulated by at least three signalling cascades. A detailed discussion of all the signalling cascades and mechanisms by which Sox9 expression is regulated, and how it in turn regulates chondrogenesis is beyond the scope of this introduction. This section focuses, rather, on data supporting the conclusion that Sox9 is required for chondrogenesis and that its expression is regulated by a number of extracellular molecules.

Sox9 is one of the earliest genes expressed during chondrogenesis, its expression is detected prior to and during cartilage matrix formation (Wright et al., 1995). The requirement for Sox9 for chondrogenesis is supported by at least three lines of evidence. First, analysis of the Prx1-cre:*Sox9*\textsuperscript{flox/flox} mouse, in which Sox9 is conditionally knocked-out in condensing mesenchymal cells expressing Prx1, revealed that no cartilage formed in the limbs of this compound mouse (Akiyama et al., 2002). Second, characterisation of the mouse chimeras made by the injection of embryonic stem cells null for Sox9 (*Sox9*\textsuperscript{−/−}), heterozygous for Sox9 (*Sox9*\textsuperscript{+/−}) or wildtype (*Sox9*\textsuperscript{+/+}) into wildtype blastocysts, revealed important differences. Sox9\textsuperscript{−/−} cells were present in mesenchymal condensates, but did not adopt a round morphology and were not part of cartilage structures, while Sox9\textsuperscript{+/−} and wildtype cells were present in these structures (Bi et al., 1999). Last, in a teratoma formation assay, in which the ability of these embryonic stem cells to
form cartilage was assayed in vivo, no cartilage structures were observed with the \( \text{Sox9}^{-/-} \) cells. In contrast, cartilage was seen in teratomas formed by the wildtype and \( \text{Sox9}^{+/-} \) cells (Bi et al., 1999). Taken together these reports argue convincingly for a requirement of \( \text{Sox9} \) in chondrogenesis.

Consistent with this conclusion, \( \text{Sox9} \) was shown to be important in the regulation of many genes involved in chondrogenesis. For example, \( \text{Sox9} \) is required for \( \text{Sox5} \) and \( \text{Sox6} \) expression (Akiyama et al., 2002) and these three \( \text{Sox} \) genes are also implicated in the expression of two cartilage matrix proteins, aggregan and type-II collagen (Han and Lefebvre, 2008; Lefebvre et al., 1997; Lefebvre et al., 1998). Regulation of the expression of \( \text{Sox9} \) is required to ensure the differentiation of mesenchymal cells into chondrocytes, and for their maturation into hypertrophic chondrocytes. The hypertrophy of chondrocytes is important for endochondral ossification. Forced expression of \( \text{Sox9} \) demonstrated that its levels must be down-regulated to permit this differentiation program (Zhao et al., 1997). A 20% increase in mRNA levels resulted in a decreased chondrocyte proliferation, and a delayed in both chondrocyte hypertrophy and endochondral ossification (Akiyama et al., 2004a). Thus, the temporal expression of \( \text{Sox9} \) must be precisely regulated for the proper development of cartilage and ossification.

The TGF-\( \beta \), BMPs, and Wnt/\( \beta \)-catenin signalling cascades have been shown to be important in the regulation of \( \text{Sox9} \) expression. TGF-\( \beta \) is normally expressed at the time of mesenchymal condensation in limb buds. Treatment of limb buds with TGF-\( \beta \) induces the expression of \( \text{Sox9} \) within 30-60 minutes of treatment, and induces cartilage formation (Chimal-Monroy et al., 2003). This very short delay in \( \text{Sox9} \) induction, suggests that it is a direct target of TGF-\( \beta \) signalling (Kawakami et al., 2006). BMP signalling is also important for cartilage differentiation. Conditional loss of BMPR1A and BMPR1B in type-II collagen expressing cells resulted in chondrodysplasia (Yoon et al., 2005). In this mouse, the differentiation, proliferation and survival of chondrocytes were decreased (Yoon et al., 2005). The mutant cells expressed very low to undetectable levels of \( \text{Sox9}, \text{Sox5}, \text{Sox6}, \) and the cartilage matrix proteins type-II collagen, link protein and aggregan (Yoon et al., 2005). This demonstrates that BMP signalling is required for the complete differentiation of chondroprogenitor cells. A third mechanism by which chondrogenesis is regulated is that of the Wnt/\( \beta \)-catenin pathway. \( \beta \)-catenin has been shown to physically interact with the \( \text{Sox9} \) protein, which resulted in their degradation (Akiyama
et al., 2004b). This caused a decreased in Sox9 activity and in reduced differentiation into chondrocytes (Akiyama et al., 2004b). Thus, the temporal regulation of these three signalling cascades may ensure the appropriate regulation of Sox9 during both endochondral ossification, which involves the hypertrophy of chondrocytes, and the formation of joint cartilage for which hypertrophy must be avoided.

The mechanisms involved in the formation of joint cartilage have not been fully characterized but a requirement for the presence of TGF-β, GDF-5 and a truncated form of the ets related gene (ERG) named C-1-1, has been demonstrated. TGF-β is important for the initiation of joint cartilage formation as it can induce GDF-5 expression, whereas deletion of TGF-β signalling abrogates the process (Spagnoli et al., 2007). GDF-5 is present at the location of joint formation and insertion of beads soaked with GFD-5 in developing mouse limb buds induce the expression of ERG, possibly C-1-1, within six hours (Iwamoto et al., 2007). C-1-1 expression also localises to the joints (Iwamoto et al., 2001; Iwamoto et al., 2000), and its over-expression prevents hypertrophy of mouse and chicken chondrocytes in vitro and in vivo, and induces the expression of the joint cartilage marker, tenacin-C (Iwamoto et al., 2000; Iwamoto et al., 2007). In contrast, the forced expression of the full-length form of ERG in cultured chondrocytes, induced their maturation as characterised by the expression of the hypertrophic chondrocyte marker ALP and the formation of mineral deposits (Iwamoto et al., 2001; Iwamoto et al., 2000). Taken together, these data suggest a key role for a TGF-β – GDF-5 – C-1-1 pathway in the determination of joint chondrocyte identity.

During endochondral ossification, chondrocytes initiate a program of terminal differentiation; they cease proliferation; they change their gene expression profile; and they start to enlarge (hypertrophy). The changes in the gene expression include the down-regulation of chondrocyte-associated genes such as those encoding Sox9 and COL2A1 (type-II collagen), and the up-regulation of hypertrophic chondrocyte- and osteoblast-associated genes which include Dlx5, Dlx6, SHOX/Shox2, MMP13, the hypertrophic chondrocyte specific marker type-X collagen, and the osteogenic gene Runx2 (Solomon et al., 2008). The osteogenic markers ALP and osteopontin, and the angiogenic factor vascular endothelial growth factor (VEGF) are also expressed by hypertrophic chondrocytes (Gerstenfeld and Shapiro, 1996; Leboy et al., 1989; Solomon et al., 2008). One net result of this change in gene expression profile is the
mineralization of the cartilage matrix and the recruitment of blood vessels. Proliferation and hypertrophy of chondrocytes is well regulated by a number of factors including parathyroid hormone-related protein (PTHrP) and Indian hedgehog (Ihh), and will be presented in details below (Section 1.3.2).

1.2.2.2 Osteoblast and osteocyte differentiation

Osteoblasts are the cells that synthesise the collagen-rich matrix that undergoes mineralisation to generate the actual bone tissue. In the formation of flat bones such as those of the face, osteoblasts originate from the osteogenic differentiation of cells of the mesenchymal condensate (Hall and Miyake, 2000). Osteoblasts are also generated by osteoprogenitor cells present in the periosteum, a layer of connective tissue composed of several different cell types that constitutes an envelope around the outer surface of the bone tissue (Augustin et al., 2007).

Historically, the first osteoblast-specific marker to be recognised was osteocalcin. Characterisation of the regulation of the osteocalcin gene identified the transcription factor Cbfa1/Runx2 that can bind to regulatory elements in the promoters of all major genes expressed by osteoblasts (reviewed in (Ducy, 2000)). Cbfa1/Runx2 is required for osteogenesis, as the Cbfa1/Runx2 knock-out mouse had no mineralized bones, and osteoblasts appeared immature and expressed low levels of alkaline phosphatase and undetectable levels of osteopontin and osteocalcin (Komori et al., 1997; Otto et al., 1997). It is important to note that, in these animals, the cartilage present in the long bones appeared normal. This study demonstrated the requirement of Runx2 for the differentiation of mesenchymal cells into mature mineralizing osteoblasts. Moreover, forced Runx2 expression is sufficient to induce the osteoblast phenotype in fibroblasts (Ducy et al., 1997). One downstream effector of Runx2 is the transcription factor Osterix, which is also necessary for osteoblast differentiation and for the expression of many osteoblast genes (Cohen, 2006).

A number of transcription factors and signalling molecules have been identified as regulators of Runx2 expression or function (Marie, 2008). Sox9, Sox8 and the Hox homeodomain transcription factor Hoxa2 negatively regulate the expression or function of Runx2, whereas β-catenin, BMP-2, TGF-β, PTH, and glucocorticoids are positive regulators of Runx2 expression and function (Marie, 2008). While a complete discussion of all the various regulators of Runx2 function is beyond the scope of this introduction, it is interesting to note that
those having a positive regulatory effect on chondrogenesis have negative effects on the osteogenic differentiation program and *vice versa*.

Runx2 expression results in the induction of many osteogenic genes, including osteocalcin, osteopontin, bone sialoprotein (BSP), and type-I collagen (Ducy et al., 1997). The expression profile of these genes correlates with, and is used as a marker for the maturation stages of the differentiating osteoblasts. Type-I collagen is the earliest osteoblast marker, followed by ALP, and then by osteopontin as the pre-osteoblast matures into an osteoblast, while osteocalcin is expressed by mature osteoblasts (Aubin et al., 1995; Liu et al., 1994). *In vitro*, expression of osteocalcin coincides with the presence of mineral deposits. Osteoblasts also express many more extracellular matrix proteins, receptors, and secreted proteins (Aubin et al., 1995; Franz-Odendaal et al., 2006) that may play important role in the regulation of osteoblast differentiation.

The main sequence of events during the differentiation of mesenchymal cells into osteocytes, the most mature bone cells, has been elucidated (Aubin et al., 1995). It starts with the differentiation of mesenchymal cells into osteoprogenitor cells, which express type-I collagen. These differentiate into pre-osteoblasts that maintain proliferative capabilities and express ALP, type-I collagen, osteonectin, and the PTH/PTHrP receptor. Pre-osteoblasts then differentiate into active osteoblasts, which have exited the cell cycle, express ALP, osteopontin, osteocalcin as well as type-I collagen, and induce the mineralization of the extracellular matrix (Franz-Odendaal et al., 2006). As the osteoblasts secrete bone matrix proteins, about 10-20% of them become trapped in the resulting matrix and differentiate into osteocytes (Aubin, 1998). In the mature bone, there are approximately ten times more osteocytes than osteoblasts (Franz-Odendaal et al., 2006). Osteocytes form gap junction connections with one other and with the overlaying osteoblasts, ensuring the supply of nutrients from nearby blood vessels to the cells embedded into the bone matrix. Osteocytes are replaced during bone turnover which occurs at a rate of 4-10% a year in humans (Franz-Odendaal et al., 2006).

**1.2.2.3 Osteoclast differentiation**

Osteoclasts are multinucleated cells of hematopoietic origin that are specialized in bone-resorption (Suda et al., 1992). The hematopoietic precursor of osteoclasts is the granulocyte-macrophage colony-forming cell (CFU-GM), which also gives rise to macrophages (Menaa et
The differentiation of osteoclast-committed precursors and their fusion to generate multinucleated osteoclasts is regulated by the receptor activator of NF–κB (RANK) ligand (RANKL) protein, a member of the TNF family. Other factors such as 1α,25-dihydroxyvitamin D₃, interleukin-11 (IL-11), and prostaglandins (PGE₂) induce the expression of RANKL on the surface of immature osteoblasts and bone marrow cells thereby prompting the differentiation of the hematopoietic osteoclast precursor cells into osteoclasts (Roodman, 2006). RANKL is also important for the activation of osteoclasts. Their differentiation and activation are negatively regulated by expression of the decoy receptor osteoprogerin (OPG) which binds RANKL and prevents its interaction with the RANK receptor (Roodman, 2006). Another inducer of osteoclasts is IL-6 via the expression of IL-1β (Kurihara et al., 1990), which promotes the differentiation, multinucleation, activation and survival of osteoclasts (reviewed in (Nakamura and Jimi, 2006)).

Osteoclasts can be identified histologically by their multinucleated phenotype and by their marked staining for tartrate-resistant acid phosphatase (TRAP) (Suda et al., 1992). One characteristic organelle of osteoclasts is the “ruffled border”, a complex structure composed of finger-like folds of the plasma membrane found adjacent to the bone surface and surrounded by a clear zone (Suda et al., 1992). The clear zone, or the sealing zone, is a smooth area of the plasma membrane that makes contact with the bone forming a seal around the ruffled border. Within this sealed “pocket”, protons and proteolytic enzymes are secreted which acidify the local environment, resulting in demineralization of the bone, and degradation of the bone matrix proteins (Vaananen et al., 2000). This function of osteoclasts is important for bone formation, remodelling, turn-over, and fracture repair. Loss of osteoclast function can result in osteopetrosis which is characterized by an increase in susceptibility to fractures, dental malformation, visual and hearing problems and, in more severe cases, increases in bone mass resulting in the narrowing of the bone marrow cavity and severe haematological malfunction (Askmyr et al., 2008). Conversely, hyperactivation of osteoclast activity can result in decreased bone mass and osteoporosis.

1.3 Bone formation

Bone tissue can be formed by two types of ossification: intramembranous and endochondral. Newly formed bone subsequently undergoes bone modelling and remodelling. These two
processes are responsible for the shaping of the bone, increasing its mechanical strength and contributing to the normal bone tissue turn-over.

1.3.1 Intramembranous ossification

This type of ossification is responsible for the formation of flat bones, such as those of the face. In these locations, the cells present in the mesenchymal condensates differentiate into osteoblasts. The cells at the edge of the condensates form the periosteum, the connective tissue structure that envelops the bones and contains osteoprogenitor cells (Augustin et al., 2007). The newly differentiated osteoblasts immediately start producing the bone matrix and some of them become entrapped in this matrix to become osteocytes as described in section 1.2.2.2. The fates of the remaining osteoblasts include apoptosis or becoming bone-lining cells (Franz-Odendaal et al., 2006). The number of osteoblasts that become osteocytes has been estimated to be 10-20% (Aubin, 1998), while apoptosis is thought to eliminate approximately 60-80% of these osteoblasts (Jilka et al., 2007). In the fully formed bone, the osteocytes constitute 95% of the cells in the bone (Franz-Odendaal et al., 2006).

1.3.2 Endochondral ossification

Endochondral ossification differs from intramembranous ossification by the initial formation of a cartilage mould that is then replaced with bone matrix. This type of ossification is used to form most of the bones of the body. It starts with the differentiation of mesenchymal condensate cells into chondrocytes that secrete a cartilage matrix rich in type-II collagen and aggrecan. At the border of the condensate, the perichondrium is formed (Kronenberg, 2003), and at the centre, the chondrocytes further differentiate to become hypertrophic.

These hypertrophic chondrocytes then produce type-X collagen, the angiogenic factor VEGF and a number of osteoblast-specific proteins including ALP and osteopontin. This results in the mineralisation of the cartilage matrix and in the recruitment of new blood vessels to the avascular cartilage. Hypertrophic chondrocytes also signal to the cells of the perichondrium, layers of cells and connective tissue surrounding the cartilage, to induce their osteogenic differentiation, resulting in the “transformation” of the perichondrium into the periosteum (Kronenberg, 2003). The invasion of blood vessels into the mineralized cartilage mould results in the recruitment of osteoclasts and osteoblasts. The hypertrophic chondrocytes eventually
undergo apoptosis, whereas the osteoclasts digest the mineralised cartilage matrix and the osteoblasts lining the edge of the digested matrix where they produce the first true bone matrix, also called primary spongiosa or primary ossification (Kronenberg, 2006). At the centre of the primary spongiosa a cavity is formed. This becomes the marrow cavity and is colonised by bone marrow cells, including both the hematopoietic and mesenchymal stem cells. The mechanism by which mesenchymal stem cells colonise the bone marrow cavity remains to be determined. Two current theories propose either their transport from a distal source by the vascularisation of the bone, or the presence of residual precursor cells from the pool that generates the embryonic bone, that remain undifferentiated and subsequently colonise the bone marrow.

At the extremities of the forming bones, chondrocytes continue to proliferate and are organized into columns. These columns are oriented parallel to the direction of bone growth with the less differentiated chondrocytes at the end distal to the primary spongia. As these cells proliferate, thereby extending the columns, they flatten and hypertrophy. The ends of the columns proximal to the primary spongia are characterised by hypertrophic chondrocytes and ongoing endochondral ossification. The structure composed of these columns of chondrocytes is referred to as the growth plate. It is the continuous proliferation of the chondrocytes that generates the driving force of the longitudinal growth of the bone. In humans, these growth plates disappear at puberty and, as a consequence, the longitudinal growth of bone ceases. The length of the columns of hyperproliferating chondrocytes, as well as the location at which differentiation into hypertrophic chondrocytes occurs, is regulated by the interplay of Indian hedgehog (Ihh) and parathyroid hormone and parathyroid hormone related protein (PTH/PTHrP) signalling (Kronenberg, 2006). Ihh is expressed by pre-hypertrophic and hypertrophic chondrocytes, and indirectly induces the expression of PTHrP by certain chondrocytes and other cells of the perichondrium (Kronenberg, 2006). The net result of PTHrP signalling is to inhibit hypertrophy by inducing the stabilisation of Sox9 and suppressing the expression of Runx2 (Kronenberg, 2006).

1.3.3 Bone modeling and remodelling

The next step, following the formation of the new bone, is its modeling and remodelling. Bone modeling is the process of apposition or removal of bone matrix from different locations to define the appropriate shape of the bone and to ensure its mechanical strength. This permits a
large increase in bone strength with minimal gain in bone mass. This process normally occurs in children or following major changes in body weight or activity (Robling et al., 2006). *Bone remodelling* is the process of bone removal coupled with the synthesis of new bone at the same location. Bone remodeling is achieved by multicellular units composed of osteoclasts and osteoblasts. The osteoclasts “drill” a tunnel with a diameter of about 250-300 micrometers into the bone following the lines of mechanical stress (Hert et al., 1994). These tunnels are then filled with newly formed bone secreted by the osteoblasts which follow behind the osteoclasts. This structure composed of osteoclasts degrading the bone matrix at its leading edge and of osteoblasts subsequently forming new bone matrix is referred to as a “cutting cone” (Einhorn, 1998). The newly formed bone is called osteon and has a circular cross section (Robling et al., 2006). This process results in the formation of lamellar bone, which contributes to an increase in the mechanical strength of the bone, the turnover of the bone matrix, the removal of damaged areas and the maintenance of appropriate levels of serum ions (Robling et al., 2006).

1.4 Fracture repair

Bone formation is not limited to the embryo and the first decade of life. The skeleton is exposed to a series of mechanical stresses throughout an organism’s life, and in some cases the structural integrity fails. In contrast to injured cartilage that has a limited capacity for regeneration; bone tissue has the ability to repair itself following the incidence of a fracture. The injured bone can be repaired by either of two processes: primary or secondary healing (Einhorn, 1998). Primary healing is characterized by the direct formation of bone matrix by intramembranous ossification through a mechanism analogous to bone remodelling. Cutting cones generated on each side of the fracture traverse the site to restore the mechanical continuity of the bone (Einhorn, 1998). This type of bone healing occurs when there is no mechanical movement of the bone fragments such as, in the case of rigid fixation of the fracture site, a partial fracture, or micro cracks.

Secondary healing is characterized by the formation of bone matrix by the combination of both endochondral and intramembranous ossification (Einhorn, 1998). This type of healing is observed in cases where minimal movements and forces are applied to the fracture site, such as in most clinical settings when a fractured limb is stabilized by a cast. To study the different molecular and cellular mechanisms involved during the healing process, a rodent model of stable fracture has been generated (Greiff, 1978; Hiltunen et al., 1993). In this model the fractured tibia
or femur is stabilized by the presence of an intramedullary pin, which allows only minimal movements between the two fractured bone fragments. This type of fracture heals by secondary bone healing. This model has several advantages: the healing bone recapitulates the sequence of events that take place in the regeneration of structural integrity in a highly reproducible manner; this model reproduces molecular events that are also involved in human bone healing, and it allows the use of transgenic animals to characterize the underlying molecular mechanisms essential for proper healing. This model has enabled many groups to study the key cellular and molecular events involved in long bone healing. While many of these events may occur simultaneously and/or overlap in their timing, for simplicity they will be presented as four phases, the inflammatory response, the formation of the soft callus, the formation of the hard callus and the bone remodelling stage.

### 1.4.1 The inflammatory response

The initial event following the onset of bone fracture is the inflammatory response, which includes the formation of a hematoma, the release of numerous cytokines and the recruitment of immune cells. Monocytes have been observed to be associated with the hematoma at 24 hours post-fracture and macrophages are observed in the proximity of the periosteum at day two post-fracture (Bourque et al., 1993). Other immune cells present at the fracture site include polymorphonuclear leukocytes and lymphocytes (Bourque et al., 1993; Schindeler et al., 2008). One role of these cells is to clear potential infections. The formation of the hematoma results in the activation and degranulation of platelets, thereby releasing a cocktail of cytokines that includes PDGF (platelet-derived growth factor-αα, ββ and αβ isoforms), TGFβ, fibroblast growth factor (FGF), and IL-1 (Mehta and Watson, 2008). In addition, the transcription of many pro-inflammatory cytokines, growth factors and morphogens such as TGFβ1, BMP-2, BMP-5, GDF8, BMP-3b/GDF10, IL-1, IL-6, IL-11, OPG, RANKL, M-CSF, LT-β, prostaglandins and tumour necrosis factor-alpha (TNF-α) is upregulated at the fracture site (Cho et al., 2002; Einhorn, 1998; Gerstenfeld et al., 2003b; Kon et al., 2001) (Dekel et al., 1981). The presence of these cytokines plays a crucial role in proper bone healing as is clearly demonstrated by studies determining the role of TNFα, prostaglandins and cyclooxygenase during bone healing in rodents and human (see below).
TNF-α mRNA levels peak at 24 hours post-fracture, decrease to basal levels by day 7 and increase again by days 21 and 28, at which time osteogenesis is ongoing (Kon et al., 2001). Macrophages are among the first cells to express TNF-α in response to a fracture (Kon et al., 2001). At day 7 and 14 TNF-α expression is seen in osteoblasts, chondrocytes and hypertrophic chondrocytes. TNF-α signalling is important at multiple stages of the normal bone healing process as transgenic mice null for the TNF-α receptors p75 and p55 displayed impaired fracture healing (Gerstenfeld et al., 2003a). In these animals, chondrogenesis is delayed, formation of trabecular bone is decreased, apoptosis of hypertrophic chondrocytes is delayed and the invasion of osteoclasts, which are responsible for the digestion of calcified cartilage, is also delayed (Gerstenfeld et al., 2003a). It remains to be determined whether the actions of TNF-α in the initiation of bone repair are direct or indirect. TNF-α signalling could exert its effect through the induction of the signalling of other differentiation factors present at the fracture site.

Prostaglandins are also mediators of inflammation. The synthesis of these molecules is mainly controlled by the rate-limiting cyclooxygenases enzymes. Cyclooxygenase-1 (Cox-1) is constitutively expressed in many tissues, while cyclooxygenase-2 (Cox-2) expression is only induced following inflammation or injury, including following bone fracture (Gerstenfeld et al., 2003b). A role for Cox activity in bone repair was supported by the observation that the inhibition of Cox-1 and Cox-2 using nonsteroid anti-inflammatory drugs (NSAIDs), resulted in delayed union or non-union of the fractured fragments (Simon et al., 2002). Cox-2 seems to play a major role in this process as treatment with NSAIDs specific for Cox-2, and transgenic animals lacking Cox-2 expression, exhibited profound inhibition of appropriate bone healing with an increased frequency of non-union fractures and/or the absence of bone continuity at the fracture site (Simon et al., 2002; Zhang et al., 2002). It is also important to note that this effect of NSAIDs could be reversed by simply terminating the treatment (Gerstenfeld et al., 2007). These negative effects of NSAIDs on bone healing were also observed in humans (Giannoudis et al., 2000). Taken together, these observations clearly demonstrate that the inflammatory response plays a crucial role in the normal healing process of fractured bone.

The expression patterns of the many of the cytokines present at the fracture site are currently being elucidated. Some of them have very specific expression patterns, suggesting specific roles during bone repair. It is not surprising that a number of these factors are
implicated in the differentiation of chondrocytes, osteocytes and osteoclasts (Section 1.3.4). The expression of these cytokines falls into three broad patterns. The first is characterised by a peak of expression at 24 hours and a return to basal levels by day 3. Cytokines exhibiting this pattern include GDF8, TGFβ1, TNF-α, LTβ, IL-1β, IL-6 (Cho et al., 2002; Kon et al., 2001). The second pattern is bimodal, where the expression reaches a maximum at 24 hours, decreases by days 3-7 and is then up-regulated again at a later stage when osteogenesis is ongoing. BMP-2, RANKL and M-CSF follow this pattern (Cho et al., 2002; Kon et al., 2001). The third pattern is exemplified by the expression of BMP-3b/GDF10, and BMP5 which are up-regulated at 24 hours post-fracture and remained elevated throughout the healing process (Cho et al., 2002).

The presence of these cytokines and growth factors induces the recruitment and proliferation of precursor cells. The precursor cells involved in bone repair have been suggested to originate from four main sources, the periosteum, the endosteum, the surrounding soft tissues and vasculature (Schindeler et al., 2008). PDGF and TGF-β1 are potent cytokines that induce expansion of regenerating cells. For example, PDGF can induce the proliferation of mesenchymal stem cells, osteoprogenitors and endothelial cells (Mehta and Watson, 2008). Consistent with this role, PDGF expression is observed in macrophages located in the vicinity of the periosteum when cell division is initiated in its cambium layer, the layer adjacent to the bone which is a site of osteoprogenitor cells (Bourque et al., 1993).

Taken together these results demonstrate that the fracture site contains a cocktail of cytokines capable of modulating the recruitment, proliferation, differentiation and cell survival of cells located at the vicinity of the fracture during the first three days following injury.

1.4.2 The formation of the soft callus

This stage of bone fracture healing starts with the proliferation and differentiation of precursor cells into chondrocytes, and the formation of a cartilaginous matrix. This step is analogous to endochondral ossification. At the histological and cellular levels, the callus mass is visible by day 5 and is seen to bridge the fracture site with soft tissue (Hiltunen et al., 1993). At this time, many fibroblast-like mesenchymal cells are present and represent approximately 80% of the callus area, a proportion that will decrease to 40% by day 7 (Hiltunen et al., 1993). The presence of PDGF and TGF-β within the callus stimulates their proliferation (Mehta and Watson, 2008).
TGF-β2 and TGF-β3 are up-regulated at day 7, TGF-β2 decreases by day 21, while TGF-β3 returns to basal levels by day 28 (Cho et al., 2002). Some of these mesenchymal cells differentiate into chondrocytes expressing type-II collagen, a marker for chondrogenic differentiation, by day 5 (Le et al., 2001). Cartilage matrix is detectable from day 5 onward and increases to reach a maximum of 46% of the callus area on day 9 (Hiltunen et al., 1993; Le et al., 2001). Starting at day 7, hypertrophic chondrocytes are observed and type X collagen becomes detectable (Cho et al., 2002; Kon et al., 2001; Le et al., 2001). Mineralization of the callus increases from day 9 to day 14. At day 14, the callus reaches a maximum in mass and volume.

1.4.3 Formation of the hard callus

The hard callus refers to the bone structure of the callus (Einhorn, 1998). The initial bone formation occurs by intramembranous ossification and is concomitant with chondrogenesis occurring in other parts of the callus. Located in the cambium layer of the periosteum, pre-osteoblasts are activated and proliferate. Macrophages have been suggested to play an important role in this process as they are located adjacent to the periosteum and they express mitogenic factors such as PDGF and FGF (Barnes et al., 1999; Bourque et al., 1993). Based on the expression of type-I collagen, a marker for osteogenic differentiation, osteogenesis starts at day one post-fracture (Kon et al., 2001). By day 7, new bone is observed at the periosteal surface of the bone adjacent to the fracture site (Kon et al., 2001).

The major peak in ossification occurs around the third week post-fracture when the calcified cartilage is replaced with woven bone, by the process of endochondral ossification. This stage requires the coordination of anabolic (matrix formation) and catabolic (matrix removal) events. As the chondrocytes hypertrophy, they activate a genetic program similar to that of osteogenesis and start to express the angiogenic factor VEGF. The invasion of blood vessels into the cartilage matrix recruits osteoclasts that degrade the calcified cartilage matrix. By day 14 post-fracture, osteoclasts have been identified in the callus, based on their tartrate acid phosphatase (TRAP) expression (Kon et al., 2001). Consistent with this observation, activators of osteoclastogenesis, RANKL and M-CSF are up-regulated and OPG, an inhibitor of osteoclastogenesis is downregulated at this point (Kon et al., 2001). From day 14 to 21, factors such as BMP-2, -3, -4, -6, -7, and -8, GDF5, and TGF-β2 and TGF-β3 are expressed. The expression of this cocktail of osteogenic factors coincides with the formation of woven bone by
osteoblasts at sites where cartilage is removed by osteoclasts. By day 28 the callus is completely mineralized and has started to decrease in volume (Hiltunen et al., 1993).

1.4.4 The bone remodelling phase

The last phase of bone healing is bone remodelling. By day 28, the callus has completely calcified but its calcium content per dry weight is only 60% of intact bone (Hiltunen et al., 1993). At this time, the size of the callus has started to decrease (Hiltunen et al., 1993). The weight bearing capacity is about 74% of intact bone (Hiltunen et al., 1993). It is at this stage that lamellar bone is formed, through the process of bone remodelling, resulting in a substantial increase in the mechanical strength of the bone.

1.5 Mesenchymal stem cells

Mesenchymal stem cells (MSCs), also referred to as mesenchymal stromal cells or marrow stromal cells, are multipotent, non-hematopoietic precursor cells originally isolated from bone marrow. They can be expanded in vitro while retaining their ability to differentiate into a variety of mesodermal cell types. The following sections discuss their characterisation, differentiation and therapeutic potential.

The isolation of MSCs was first reported by Friedenstein and colleagues (Friedenstein et al., 1970). They isolated a fibroblast-like cell from guinea-pig bone marrow by selecting for the adherent cells when the marrow cells were plated in glass dishes. Among these adherent cells, some formed colonies after a few days in culture. The number of colonies per $10^6$ plated bone marrow cells ranged from 3.4 to 4.7 (Friedenstein et al., 1970). These cells were different from other fibroblasts, as they could differentiate into osteoblasts and chondrocytes in vivo once transplanted in diffusion chambers (Ashton et al., 1980; Friedenstein et al., 1970; Friedenstein et al., 1987). Due to their morphology and their ability to form colonies in culture, these cells were first named fibroblast colony-forming cells (FCFCs) or colony forming unit-fibroblast (CFU-F) (Friedenstein et al., 1974b; Friedenstein et al., 1976). Since their discovery, MSCs/CFU-F have been isolated from multiple species including mouse (Friedenstein et al., 1976), rabbit (Ashton et al., 1980), human (Castro-Malaspina et al., 1980; Friedenstein et al., 1974a), rat (Maniotopoulos et al., 1988), and dog (Kadiyala et al., 1997b).
The number of CFU-F per bone marrow cell varies from species to species and also among different strains of the same species. For example, nude and (CBA x C57Bl)F₁ mouse strains have respectively 0.25 and 1.25 CFU-F per 10⁵ bone marrow cells (Friedenstein et al., 1976). In humans they represent 0.0008-0.0019 % (95% confidence interval) of unfractionated bone marrow cells (Castro-Malaspina et al., 1980) or 0.001-0.01% of mononucleated cells isolated from a ficoll/percoll density gradient (Pittenger et al., 1999). In spite of their low frequency, once isolated they can be expanded considerably (20-30 population doublings) and retain their capacity to differentiate into osteoblasts and osteocytes when transplanted in vivo (Friedenstein et al., 1987).

Based on their ability to self-renew and to differentiate into osteoblasts, chondrocytes and adipocytes these colony-forming adherent bone marrow cells were subsequently named multipotent mesenchymal stroma cells or mesenchymal stem cells (Caplan, 1991; Pittenger et al., 1999). For the purpose of this introduction these cells will be referred as mesenchymal stem cells (MSCs). While the marker profile of MSCs has been extensively characterized (see below), these markers are not specific to MSCs. Thus, MSCs are mainly defined based on their fibroblast morphology, their ability to form CFU-F, their lack of hematopoietic markers, and their capacity to differentiate into these three lineages, osteoblasts, chondrocytes and adipocytes (Pittenger et al., 1999).

1.5.1 Mesenchymal stem cell markers

An extensive discussion of the different markers expressed by MSCs is beyond the scope of this introduction. Instead, the following section summarises the work on the identification of MSC immunogenic markers, their limitations and their potential uses.

Early work showed that human bone marrow-derived MSCs express type-I and type-III collagen and fibronectin, and are negative for the chondrocyte marker type-II collagen, the endothelial marker factor VIII and the hematopoietic marker Ia antigen (Castro-Malaspina et al., 1980). This supports the conclusion that MSCs are distinct from hematopoietic stem cells and endothelial cells. However, these markers are not specific to MSCs. Simmons and colleagues have generated an antibody, STRO-1, raised against cultured human MSCs and showed that the STRO-1⁺ve / glycophorinA⁻ve fraction of the bone marrow cells contains the cells that form CFU-F (Simmons and Torok-Storb, 1991). This subpopulation of sorted cells formed CFU-F at a
frequency of 1 out of 100 plated cells compared to $1/10^4$ of unsorted cells (Simmons and Torok-Storb, 1991). The epitope recognised by STRO-1 antibody remains to be determined. Since these early studies, a wide array of extracellular markers has been shown to be differentially expressed on MSCs. MSCs, derived from the indicated species, are positive for the following markers: CD29 (murine), CD44 (murine), CD73 (SH3 and SH4) (human), CD90 (Thy-1) (human), CD105 (SH2, endoglin) (human), CD106 (VCAM-1) (murine and human), CD117, CD166 (human), CD271 (LNGFR) (human), STRO-1 (human), and Sca-1 (murine) and negative for CD11b (murine), CD14 (human), CD31 (endothelial marker) (murine and human), CD34 (murine and human), and CD45 (human) (reviewed in (Bobis et al., 2006; Jones and McGonagle, 2008)).

The absence of CD14 (monocyte marker), CD34 (endothelial cell marker) and CD45 (hematopoietic marker) confirms that MSCs are distinct from hematopoietic cells. Among these markers, CD10, CD73 and CD90 are the minimal positive and CD34, CD45, HLA-DR, CD14 or CD11b, and CD79a or CD19 are the minimal negative markers to characterize human MSCs (Psaltis et al., 2008). Consistent with this observation, Boiret and colleagues reported that CD73+ or CD49+ populations contained more than 95% of CFU-F (100% for CD73 and 95.2% for CD49a), whereas at most 50% of the CFU-F were present in the CD105 or CDw90 positive population (Boiret et al., 2005). It will be interesting to determine what proportion of the CD73+ fraction is also marked with the STRO-1 antibody. Cell sorting based on the combination of these two positive markers might further enrich for the human MSC population.

It is important to note that the expression of these markers is modulated by their culture in vitro. Simmons and colleagues showed that the percentage of STRO-1 positive cells decreases in culture over time (Simmons and Torok-Storb, 1991). Culturing these cells can also result in the induction of certain markers. For example, the expansion of the sorted CD45−CD14+/CD105− or CD45−CD14+/CDw90− populations generated CFU-F that stained positive for CD105 and CDw90 (Boiret et al., 2005). This shows that culturing results in the modulation of the human MSC molecular phenotype. One limitation to the use of these markers is exemplified by the reports that many of these markers are also expressed on fibroblasts that do not have the cell differentiation capability of MSCs (Jones et al., 2004; Wagner et al., 2005). This lack of MSC-specific markers has hindered the identification of presumptive MSCs in bone marrow.
1.5.2 Multiple sources of MSCs

Partly due to their low frequency in bone marrow, multiple investigators have asked whether other adult and neonatal tissues contain MSC-like cells. Based on their morphology, ability to form CFU-F, self-renewal, differentiation potential, and surface markers many other tissues have been identified as sources of MSCs. These tissues include adipose tissue, skeletal muscle, umbilical cord blood, periosteum, synovium, amniotic membrane, and limbal stroma of the eyes (Alviano et al., 2007; De Bari et al., 2001; Erices et al., 2000; Polisetty et al., 2008; Sakaguchi et al., 2005; Zuk et al., 2002; Zuk et al., 2001). The presence of these cells in so many tissues raises the question of whether MSCs derived from these different sources are the same cells or are different cells that share similar properties. A few studies have started to address that question using multiple strategies.

Wagner and colleagues compared the expression of cell surface markers, the differentiation potential and the gene expression profiles of human MSCs derived from bone marrow, adipose tissue and umbilical cord blood (Wagner et al., 2005). Cell surface marker analysis revealed that MSCs from these three tissues as well as the fibroblast cell line HS68, all expressed CD13, CD29, CD44, CD73, CD90, CD105, CD166, and HLA-ABC, while they were negative for CD10, CD14, CD24, CD31, CD34, CD36, CD38, CD45, CD49d, CD117, CD133, SSEA4 and HLA-DR. This exemplifies the limitation of the use of these markers to identify MSCs. Osteogenic differentiation of these cells showed that MSCs from these three sources could generate mineral deposits when grown in osteogenic conditions while the fibroblast line failed to produce these deposits. When assessed for adipogenic differentiation, bone marrow or adipose tissue-derived MSCs were able to generate adipocytes as assessed with oil-red-O staining, whereas none were generated by cord blood-derived MSCs and the fibroblast line. This suggests that MSCs derived from different tissues vary in their differentiation potential. The genetic analysis of the transcriptome of the MSCs derived from the same three sources showed that some genes are differentially expressed among these MSCs, adding support to the conclusion that MSCs deriving from different sources are not identical. However, the biological significance of the differentially expressed reported genes remains to be established.

In a separate study, the expandability, efficiency of differentiation, and cell marker profile of human MSCs derived from bone marrow, synovium, periosteum, skeletal muscle and
adipose tissue were compared (Sakaguchi et al., 2005). Differences among these MSCs were observed for each of these criteria. The comparison for the number of serial passages until cessation of growth showed that MSCs derived from muscle could be passaged up to four times, from adipose tissue up to seven passages and from synovium, periosteum and bone marrow for at least ten passages (Sakaguchi et al., 2005). This shows that under the same culture conditions, MSCs deriving from different organs have different self-renewal capacity. To quantitatively compare the efficiency of cell differentiation, MSCs derived from these five sources in three patients (patient matched) were differentiated under chondrogenic, adipogenic, and osteogenic conditions and various parameters were analyzed. To assess chondrogenesis, MSCs were differentiated as pellets and the quantitative parameters of chondrogenic differentiation were the size, and the mass of each pellet and intensity of cartilage staining. This analysis showed differences, among MSCs of diverse origin, in their efficiency to differentiate into chondrocytes. The most efficient MSCs were derived from the synovium, followed by those from the bone marrow and periosteum and then by those from adipose tissue and muscle (Sakaguchi et al., 2005). Adipogenic and osteogenic differentiations were determined by plating MSCs at low densities to generate discrete colonies that were then differentiated under either adipogenic or osteogenic conditions. Differentiation efficiency was determined by calculating the number of samples that contained a certain percentage of colonies that have differentiated into the appropriate cell type. For adipogenic differentiation, the cut off was set at 80%. The most efficient MSCs were those derived from the synovium and adipose tissues, followed by those from the bone marrow, whereas the least efficient were those from the periosteum and muscle. For the osteogenic differentiation, the cut off was set at 30%. The most efficient samples were those derived from the synovium and bone marrow, followed by the periosteum, then adipose tissue and least from the muscle. Taken together, this suggests that skeletal muscle-derived MSCs differentiate the least efficiently into any of the three cell types followed, in increasing order of efficiency, by adipose tissue-, periosteum-, bone marrow- and synovium-derived MSCs.

Analyses of cell surface marker expression showed that MSCs derived from these 5 sources express NGFR, Stro-1, CD106, CD10, CD44, CD90, CD105, and CD147 (Sakaguchi et al., 2005). However, differences in terms of intensity and proportion of cell expressing these markers were observed among each MSC group. It was found that MSCs derived from skeletal
muscle expressed higher levels of NGFR while periosteum-derived MSCs expressed higher levels of CD10 (Sakaguchi et al., 2005).

Using rat MSCs, Yoshimura and colleagues performed similar growth assay and cell differentiation experiments to those described above (Yoshimura et al., 2007). They observed similar results in cell differentiation, but found that the growth rate of rat MSCs derived from bone marrow declined after passage 5 (Yoshimura et al., 2007). It remains to be determined whether this result reflects the variability between species or simply differences between culture methods.

Taken together results from these three studies show that differences exist among MSCs isolated from different tissues. This raises a semantics question: should MSCs derived from different tissues all be called MSCs? This may call for a new definition of an “MSC”. On the other hand, if the current definition of MSC, based on expression markers, self renewal and differentiation potential is retained, these results show that heterogeneity exists amongst different populations of MSCs. The recognition of these differences may have impact in the clinical use of MSCs.

### 1.5.3 In vivo location of MSCs

MSCs can be isolated from a variety of tissues. However, one question that remains unanswered is the location of endogenous MSCs within these different tissues. The answer to this important question will help to ascertain whether multipotent MSCs truly exist in situ, or whether many of their properties arise in culture. The many markers expressed by MSCs may have helped in this quest, had it not been for their lack of specificity. Also, because the use of multiple labeling techniques in situ is technically challenging, the location of MSCs in most tissues remains to be determined. Within the bone marrow, MSCs express similar markers and have morphologies similar to the bone marrow adventitial reticular cells, which are stromal cells similar to pericytes (Jones and McGonagle, 2008). Based on these observations, Jones and McGonagle have proposed that bone marrow-derived MSCs are adventitial reticular cells (Jones and McGonagle, 2008). However, it remains to be determined whether cultured adventitial reticular cells will show characteristics identical to MSCs isolated from bone marrow. Transplantation experiments will be required to show that MSCs can home to locations where adventitial reticular cells are present and that they can become functional adventitial reticular cells.
MSCs derived from tissues other than bone marrow were recently suggested to localize in blood vessel walls (Crisan et al., 2008). In this study, Crisan and colleagues showed that, similar to MSCs, pericytes isolated from different organs, cultured in vitro and differentiated in culture can generate myocytes, osteocytes, adipocytes and chondrocytes. Their myogenic and osteogenic differentiation was also achieved in vivo. It is interesting to note that pericytes deriving from different tissues had variable differentiation efficiency. For example, pericytes derived from adipose tissue had a higher index of muscle differentiation than pericytes derived from skeletal muscles. This suggests that pericytes isolated from various tissues display heterogeneity in their ability to differentiate, which is reminiscent of the heterogeneity observed in MSCs derived from different tissues (Section 1.5.2).

The immunophenotype of cultured pericytes derived from adipose tissue, pancreas, skeletal muscle and bone marrow was performed to compare their immunogenic profiles to that of MSCs. This analysis showed that all the markers normally expressed by MSCs, which include, but are not limited to CD10, CD13, CD44, CD73, CD90, and CD105 were also expressed on cultured pericytes (Crisan et al., 2008). The expression of CD44, CD73, CD90 and CD105 was also detected in freshly isolated pericytes and on pericytes in tissue sections (Crisan et al., 2008). This suggests that expression of these markers by pericytes is not an artefact of in vitro cultivation.

This study strongly suggests that, at least in certain organs, MSCs and pericytes might be the same cells in vivo, adding support to Jones and McGonagle’s hypothesis that bone marrow MSCs are adventitial reticular cells (Jones and McGonagle, 2008). However, a number of questions remain unanswered. Can MSCs be isolated from tissue depleted of pericytes or are MSCs derived solely from pericytes? Can MSCs home to the pericyte niche following transplantation and, if they do, can MSCs be isolated from the niche they have homed to? Answers to these questions will help to determine whether pericytes are the “in vivo MSCs”.

1.5.4 Embryonic origin of bone marrow MSCs

Very little is known about the embryonic origin of bone marrow MSCs. One study by Takashima and colleagues showed that a sub-population of bone marrow MSCs has a neural crest origin (Takashima et al., 2007). This was shown by genetically tagging the neuroepithelial cells and their progeny, by generating the compound mouse Sox1-Cre/ROSA26R-EYFP, which
expresses the cre recombinase in neural crest cells and induces a genetic recombination event resulting in the constitutive expression of EYFP in neural crest cells and all their derivatives. The analysis of this mouse showed that MSCs isolated from embryonic as well as postnatal tibial bone marrow contained EYFP positive cells (Takashima et al., 2007). Interestingly, the proportion of these EYFP positive cells in postnatal and aged animals decreased over time, suggesting that neural crest cells contribute to the early wave of bone marrow MSCs. The origin of the EYFP-negative MSCs remains to be identified. Other lineage tracing experiments using markers specific to other embryonic structures such as the somites may shed some light on the embryonic origin of MSCs.

1.5.5 MSC differentiation

MSCs are cultured in an undifferentiated state in monolayer conditions in the presence of 10-15% serum. It is important to note that the lot of serum is important for optimal growth and a lot suitable for culturing MSCs derived from one species may not support the same optimal growth of MSCs derived from another species (Caplan, 2005). Cultured MSCs can be differentiated into multiple cell types including, but not limited to, osteocytes, chondrocytes, adipocytes, tendocytes, myocytes and stromal cells (Caplan, 2005). As a population, MSCs are multipotent (Pittenger et al., 1999). However, at a single cell level, when clonal colonies are isolated and expanded prior to testing their capacity to differentiate into osteoblasts, chondrocytes and adipocytes, the differentiation potential among the clones varied. Some clones generated these three cell types while others had a more restricted differentiation potential (Pittenger et al., 1999). This can be explained by either the presence of cells with restricted capacity for differentiation or by a possible in vitro artefact causing a modulation of the differentiation potential of MSCs cultured for a long period of time (Pittenger et al., 1999). The second possibility is supported by a number of studies using human MSCs that showed a change in differentiation potential over time in culture (Baxter et al., 2004; Bonab et al., 2006). Nonetheless, low passage human MSCs can be differentiated into various cell types. The culture conditions for differentiating MSCs into these cell types were established in part based on the results of studies on embryo limb buds that identified a number of factors capable of inducing osteogenic and chondrogenic differentiation (Caplan, 2005). This section highlights some of the studies reporting the use of these factors for the differentiation of MSCs into osteoblasts and chondrocytes in vitro.
1.5.6 Osteogenic differentiation

The osteogenic differentiation of MSCs was first shown in vivo. Thereafter multiple groups showed that MSCs deriving from different vertebrate species can be differentiated into osteoblasts and osteocytes in vitro. In these early in vitro studies, MSCs were derived from bone marrow. The presence of serum and vitamin C in the culture media of rabbit MSCs resulted in their differentiation into osteogenic cells as assessed by the capacity to form mineral deposits and to up-regulate ALP activity (Howlett et al., 1986). Using these differentiation criteria combined with electron microscopy analysis, mouse MSCs were also shown to be capable of differentiating into osteoblasts in vitro in presence of serum, vitamin C and β-glycerophosphate (Luria et al., 1987). Subsequently, rat MSCs cultured in presence of culture media supplemented with synthetic glucocorticoid, dexamethasone, and β-glycerophosphate were shown to form mineralised bone nodules in vitro (Maniatopoulos et al., 1988). These nodules were also positive for ALP and the bone matrix proteins type-I collagen and osteonectin. Moreover these nodules contained peripheral cells with osteoblast morphology and cells with osteocyte morphology at their center (Maniatopoulos et al., 1988). The osteogenic differentiation capability of human MSCs was further shown by Gronthos and colleagues who showed that STRO-1+ human bone marrow cells that formed CFU-F were capable of differentiating into osteoblasts in the presence of serum, dexamethasone, ascorbic acid and inorganic phosphate (Gronthos et al., 1994). They also showed that dexamethasone was required and sufficient for the up-regulation of alkaline phosphatase levels and activity, and for the formation of mineral deposits (Gronthos et al., 1994). These studies showed that bone marrow MSCs, derived from multiple species, have the potential to differentiate into osteogenic cells.

1.5.7 Chondrogenic differentiation

The chondrogenic differentiation potential of MSCs was first observed in transplant experiments. In these experiments, expanded MSCs were transferred into diffusion chambers, made of silicon ring bounded with membrane filter, and then transplanted intraperitoneally into rabbits for three to six weeks. Histological analyses of these chambers revealed the presence of cartilage, hypertrophic chondrocytes and bone (Ashton et al., 1980; Friedenstein et al., 1987). Almost two decades later, Johnstone and colleagues showed that rabbit-derived MSCs can differentiate into chondrocytes in vitro (Johnstone et al., 1998). This was achieved by differentiating these cells in
aggregate cultures, which were obtained by centrifugation in polypropylene tubes, in the absence of serum, but in the presence of dexamethasone or TGF-β1. The presence of dexamethasone was sufficient to induce chondrogenic differentiation in a fraction of the cultures. The efficiency of the differentiation was increased by TGF-β1, where the combination of TGF-β1 with dexamethasone resulted in a further increase in chondrogenic differentiation and the formation of larger aggregates (Johnstone et al., 1998). The presence of cartilage in these aggregates was determined by toluidine blue staining, which stains cartilage proteoglycans, and by immunostaining and RT-PCR to detect the presence of type-II collagen. A portion of these MSCs differentiated into hypertrophic chondrocytes, as demonstrated by the expression of type-X collagen in these aggregates (Johnstone et al., 1998). These results show that rabbit MSCs can differentiate into chondrocytes that can mature into hypertrophic chondrocytes, the type of chondrocytes endogenously found during endochondral ossification, but normally absent in joint cartilage.

Using a similar assay, human MSCs were shown to differentiate into chondrocytes in vitro. One difference in this assay was the requirement for both dexamethasone and TGF-β1 or TGFβ3 in the media to induce the chondrogenic differentiation of human MSCs (Mackay et al., 1998; Yoo et al., 1998). TGF-β3 was a more potent inducer than TGF-β1 (Barry et al., 2001; Mackay et al., 1998). As a result of cell differentiation, the size of the aggregates increased due to accumulation of extracellular matrix and not from increased cell proliferation (Barry et al., 2001; Mackay et al., 1998). After two weeks of chondrogenic differentiation, the cartilage matrix proteins type-II collagen and aggrecan were present in these masses of differentiating cells (Mackay et al., 1998; Pittenger et al., 1999; Yoo et al., 1998). Type-I collagen was also present and detected more intensively on the periphery of the pellets (Mackay et al., 1998; Yoo et al., 1998). Interestingly, the chondrocytes present in these cultures continued to differentiate into hypertrophic chondrocytes over time (Barry et al., 2001; Pelttari et al., 2006; Yoo et al., 1998) or following their induction by adding thyroxine to the media, decreasing the concentration of dexamethasone, and omitting TGF-β3 (Mackay et al., 1998). The hypertrophic phenotype was determined by morphology and by the expression of type-X collagen and MMP13 (Barry et al., 2001; Pelttari et al., 2006; Yoo et al., 1998).
The maturation of these MSC-derived chondrocytes into hypertrophic chondrocytes could be due to the differentiation conditions. This possibility was addressed by Pelttari and colleagues, who compared the differentiation of MSCs with human articular chondrocytes that were expanded or not, in culture (Pelttari et al., 2006). Cells were differentiated in aggregate culture in presence of dexamethasone and TGF-β3. Under these conditions, human MSCs differentiated into chondrocytes and hypertrophic chondrocytes. Interestingly, these hypertrophic markers MMP13 and type-X collagen were expressed prior to type-II collagen (Pelttari et al., 2006). In contrast, human articular chondrocytes differentiated under the same conditions generated chondrocytes that did not express any of the hypertrophic markers. This shows that these in vitro differentiation conditions are sufficient to induce human articular chondrocyte differentiation, but not MSC differentiation into articular chondrocytes. This might be due to intrinsic signalling mechanisms in MSCs. Therefore, modifications to this protocol are required to direct MSC differentiation towards the articular chondrocyte phenotype. This is important if the use of MSCs as a source of cells for cartilage repair is considered.

Other chondrogenic conditions have been used to differentiate human MSCs. The presence of BMP-6 was found to enhance the chondrogenic differentiation of human MSCs. It is important to note that, in these experiments, BMP-6 alone had no chondrogenic inducing activity per se and chondrogenic differentiation required the presence of both TGF-β3 and dexamethasone (Sekiya et al., 2001). In the presence of these three factors, MSCs up-regulated the expression of Sox9, Sox5, Sox6, COL2A1 and COMP, which are genes normally expressed in chondrocytes (Sekiya et al., 2002)(Section 1.2.2.1). BMP-2 and BMP-6 were also induced by this chondrogenic treatment. However, the presence of BMP-6 in the differentiation media did not prevent the hypertrophy of these chondrocytes (Sekiya et al., 2002).

BMP-2 was also shown to induce chondrogenesis in human MSCs. In contrast to BMP-6, a combination of dexamethasone and BMP-2 was sufficient to induce the chondrogenic differentiation of MSCs in aggregate cultures (Schmitt et al., 2003). BMP-2 induced the production of an extracellular matrix that stained positive for Alcian blue and the transcription of COL2A1, aggrecan, cartilage oligomeric matrix protein (COMP) and cartilage link protein as determined by RT-PCR (Schmitt et al., 2003). Gene analysis following treatment with BMP-2, TGF-β3 or a combination thereof, showed that BMP-2 induced higher levels of aggrecan and
**cartilage link protein** than TGF-β3, and that TGFβ3 induced higher levels of **cartilage oligomeric matrix protein** and **COL2A1** than BMP-2. The combination of BMP-2 and TGFβ3 resulted in the induction of all these gene transcripts at levels equal to or higher than those observed with BMP-2 or TGF-β3 alone (Schmitt et al., 2003). Consistent with this result, addition of BMP-2 to the chondrogenic media containing dexamethasone and TGF-β3 resulted in larger and heavier aggregates compared to those treated with either TGF-β3 and dexamethasone alone or in combination with either BMP-4 or BMP-6 (Sekiya et al., 2005). It is of interest to point out that in presence of low levels of BMP-2 (50 ng/ml) the maturation into hypertrophic chondrocytes over 28 days of differentiation was not observed (Schmitt et al., 2003). In contrast, the use of high levels of BMP-2 (500 ng/ml) resulted in the hypertrophic differentiation of some chondrocytes in these cultures (Sekiya et al., 2005). This suggests that the final phenotype of these chondrocytes could be affected by the concentration of the factors used to induce chondrogenesis.

On a final note, the chondrogenic potential of MSCs has been observed to either decrease (Sekiya et al., 2001), or not be affected (Yoo et al., 1998) by passaging. It remains to be determined whether small changes in the culture conditions, the donor source or yet another unidentified factor(s) may be responsible for these conflicting observations.

### 1.5.8 Therapeutic applications of MSCs

MSCs are good candidates for cell therapy due to their capacity to expand *in vitro*, while retaining their ability to differentiate into various cell types and modulate the activity of a number of immune cells (see below). These properties allowed the use of MSCs in a number of pre-clinical and clinical studies for the treatment of various conditions. This section discusses the use of MSCs in graft-versus host disease, in joint cartilage repair, bone repair and reconstruction, and in genetic bone defect corrections.

#### 1.5.8.1 Immunoregulatory action of MSC

In a number of studies, MSCs were shown to have immunoregulatory effects. These effects can be the stimulation of certain immune cells or the suppression of the immune response (Patel et al., 2008). This dual effect of MSCs was seen both *in vitro* and in animal model experiments. Reports from human experiments are more consistent with an immunosuppressive action of
MSCs. The purpose of this section is to highlight the in vivo immune regulatory potential of MSCs in the context of transplantation. MSCs were shown to modulate many of the immune effector cells, including T and B lymphocytes, Natural Killer (NK) cells, dendritic cells which are antigen-presenting cells and neutrophils. However, a discussion on the mechanisms by which MSCs interact with these cells to modulate the immune system is beyond the scope of this introduction and readers are referred to recent reviews on the subject (Patel et al., 2008; Uccelli et al., 2008).

In one of the first reports showing the immunomodulatory effects of MSCs, the investigators used a mouse strain that naturally develops an autoimmune disease (Ishida et al., 1994). To treat these mice they performed bone marrow transplant using bone marrow from a different mouse strain. This treatment delayed the onset of the disease. When bone marrow derived MSCs from the same bone marrow donors were administered to the treated mice, their survival increased and the onset of the autoimmune disease was further delayed. To increase the yield of injected MSCs, the recipient mouse was transplanted with bones deriving from the same bone marrow donor. This resulted in no occurrence of the disease for the entire time of the study (48 weeks) and the stromal cells present in the host bone were predominately derived from the donor origin (Ishida et al., 1994). The precise interpretation of this study is complex due to the presence of a complete bone marrow of donor origin in the host animal, but it suggests that MSCs may have a role in either modulating the immune system or supporting better survival or engraftment of the transplanted hematopoietic stem cell. Since the publication of this study, many other reports have shown that MSCs have immunoregulatory potential in vivo and in vitro. However the source of MSCs may be important for the beneficial effects. In a bone marrow transplant mouse model, the injection of cultured syngeneic or third party MSCs resulted in better or neutral effects on bone marrow engraftment, while the injection of MSCs derived from the same donor as the bone marrow decreased the efficiency of bone marrow engraftment (Nauta et al., 2006). It is important to note that in this experiment mice were irradiated at doses resulting in 50% of bone marrow engraftment. This suggests that under conditions where blood cells are derived from both donor and host bone marrow, the origin of MSCs could play opposite roles in the maintenance of graft tissue.

Work by Di Nicola and colleagues (Di Nicola et al., 2002), showed in vitro that MSCs can suppress proliferation of activated T-lymphocyte even when MSCs were added five days
after T-cell activation. This was confirmed by Bartholomew and colleagues (Bartholomew et al., 2002). In their study Bartholomew and colleagues showed that MSCs can inhibit T-lymphocyte activation in vitro in a dose dependent manner and this inhibition could be partially relieved by the presence of the T-lymphocyte mitogen IL-2. To test whether MSCs can be immunosuppressive in vivo, in the context of skin grafts, MSCs from the skin donor or from a different donor were injected intravenously into the host at the time of skin graft. The immunosuppressive effect was determined by evaluating the time to graft rejection. In the absence of MSC injection, the average time to skin rejection was seven days. In animals injected with autologous or allogenic MSCs, the time to rejection was 11.3 and 11.8 days with a range of 10 to 13 days (Bartholomew et al., 2002). Secondary injection did not prolong the time to rejection in this model. This delay in skin rejection associated with the MSC treatment is a significant result, as the daily injection of immunosuppressant such as cyclosporine or fludarabine can delay skin rejection to 14 days (Bartholomew et al., 2002). This also shows that in the context of skin graft, the source of MSCs had little importance. It would also be interesting to determine whether co-treatment with MSCs and immunosuppressive drugs would result in prolonged time to graft rejection. The results of this experiment, in light of the mechanism by which these immunosuppressive drugs exert their effect, may help elucidate the mechanism by which MSCs exert their immunosuppressive response.

There are also some case reports describing the use of MSCs to treat human patients with severe graft-versus-host-disease (GVHD). In two case reports, patients suffered from drug resistant graft-versus-host-disease following bone marrow transplant (Fang et al., 2007; Le Blanc et al., 2004). In these two cases allogenic donors were used. In one of the studies, MSCs were derived from adult bone marrow and, due to the severity of the GVHD, two MSC injections were administered (Le Blanc et al., 2004). After the first intravenous injection, the condition of the affected 9-year-old greatly improved. However, some remnant of the disease remained and the patient became ill again. Following the second injection of MSCs from the same culture, the patient recovered and was disease free for the next year (Le Blanc et al., 2004). In the second case, adult MSCs were generated from adipose tissue. Following the injection, the 43-year-old patient recovered and was free from disease for over 1 year without the need for medication (Fang et al., 2007). This suggests that the immunosuppressive property of MSCs may be shared by MSCs derived from various tissues.
1.5.8.2 Cartilage repair

Joint cartilage is avascular and has limited regenerative capacity. Two common causes of cartilage degeneration are osteoarthritis and rheumatoid arthritis. The exact aetiology of both osteoarthritis and rheumatoid arthritis is not fully understood, but a number of factors have been associated with these conditions. Injury, age, and genetics have been considered risk factors for osteoarthritis (Chen and Tuan, 2008). Activation of an autoimmune response against antigens present in cartilage is associated with rheumatoid arthritis. To treat or prevent these conditions, cartilage tissue needs to be repaired. In the case of cartilage injury, the repair of localised damage may prevent the occurrence of osteoarthritis. Following the onset of osteoarthritis and rheumatoid arthritis, an inflammatory or immune response is initiated and multiple tissues of the joint can be affected. The therapeutic approach to successfully treat these two conditions may differ. For localised damage, the use of scaffold-embedded MSCs can be effective, whereas synovial injection of MSCs may be more effective following the onset of osteoarthritis or rheumatoid arthritis, as they may contribute to cartilage healing and modulation of the immune or inflammatory response. The following discussion will highlight some studies that demonstrate the feasibility of these two approaches.

1.5.8.2.1 Scaffold-embedded MSCs

The use of scaffolds to regenerate cartilage requires materials that have specific properties. For example, they have to be biocompatible, biodegradable, and highly porous to allow transfer of cells, nutrients and gas exchange. They must resist mechanical stresses present at the transplant site, and promote chondrogenic differentiation and formation of appropriate extracellular matrix (Noth et al., 2008). A discussion of the properties and advantages and disadvantages of different matrices used is beyond the scope of this introduction. Instead, this section will highlight some studies that used MSCs embedded in scaffolds in the treatment of cartilage defects. The strategy involves the embedding of naïve MSCs into a matrix that is then either directly transplanted into defective cartilage or pre-differentiated in vitro before transplantation. One of the early studies studying knee cartilage defects in rabbits, showed that transplantation of autologous bone marrow- or periosteum-derived MSCs mixed with a collagen gel resulted in the formation of cartilage tissue that could be observed as early as two weeks post-implantation (Wakitani et al., 1994). At four weeks, the edges of the transplant were barely discernable and the cartilage was thicker than the surrounding articular cartilage. However, by 12 weeks, the newly formed
cartilage had thinned so that it was thinner than the surrounding tissue. The thinning was even more apparent by 24 weeks (Wakitani et al., 1994). This showed that while the initial repair is promising, the newly formed cartilage layer decreases over time. However, Nöth and colleagues reported that using such a strategy in mini-pigs resulted in the formation of cartilage that filled the defect and showed bonding with surrounding joint cartilage at 6 months post implantation (Noth et al., 2008).

This cartilage repair strategy has also been used in human patients. Kuroda and colleagues reported that autologous transplantation of bone marrow-derived MSCs embedded into a collagen gel, regenerated the damage knee cartilage of a judo athlete (Kuroda et al., 2007). In this case report, the MSC-embedded collagen gel was covered by an autologous periosteal flap to maintain the transplanted cells at the proper location. This may complicate the interpretation of the results, as periosteum also contains osteochondroprogenitor cells. Nonetheless, at seven months post-surgery, the defective cartilage was filled with smooth tissue that resembled hyaline cartilage and covered with fibrous tissue rich in type-I collagen. The authors suggested that the presence of this fibrous tissue is attributable to the periosteal flap. One year post surgery, some chondral and subchondral irregularities within the regenerated cartilage were detected by MRI, but the overall clinical symptoms had improved and the patient had resumed his regular activities. However, it remains to be determined whether the regenerated tissue will remain functional over a long period of time.

1.5.8.2.2 Synovial injections of MSCs

The synovial injection of naïve MSCs allows the injected cells to be in contact with the different tissues and cells of the joint. This may have the advantage of facilitating the healing contribution of MSCs to multiple tissues and possibly regulating inflammation with a single injection. This therapeutic strategy could be well suited for the treatment of osteoarthritis and rheumatoid arthritis, in which tissues at multiple positions within the joint can be affected. This was tested in a goat model of osteoarthritis (Murphy et al., 2003). These animals had unilateral medial mesiscectomy surgery with transection of the anterior cruciate ligament. Animals were allowed to recover and exercise to induce osteoarthritis. Six weeks post-injury autologous MSCs mixed in hyaluronan solution were injected into the damaged joint. Macroscopic analysis at six weeks post-injection showed that treated joints had formed a meniscal-like tissue of hyaline appearance
that contained type-I and type-II collagen. This was also seen in 7 of 9 treated joints at 20 weeks post-injection. However, no regeneration of the anterior cruciate ligament was observed (Murphy et al., 2003). The MSC treatment reduced the severity of the damages associated with osteoarthritis in most animals (Murphy et al., 2003). It is important to note that at 20 weeks, joints treated with MSCs displayed osteoarthritic lesions. The authors suggested that this may be due to the cumulative effects of the abnormal load distribution resulting from the anterior cruciate ligament severing (Murphy et al., 2003). At earlier time points, in joints that benefited from the injections, no injected cells were present in the articular cartilage (Murphy et al., 2003). It is possible that one of the positive effects of MSCs in this model is the induction of the formation of the meniscus which would reduce articular cartilage damage. Since the regenerated meniscus contained injected and host cells, it showed that injected MSCs could recruit endogenous cells to regenerate the tissue. It is also possible that the ability of MSCs to modulate the inflammatory response may have favored the regeneration of the tissues. Based on the results of this study, a phase I/II clinical study is currently ongoing for meniscus repair and prevention of osteoarthritis (Chen and Tuan, 2008).

Similar regenerative results were observed in a mini-pig model for large joint damage (Lee et al., 2007). The cartilage defect was 8.5 mm in diameter with a depth of 1 mm in a weight baring area of the knee. In most treated animals, direct injection of autologous MSCs, in hyaluronic acid solution, into the damaged joint one week post-damage resulted in the complete healing of cartilage defect at 12 weeks post-injection. In this study, the injected cells were present in the newly regenerated cartilage. These results show that injection of MSCs in hyaluronic acid solution can contribute to the regeneration of cartilage when osteoarthritis may not have yet fully developed. It would be interesting to address whether it is the knee conditions or the models used by these two studies that resulted in the regeneration of cartilage tissue in one study but not in the other. It is possible that in the study by Murphy and colleagues (Murphy et al., 2003), the severing of the ligament may have resulted in abrasive forces acting on the cartilage tissue, thereby preventing or removing MSCs that have started to integrate into the cartilage tissue.

Similar strategies were tested in the treatment of rheumatoid arthritis. In a mouse model of rheumatoid arthritis, collagen immunization-induced arthritis, in which mice are immunized for type-II collagen, the peritoneal injection of allogenic MSCs at either the time of
immunization or at the time of the boost decreased the incidence and severity of the disease (Augello et al., 2007). In these animals, the allogenic MSCs were not present at the joint nor the site of injection or the spleen at day 11 post-injection. The MSC treatment resulted in the hyporesponsiveness of T cells and in the reduction of inflammatory cytokines IL-4, IL-10, IFN-γ, and TNFα, but the serum levels IL-2, a T-lymphocyte mitogen, were not affected in the treated animals (Augello et al., 2007). The authors also showed that injected MSCs increased the number of regulatory T-lymphocytes, which inhibit the activity of T-cells in an antigen specific manner.

More research is required to fully explore the potential and to optimize the therapeutic use of MSCs in the treatment of cartilage defects. It is important to note that stem cell therapy can be used to target different aspects of diseases affecting joint cartilage.

1.5.8.3 Bone repairs

Common sources of skeletal defects with clinical importance are bone fractures that result in non-union (5% of fracture cases (Silkstone et al., 2008)), tumour resections, genetic disorders such as osteogenesis imperfecta, or defects caused by the degeneration of bone tissues associated with metabolic bone diseases such as osteoporosis. Orthopaedic surgery is often required to treat these conditions, often requiring a source of osteogenic cells. For the reasons enumerated above, MSCs are potential candidates for such therapies. This section focuses on the use of MSCs for bone reconstruction and for the treatment of osteogenesis imperfecta.

1.5.8.3.1 Bone reconstruction

Osteogenic cells are needed to fill gaps of missing bone or areas of non-healing bone. Early studies on bone repair used bone marrows as osteogenic cell sources. A preclinical study showed that autologous bone marrow injection into bone fractures improved the extent of healing and decreased the healing time in rabbits (Paley et al., 1986). This beneficial effect of bone marrow injection was also observed in a clinical setting. Autologous bone marrow injected in non-union fractures of patients who did not respond to traditional treatments, resulted in bone union in most cases (Connolly et al., 1991). While patients’ conditions improved, it is not clear which population of bone marrow cells was responsible for this effect.
Results from the early characterisation of MSCs showed that osteogenic differentiation of MSCs in vivo in ectopic locations requires the presence of a substrate (Friedenstein et al., 1982). This was shown by using a biodegradable sponge that degraded prior to the osteogenic differentiation of MSCs, which resulted in the failure of MSCs to generate bone tissue (Friedenstein et al., 1982). To solve this issue, two approaches or a combination thereof were used. One was to pre-differentiate MSCs towards the osteogenic lineage prior to their injection into a fracture gap. Shao and colleagues showed that the injection of MSCs, cultured in osteogenic conditions for more than 1 week, into the distracted callus of a rabbit mandible bone resulted in a decrease in healing time compared to the uninjected side (Shao et al., 2007). However, whether the newly formed bone was composed of injected cells or whether the transplanted cells induced the recruitment of endogenous cells to regenerate the bone was not distinguished in this study.

A second approach is to combine the bone forming ability of MSCs with biomaterials, that have been experimentally used to replace the missing bone tissue in order to help filling large gaps that would normally not heal without intervention (for a review of such material see (Mauney et al., 2005)). Such scaffolds can be designed with properties as described above (see section 1.5.8.2.1) in addition to being osteogenic, osteoconducive or osteoinductive. However, due to the large mechanical stress that long bone must endure due to load bearing, the materials that make up the matrix must withstand these forces until the bone is formed or require supplemental support. One such cell carrier is hydroxyapatite (HA) or a mixture of HA and tricalcium phosphate (HA/TCP). They are osteoconductive and support osteogenic differentiation of MSCs in heterotropic locations in vivo. This was shown by transplanting expanded canine MSCs embedded into porous HA/TCP intramuscularly in dog or subcutaneously in athymic mouse (Kadiyala et al., 1997b). Osteogenic differentiation was also observed when using human MSCs loaded into HA/TCP and then transplanted subcutaneously in nude mice (Haynesworth et al., 1992). These experiments showed that MSCs can differentiate into osteogenic cells in these matrices and suggested that the use of these materials might be beneficial in the reconstruction of bone defects.

In a preclinical trial study, using femoral segmental defects in dogs, the implantation of autologous MSCs loaded into HA/TCP ceramic scaffolds resulted in the formation of bone matrix at defective sites (Bruder et al., 1998). Similar results were obtained when scaffolds
composed of different materials were used. Coral scaffolds loaded with MSCs were more effective at repairing a 25 mm defect in sheep metatarsals than those loaded with fresh bone marrow (Petite et al., 2000). No bone unions were observed in animal implanted with coral scaffolds loaded with fresh bone marrow cells or no cells. In contrast, about half of the animals treated with MSC-loaded scaffolds displayed bone union, where cortical bone was present with a marrow cavity. Similar results were also reported in rats transplanted with HA/TCP implants loaded with either syngeneic MSCs, fresh bone marrow or no cells (Kadiyala et al., 1997a). In the Petite and Kadiyala studies the difference in bone formation between the MSCs and bone marrow cells can be attributed to an underestimate in the actual number of MSCs present in the carrier. Goshima and colleagues showed that increases in cell density of the loaded cells resulted in increased bone formation in calcium phosphate ceramics (Goshima et al., 1991). Taken together, these results suggest the clinical potential of MSCs in generating new bone tissue in patients with bone defects. A phase I/II clinical study is currently looking at the use of autologous human MSCs loaded into a carrier that are transplanted at the site of fracture (NCT00250302, clinicaltrials.gov). Results from this study will help to determine the safety and efficacy of this approach in humans.

The third approach is the combination of the pre-differentiation of MSCs and the use of a scaffold. van den Dolder and colleagues (van den Dolder et al., 2002) showed that in vitro differentiation of rat MSCs loaded into a titanium scaffold prior to subcutaneous transplantation for four weeks in syngenic rats resulted in the formation of bone tissue when cells were pre-differentiated for one or four days but not when pre-differentiated for eight days. In a number of implants differentiated for four days some bone marrow-like structure were also observed. Similar results were also reported by Castano-Izquierdo and colleagues (Castano-Izquierdo et al., 2007). In this study, MSCs were pre-differentiated on tricalcium phosphate for four, 10 or 16 days before being loaded on titanium scaffolds. These loaded scaffolds were then transplanted into 8-mm wide calvaria bone defects in syngeneic rat. In animals transplanted with the four day pre-differentiated cells, bone formation and bone union was observed in seven out of eight animals (Castano-Izquierdo et al., 2007). Bone union occurred in only two of eight animals when scaffolds were loaded with naïve MSCs or MSCs pre-differentiated for 10 days. No union was found when cells were pre-differentiated for 16 days. These results suggest that short pre-differentiation of MSCs can increase bone formation when transplanted in vivo, whereas this
positive effect can be lost if cells are pre-differentiated for too long. In these longer pre-differentiation, MSCs could have differentiated into osteoblasts and osteocytes, which are non-dividing cells, and this may prevent the *in vivo* expansion of the transplanted cells and thus result in a decrease production of bone matrix (Franz-Odendaal et al., 2006). In contrast, a short pre-differentiation would result in the differentiation of MSCs into preosteoblasts, which are proliferative osteogenic cells that do not produce mineralisation but can differentiate into active mineralising osteoblasts (Franz-Odendaal et al., 2006). These preosteoblasts would have the potential to proliferate in these scaffolds, thus potentially increasing the number of cells that form bone tissue.

Taken together, loading of MSCs into different types of scaffolds can increase their ability to generate the bone tissues required to repair large bone defects. The pre-differentiation of MSCs could further enhance the formation of bone if it is performed for short period of time, as long pre-differentiation abrogates this positive effect.

### 1.5.8.3.2 Osteogenesis imperfecta

Osteogenesis imperfecta (OI) is a genetic disorder characterised by an increase in bone fragility. Its prevalence is about 1 in 10 000 births (Glorieux, 2008). The severity of the phenotype varies widely from intrauterine fractures and lethality to milder forms, in which no bone fractures are observed (Rauch and Glorieux, 2004). In the most severe cases, perinatal lethality is typically associated with respiratory failure due to broken ribs. In milder cases, children can display mild bone deformities and short stature. Clinical symptoms also observed in OI patients include blue sclera, dentinogenesis imperfecta, hearing impairment, and skin hyperlaxity. In most patients with the diagnostic of osteogenesis imperfecta, at least one of the two genes encoding type-I collagen (*COL1A1* and *COL1A2*) is mutated (Rauch and Glorieux, 2004). Among cases with mutation(s) in the type-I collagen gene(s), the mutant collagen units may not form the proper triple-helical structure (two $\alpha_1$ and one $\alpha_2$ chains) resulting in defective type-I collagen fibres. Furthermore, the osteoblasts of severely affected osteogenesis imperfecta patients have been shown to express lower levels of the bone matrix protein osteocalcin and have increased *in vitro* cell proliferation capacity when compared to age-matched controls (Morike et al., 1993).

One of the current strategies aimed at alleviating the skeletal fragility of these patients is the use of bisphosphonates, such as pamidronate and neridronate (Rauch and Glorieux, 2004).
This treatment inhibits bone resorption by decreasing osteoclast activity, where the net result is an increase in cortical bone thickness. This effect is only transient and decreases after three to four years of treatment (Glorieux, 2008). Another limitation of this therapy is that mutant collagen fibres are not replaced with normal collagen, and an increase in bone mineralisation is not sufficient to restore the mechanical properties of normal bone. One interesting observation is that unaffected parents of affected children are mosaics for cells expressing the normal or affected gene (Edwards et al., 1992; Wallis et al., 1990). This suggests that the presence of unaffected cells could reduce the severity of the osteogenesis imperfecta phenotype, which puts forward the possibility that a cell therapy might be beneficial to treat affected patients.

One pre-clinical study in mice showed that the injection of MSCs can result in the integration of the transplanted cells into bone tissue. In this study, MSCs derived from a mouse expressing the human type-I collagen mini gene were combined with bone marrow cells, and injected intravenously into irradiated mouse (Pereira et al., 1995). The irradiation of the host animal was necessary for the integration of donor cells. The presence of the transplanted MSCs into bone was determined by PCR for the presence of the human type-I collagen mini gene on crushed bone and on isolated and cultured bone cells from the recipient animal. The number of donor cells peaked at one month post-transplantation (4.0 – 10.5% of bone cells) and slightly decreased at five months (2.0-5.5%). This decrease failed to be statistically significant, but this might be attributable to the very small sample size (n = 3 animals in each group). This study showed that the injection of MSCs combined with bone marrow cells into an irradiated host animal can result in the generation of MSC-derived cells that integrate into the host’s bone tissue. This result spearheaded similar studies in human.

Horwitz and colleagues reported a clinical trial using cellular therapy to improve the clinical condition of children affected with osteogenesis imperfecta (Horwitz et al., 1999). In their study, they transplanted unmanipulated bone marrow from healthy siblings in children with osteogenesis imperfecta to test whether donor MSCs could colonise the grafts, differentiate into osteoblasts, and integrate into the host bones, thereby potentially improving the clinical condition of the treated patients (Horwitz et al., 1999). They reported that three of three patients had engraftment of the donor bone marrow. At seven months post-transplantation, the percentage of osteoblasts of donor origin was evaluated at 1.5 to 2% in the culture of osteoblasts derived from bone biopsy, which suggests that the same percentages of donor osteoblasts were present in the
host bone. These authors also reported an increase in the number of osteoblasts, bone mineral density, and a decrease in fracture incidence following transplantation. These results require careful evaluation and raised some concerns. First, it remained unexplained how the presence of only 1.5-2% of normal osteoblasts (percentage of total number of osteoblast) could result in a four-fold increase in the number of bone osteoblasts and 45-78% increase in bone mineral content (Marini, 1999). A potential explanation for the increase in bone mass is the potential loss of osteoclasts due to the treatment that depleted the host bone marrow prior to the graft (Horwitz et al., 2001). Second, in this study the reference group was the patient data prior to treatment or the normal value for healthy individuals. These reference groups may not be adequate as fracture rates in young osteogenesis imperfecta patients are highest during the first and six months after birth, and then gradually fall (Bishop, 1999). Thus, the comparison between the numbers of fractures in the first 13 months of life with the number of fractures in the six months following the transplantation might not reflect the actual improvement due to the graft (Bishop, 1999). Finally, while these results show the feasibility of grafting bone marrow in osteogenesis imperfecta patients, an improved, better study design allowing statistical comparison with an appropriate control group will be required to determine the magnitude of the clinical impact of such therapies.

In a second related report, two of the patients from the study described above were included with another treated patient and two other age-matched patients that formed the control group (Horwitz et al., 2001). In this report they compared the growth and fracture frequency of the graft patients to the value of untreated control. The initial growth of the treated patients was 6.5 to 8.0 cm compares to 1.0 to 1.5 cm of the controls for the same period of time, but in longer follow-ups, the growth rate of treated patients decreased. The number of fractures reported for the control group was three to five fractures per year, whereas the transplanted patients had 0-3 fractures per year. No statistical analysis was performed on the data generated from this small sample size, but these results suggest a beneficial effect of the transplantation. However, two of the three patients that received bone marrow grafts experienced clinical complications (Horwitz et al., 2001). This raises the question of whether benefits from such treatments outweigh risks of potential complications. The results of this study also show that the beneficial effects from such treatments, especially with regard to growth, may not be sustained over time. This implies that multiple rounds of treatment may be necessary to sustain the growth of these patients.
In a third report, the same group asked whether subsequent injections of MSCs could further improve the conditions of patients enrolled in their previous studies (Horwitz et al., 2002). The initial growth velocity of these treated patients had decreased to 0-40% of normal age matched children. In this study these patients received two intravenous injections of normal MSCs. Their growth velocity, assessed three months post-treatment, had increased to 67-94% of healthy control. However, no increase in bone mineral content was observed. Two potential explanations for this observation were given by the authors (Horwitz et al., 2002). First, the follow up might not have been long enough to measure a difference. Second, bone growth and bone mineralisation can be uncoupled, and MSCs might be responsible for the former. This explanation is consistent with a previous animal study in which osteogenesis imperfecta mice were injected with either wild type MSCs or osteogenesis imperfecta-derived MSCs. Mice treated with wild-type MSCs had only a marginal increase in bone collagen and mineral content (Pereira et al., 1998).

Taken together, these results show that transplantation of bone marrow or MSCs can transiently improve the condition, but is not sufficient to cure patients affected with osteogenesis imperfecta. The transient benefits of these MSC treatments indicate that injected cells do not colonise the osteoprogenitor niche of the treated patients. This is supported by the observations that in utero injection of MSCs in osteogenesis imperfecta patients did not result in the presence of donor MSCs in the bone marrow at 9 months of age (Le Blanc et al., 2005). To colonise the osteoprogenitor niche, MSCs could be injected at the time when bones are forming. In a recent paper, osteogenesis imperfecta mice were injected with human fetal cord blood-derived MSCs at a time when bones were forming (E13.5-E15.5) and were followed for 12 months (Guillot et al., 2008). Compared to untransplanted animals, the treated mice had a decrease in fracture incidence, and an increase in bone growth, thickness and strength. The donors cells were more abundant in locations of bone formation, remodelling and at healed fracture sites. Whether these results are attributable to the age of the recipient and or the source of the MSCs remains to be tested. Given that bones are formed very early in life, a more practical approach to treat human patients may be to deplete an osteoprogenitor niche, such as the periosteum, and transplant normal MSCs into that depleted niche. This might improve the rate of MSC integration into the osteoprogenitor niche. However, the efficacy of this approach remains to be tested in animal models.
1.5.8.4 Limitations in the use of MSCs in transplantation

Due to their ability to be expanded in vitro and to retain their capacity to differentiate into cell types with therapeutic uses, MSCs are good precursor cell candidates in the treatment of multiple clinical conditions (see above sections). However, there are a number of factors that can limit their use. First is their low frequency in tissues, especially in the bone marrow. As a consequence, they need to be expanded extensively in vitro to obtain the quantity of cells required for therapeutic purposes. The second limitation is associated with artefacts resulting from their amplification in culture. Some studies have observed an increase in senescence and a decrease in differentiation potential in human MSCs cultured for long periods of time (Baxter et al., 2004; Bonab et al., 2006; Goshima et al., 1991). This may be patient specific, but this can have two important consequences. First, the increase in senescence may complicate the generation of the large number of MSCs required to treat patients. Second, the decrease in differentiation potential may either decrease their therapeutic efficiency or restrict their use to only certain types of defects. Due to these limitations new culture strategies are currently being developed. These include adding FGF-2 to the culture media to extend their proliferation capacity (Tsutumi et al., 2001). Others involve the use of transgene over-expression such as telomerase (hTERT) to increase time to senescence, thereby increasing the number of possible cell divisions (Shi et al., 2002). More work is required to determine whether MSCs cultured under these conditions will retain their differentiation potential at longer passages, will not accumulate genomic instability and be safe for therapeutic use.

An alternative to these strategies is the isolation of another source of precursor cells that can be found at higher frequencies and have a broader differentiation potential. Such a cell type has been identified in the skin. These cells are named skin-derived precursor (SKP) cells. The next sections will discuss the work characterizing these cells.

1.6 Skin-derived precursor cells: Identification and characterization

The skin is the largest organ in the human body. It is composed of three distinct layers: outermost is the epidermis, innermost is the hypodermis, and the dermis lies between the
epidermis and the hypodermis. Many other structures are present in the skin, such as hair follicles, sensory receptors, blood vessels and nerve endings. The maintenance of skin integrity relies on the presence of precursor cells that regenerate the different structures of the skin. For example, the epidermis is continually regenerated throughout life by epidermal stem cells (Blanpain and Fuchs, 2006). The cyclic growth of hair follicles is dependent on precursor cells present in the dermal papillae and the bulge (Blanpain and Fuchs, 2006), and following skin damage cells present in the dermis contribute to the process of healing (Blanpain and Fuchs, 2006). Recently, skin-derived precursor (SKP) cells with neural crest properties have been isolated from both rodent and human skin (Toma et al., 2001; Toma et al., 2005).

SKPs may be defined as cells isolated from dissociated skin that, if cultured in non-adherent conditions in the presence of epidermal growth factor (EGF), and basic fibroblast growth factor (FGF-2) will form floating spheres that can be serially passaged (Biernaskie et al., 2006; Toma et al., 2001), and that have the ability to generate both mesodermal and peripheral neural cell types.

In addition, SKPs cultured as individual single cells (Toma et al., 2001; Toma et al., 2005) or dissociated skin cells plated at low density such as 25 000 skin cells/ml (Fernandes et al., 2004) generate clonal spheres that can be amplified, thus generating clonal SKP cultures. The clonality of these low density cultures was shown by co-culturing skin cells derived from a wild-type mouse with those derived from a YFP-expressing transgenic mouse. The resulting spheres contained cells that were either all non-fluorescent or all fluorescent, thus demonstrating that under these conditions, SKP spheres are not formed from the clustering of cells, but by the division of a single cell (Fernandes et al., 2004). Once isolated, SKPs can be amplified for a long period of time. Rodent SKPs can be passaged for at least 5 months (Toma et al., 2001) and human SKPs passaged for over 1 year retain a normal karyotype, as assessed by G-banding (Toma et al., 2005). Similar results were also observed with porcine SKPs (Dyce et al., 2004). These results illustrate the self renewal capacity of SKPs.

The *in vitro* isolation of SKPs prompted an important question: in which compartment of the skin do SKPs normally reside? Details of isolation protocols suggested three candidate compartments: the epidermis, the dermis or the neural component of the skin. Early work by Toma and colleagues showed the presence of SKPs in the dermis, as cultures of dissected dermis,
but not dissected epidermis or sciatic nerve, yielded SKP-like spheres (Toma et al., 2001). SKPs can first be isolated from mouse skin at embryonic day E15 (Fernandes et al., 2004) or from rat skin at E12 (Hunt et al., 2008). The proportion of SKPs in the skin peaks at E16 in rat and E18 in mouse, reaching 0.5%-1% of skin cells, and decreases at birth by at least 10-fold, remaining low throughout adulthood (Fernandes et al., 2004; Hunt et al., 2008).

Primary SKP spheres as well as passaged spheres express nestin, an intermediate filament expressed in neural and skeletal muscle precursors, and the extracellular matrix proteins fibronectin and vimentin (Fernandes et al., 2004; Toma et al., 2001). SKPs express the transcription factors *slug*, *snail*, *twist*, *Pax3* and *Sox9*, which are also expressed in neural crest cells (see below) (Fernandes et al., 2004). In addition, SKPs express Dermo-1 and SHOX2, which are transcription factors associated with the mesenchymal capability of cranial neural crest cells (Fernandes et al., 2004; Fernandes et al., 2008). However, primary SKPs are negative for the expression of the melanoblast/melanocyte markers *trp1*, *c-kit*, and *dct*, and the Schwann cell markers *p75NTR*, *myelin associated proteins MBP*, *P0* and *Sox10*, and the neuronal markers DβH and *Peripherin* (Fernandes et al., 2004). Therefore, SKPs are unlikely to be derived from melanoblasts/melanocytes, Schwann cells or neurons. This is consistent with the conclusion that SKPs do not originate from the neural component of the skin. Interestingly, mouse skin cells positive for Sca1 (unpublished observation, K.M.S. D.R.K. and F.D.M.), a marker for hematopoietic stem cells (Ito et al., 2003a), and human skin cells positive for AC133, a marker for hematopoietic/endothelial precursor cells (Belicchi et al., 2004) form SKP-like spheres with SKP properties. Further characterization is required to confirm that these are true markers for prospective SKPs, as it is in the Sca1 low and Sca1 negative populations that the neurogenic cells are found (J.B, personal communications). Immunogenic characterization of SKP spheres by flow cytometric analysis suggests that SKPs are positive for CD29 (β1-integrin), weakly positive for CD71 (transferrin) and CD49f, and negative for E-Cadherin, Thy-1.2, and CD34 (hematopoietic marker) (Kawase et al., 2004).

1.6.1 SKPs are multipotent.

In addition to their ability to self-renew, SKPs can be differentiated into a variety of cell types, even following extensive expansion. As described below, SKPs are capable of differentiating into cell types of at least two embryonic lineages, ectodermal and mesodermal. These cell types
include neurons, Schwann cells, adipocytes and smooth muscle-like cells. Also, clonal SKP analysis showed that a single SKP cell can be amplified and that its progeny have the capacity to differentiate into both ectodermal and mesodermal cell types, illustrating their pluripotency.

1.6.2 SKPs differentiate into neuronal cells

SKPs can differentiate into neuronal cells following the withdrawal of growth factors FGF-2 and EGF in the presence of serum. Under these conditions, SKPs become adherent to the culture dish and a subset of the cells start to extend processes reminiscent of cultured neurons. Staining these cells for a variety of neural markers to determine their immunophenotype demonstrated their expression of neural markers including the pan-neuronal markers βIII-tubulin and neurofilament M, the peripheral neuron markers p75NTR, peripherin, and NCAM, and the catecholaminergic markers TH and dopamine-β-hydroxylase (Fernandes et al., 2006; Toma et al., 2001). The expression of these markers indicates that SKPs adopt a catecholaminergic phenotype in vitro. The presence of neurotrophins NGF, BDNF and NT-3 in the culture media increased the survival of these neural cells, which is also consistent with their neuronal phenotype (Fernandes et al., 2006). The neuronal differentiation of SKPs has been successfully repeated by a number of groups using rodent (Gingras et al., 2007), porcine (Dyce et al., 2004) and human skin (Hunt et al., 2008; Joannides et al., 2004; Toma et al., 2005).

To determine whether SKPs can adopt a CNS neuronal phenotype, Fernandes and colleagues hypothesized that the CNS environment contains factors that could induce differentiation of naïve SKPs into CNS neurons. To mimic the in vivo CNS environment, naïve and pre-differentiated SKPs were transplanted ex vivo into hippocampal slices. Under these conditions, naïve SKPs produced only rare cells that extended processes that stained positive for βIII-tubulin, and most transplanted cells disappeared over time probably due to cell death (Fernandes et al., 2006). Therefore, the CNS environment is not sufficient to support the survival of naïve SKPs. Alternatively, SKPs were pre-differentiated under neurogenic conditions for 1-2 weeks and then transplanted into hippocampal slices, into either the dentate gyrus (a neurogenic area) or the CA3 layer (a non-neurogenic area). Analysis, 4 to 6 weeks post-transplantation, showed no difference between the numbers of surviving transplanted SKPs in the two regions, however cells that were transplanted into the neurogenic dentate gyrus area, had migrated further from the transplant site than those in the CA3 (Fernandes et al., 2006). This is consistent with
the idea that newly born neurons of the dentate gyrus migrate out of the neurogenic zone, and suggests that pre-differentiated SKPs might be able to respond to at least some of these migratory cues. In addition, among the surviving cells 21% expressed the catecholaminergic marker TH, 39% expressed βIII tubulin, 78% expressed p75NTR and 23% expressed the P/Q-type voltage-gated calcium channel (Fernandes et al., 2006). In contrast, hippocampal cells did not express p75NTR or TH. Therefore, while the hippocampal environment does not promote the survival of naïve SKPs but allows pre-differentiated SKPs to survive, it does not promote the acquisition of a CNS phenotype by pre-differentiated SKPs as they mature into neuronal cells with the peripheral catecholaminergic phenotype. Interestingly, in vitro neuronal differentiation of SKPs in response to conditioned media from hippocampal astrocyte cultures resulted in a neural phenotype without expression of TH or choline acetyltransferase (Joannides et al., 2004). Further characterisation of these neuron-like cells is required to fully determine their phenotype, however, this finding raises the possibility that neuronal identity (peripheral vs CNS neurons) may be acquired very early during differentiation. It would be interesting to determine whether SKPs pre-differentiated in the presence of hippocampal astrocyte conditioned media would adopt a CNS neuron phenotype in either ex vivo or in vivo CNS transplants.

One key characteristic of mature neurons is their electrophysiological profile: a low voltage resting potential and the capacity to transmit electrical current in the form of an action potential (AP). To determine whether SKPs can generate mature neurons, the resting membrane potential and the capacity to generate an AP of SKP-derived neurons was compared to that of cultured sympathetic neurons (SCGs). The resting membrane potential of SKP-derived neurons was determined to be -26.4 ± 2.7 mV, which is significantly higher than that of sympathetic neurons (-45.8 ± 3.6 mV) (Fernandes et al., 2006). The injection of a step-wise current to cultured sympathetic neurons induced the generation of AP, however, no APs were observed from SKP-derived neurons - only a graded depolarization.

These results can be interpreted in two ways. First, when these recordings were performed, SKP-derived neurons were identified solely based on their morphology, which might not be sufficient to discriminate accurately between mature and immature neuronal cells. This may have resulted in the recording of a heterogeneous population of cells containing cells at different stages of differentiation, where the number of more mature neurons was potentially overestimated. The neuronal identity of these cells could have been confirmed by
immunological staining of the recorded cells for neuronal markers, or by the use of SKPs derived from an animal expressing a reporter gene such as GFP under the control of a neuronal promoter. A second explanation, that is not mutually exclusive with the first, is that SKPs can differentiate into neuron-like cells, based on their morphology and marker expression, but that they don’t generate mature neurons in vitro. This conclusion is also supported by the work of Collo and colleagues who conducted complementary experiments with cells generated using the same differentiation protocols. They demonstrated that SKP-derived neurons expressed GAD67, but not synapsin-1, which is consistent with immature synaptogenesis; lacked spontaneous or evoked APs; and were non-responsive to GABA, kainic acid and nicotine (Collo et al., 2006). Interestingly, they also showed that co-culture of these cells with astrocytes increased the number of dendritic branches and their size (Collo et al., 2006). This suggests that current differentiation protocols are deficient in factors required to induce or permit the neural differentiation of SKPs into a more mature phenotype. It would be interesting to perform the electrophysiological analyses on SKP-derived neurons transplanted into hippocampal slices as these cells have been shown to express the P/Q-type of voltage gated calcium channel, suggesting that these conditions may induce differentiation into a more mature phenotype.

The generation of neurons from SKPs is promising and provides hope that they may eventually prove useful in the cell therapy of neurodegenerative diseases. However, further work is required to optimise and improve the current differentiation protocols to obtain truly functional neurons. This research will also generate important contributions to the understanding of the processes involved in controlling the differentiation of immature neurons into electrophysiologically-mature neurons.

1.6.3 SKPs differentiate into glial cells

Only a small percentage (3-7%) of cultured SKPs can generate neurons under neuronal differentiation conditions (Toma et al., 2001). Therefore, the phenotype of other cells in these cultures was determined. It has been demonstrated that neural crest precursor cells can also give rise to glial cells both in vivo and in vitro (Le Douarin et al., 1991; Qian et al., 2000). To test this possibility, Toma and colleagues characterized the SKP cultures with regard to two glial markers, glial fibrillary acidic protein (GFAP) and CNPase. Double immunofluorescence staining for these markers revealed four populations of cells: (1) the double negative population
that represents many different cell types, including undifferentiated cells and neurons; (2) the 
GFAP positive and CNPase negative cells, which are potential astrocytes or early Schwann cells;
(3) the CNPase positive and GFAP negative cells, potentially oligodendrocytes or Schwann cells;
and (4) the double positive cells which had a bipolar morphology, potentially Schwann cells
(Toma et al., 2001). Unfortunately, these two markers in combination with the cell morphology
are not sufficient to definitively determine the phenotype of these cells. For example, Schwann
cells can adopt different morphologies in culture and express variable levels of these markers.
Staining for A2B5, a marker of oligodendrocyte precursors, revealed that some of the cells were
also positive for this marker, although this marker is not specific for this cell type. Thus, these
data indicated that a subpopulation of differentiated SKPs expresses glial markers, and the
identity and/or functionality of these cells remained to be determined. Subsequent work from the
Miller laboratory indicated that most if not all of these glial cells were peripheral Schwann cells,
consistent with the neural crest precursor phenotype of SKPs (see below). However, a study by
Belicchi and colleagues suggested that some of them may instead be astrocytes; these
investigators sorted CD133+ cells from human skin and grew them in SKPs condition until SKP-
like spheres formed. These cells were capable of differentiating into astrocytes, based on their
GFAP expression, under neurogenic differentiation conditions in vitro, and to generate GFAP+
cells in vivo when injected into NOD-SCID mouse brains (Belicchi et al., 2004). While the use of
more positive and negative markers will be required to confirm the astrocytic phenotype of these
cells, a number of questions were raised by this study. First, since immature Schwann cells
express GFAP, are these cells Schwann cells rather than astrocytes? Second, are the spheres
generated from CD133+ skin cells the same as SKP spheres? Spheres derived from these
CD133+ cells were nestin and c-kit positive (a marker expressed by melanocyte stem cells in the
skin), while SKPs are nestin positive but c-kit negative (Belicchi et al., 2004; Fernandes et al.,
2004). Therefore, a better characterisation of these CD133+ sphere forming cells and their
differentiated progeny will be required.

A substantial amount of work has been done to demonstrate that most, if not all, of the
glial cells generated by SKPs are peripheral Schwann cells. In particular, a detailed analysis of
their expression markers was carried out by Fernandes and colleagues (Fernandes et al., 2004).
In addition to expressing GFAP and CNPase, these bipolar cells were also positive for p75NTR
and S100β, which are normally expressed by Schwann cells. Moreover, elevation of
intracellular cAMP by the addition of forskolin to the media, induced the expression of myelin basic protein (MBP) and the peripheral myelin protein P0, as previously reported for cultured Schwann cells (Monuki et al., 1989). A protocol has been established to differentiate embryonic neural crest precursors into Schwann cells (Morgan et al., 1991; Shah et al., 1994). These optimised conditions, which include forskolin and neuregulin-1β in the absence of serum, were shown to induce the Schwann cell differentiation of SKPs (Biernaskie et al., 2006; McKenzie et al., 2006). Under these conditions, rodent SKPs formed arrays of bipolar cells positive for S100β, GFAP, p75NTR and the myelin proteins MBP and PMP22. SKPs have a high potential to differentiate into Schwann cells as 83% of clonal SKP spheres cultured in the presence of forskolin and neuregulin-β generated GFAP-positive bipolar cells. This differentiation is not restricted to rodent SKPs; human SKPs cultured under these conditions and supplemented with 1% serum also generated arrays of bipolar cells positive for S100β, p75NTR, GFAP with a subpopulation of these also positive for PMP22 or MBP. However, the number of differentiated cells was evaluated at 5% of the cells in culture, indicating a lower differentiation efficiency than that of rodent SKPs. While the current protocol still needs to be optimized for human SKPs, these results show that both rodent and human SKPs can generate Schwann cell in vitro.

The definitive evidence that these Schwann cell-like cells were in fact bona fide Schwann cells came from a series of functional assays. The principal in vivo function of Schwann cells is to associate with and/or myelinate the axons of peripheral neurons. SKP-derived Schwann cells (SKP-SCs) were therefore tested for their ability to myelinate axons in vitro. This was done by co-culturing SKP-SCs with peripheral neurons. Dorsal root ganglia (DRG) explants were isolated from shiverer mutant mice (McKenzie et al., 2006). These mice are genetically deficient in MBP, an important protein in myelin formation, especially in the CNS. SKP-SCs were derived from YFP-expressing transgenic mice and could, therefore, be distinguished from shiverer-derived cells. Co-cultures of these YFP-expressing SKP-SCs (YFP-SKP-SCs) with the shiverer DRG explants resulted in the association of YFP-SKP-SCs with the axons of the DRG neurons (McKenzie et al., 2006). The YFP-Schwann cells were positive for the myelin protein PMP22 and about half of the cells were also positive for MBP (McKenzie et al., 2006). This suggests that, in vitro, SKP-SCs constitute functional Schwann cells.
This *in vitro* conclusion was also supported by two *in vivo* experiments. The first experiment asked whether SKP-SCs could re-myelinate peripheral axons *in vivo*. In contrast to the central nervous system, the peripheral nervous system has the capacity for regeneration. For example, following injury of the sciatic nerve, the regenerating axons will associate with Schwann cells *de novo*. Using this model, purified YFP-SKP-SCs were injected into the sciatic nerve of adult *shiverer* mice, distal to the crush site. One to two months following surgery, many of the injected cells were present in the injured nerve, most of the YFP-cells were aligned longitudinally with the NFM positive axons, and many of these YFP-cells expressed MBP (McKenzie et al., 2006). The YFP-Schwann cells that were negative for MBP were GFAP-positive, indicating that these were non-myelinating Schwann cells. Similar results were obtained when naïve SKPs were injected into the crushed sciatic nerve. To confirm that the MBP-expressing cells were making *bona fide* compact myelin, electron microscopy was performed on sections of sciatic nerves four weeks following transplantation with naïve YFP-SKPs or YFP-SKP-SCs. In these sections, cells differentiated from both naïve SKPs and SKP-SCs formed MBP-positive compact myelin sheaths (McKenzie et al., 2006). These results show that SKPs can be differentiated into Schwann cells that can myelinate axons *in vivo*, and thus are truly functional Schwann cells.

A second set of experiments was carried out to determine whether the capacity of SKP-derived Schwann cells to myelinate axons was restricted to those of peripheral neurons. YFP-SKP-SCs were injected into *ex vivo* cerebellar slices of *shiverer* mice. In contrast to the PNS, in *shiverer* mice, the CNS is characterized by dysmyelination, therefore any myelin observed in these brains must result from the transplanted cells. In fact, the injected YFP-cells expressed the Schwann cell markers S100β and MBP, indicating the presence of myelinating Schwann cells in these slices (McKenzie et al., 2006). To address whether or not naïve SKPs could differentiate into Schwann cells and myelinate axons *in vivo* in the CNS environment, naïve YFP-SKPs were injected into the brains of newborn *shiverer* mice, at a developmental stage when myelination occurs in normal mice. YFP cells were still present in these brains four weeks post-transplantation. Electron microscopy of slices of the brains revealed the presence of compact myelin in the region where the YFP cells were transplanted, but not in adjacent regions (McKenzie et al., 2006). Interestingly, the same results were obtained from a parallel experiment in which human foreskin-derived naïve SKPs were injected into the brains of *shiverer* mice.
These results demonstrate that both human and rodent naïve SKPs can survive and differentiate into myelinating cells in the mouse brain, in contrast with the results from the attempts to induce neuronal differentiation of naïve SKPs in rat hippocampal slices (see above section). This difference might be explained by an absence of survival factors in the 7 day old-rat hippocampal slices that are present in the brains of newborn mice. These survival factors might only be available to SKPs which associate with unmyelinated axons and then differentiate into Schwann cells. In this scenario, those that fail to associate with axons would not receive these factors and thus would likely undergo apoptosis.

1.6.4 SKPs differentiate into Mesodermal cells

The embryonic mesoderm gives rise to a variety of structures and cell types that include adipocytes, skeletal muscle, smooth muscle, bone and cartilage cells. To test whether SKPs can differentiate into mesodermal cell types, SKPs cultured in the presence of serum but no growth factors, were stained for adipogenic and smooth muscle markers. Under these conditions, rodent SKPs differentiated into two mesodermal cell types: adipocytes and smooth muscle cells. The adipogenic phenotype of rodent SKP-derived adipocytes was characterized by the presence of lipid vesicles that stained positive with the lipophilic dye, oil-red-O (Toma et al., 2001). The adipogenic differentiation of human SKPs was only characterized by the presence of vesicles within the cells (Toma et al., 2005). To confirm these vesicles as lipid vesicles, oil-red-o staining would also need to be performed on these cells. In addition, staining for other markers such as FABP is required to confirm the adipogenic phenotype of the cells.

Smooth muscle cell differentiation was characterized by the expression of smooth muscle actin (SMA) and cellular morphology (Toma et al., 2001). However, staining for this marker alone can not distinguish between smooth muscle cells, pericytes that are perivascular cells associated with small blood vessels, and myofibroblasts, which are fibroblasts implicated in wound healing that express SMA (Eyden, 2008). Further characterisation of these putative smooth muscle cells is required and might include staining with other smooth muscle markers such as SM22α, smooth muscle myosin heavy chain, h-caldesmon and smoothelin (Owens et al., 2004). Their activity should also be tested in vivo by assessing their ability to associate and be functional in tissue where smooth muscles are normally present such as blood vessels and the intestine wall muscles. A complete evaluation of the ability of SKPs to generate mesodermal
cell types, would have to include an assay of whether SKPs can be differentiated into chondrocytes and osteocytes, two main cell types that are typically derived from the mesoderm in the trunk.

1.6.5 SKPs are located in the dermis and associated with hair follicles.

Having shown that SKPs can be isolated from the dermis compartment of the skin, the question of their precise location amongst the various structures present in the dermis remained. One approach to addressing this question is to determine which cells in the dermis express SKP markers, which include nestin, snail, slug and twist. To do this, embryonic and adult skin were analysed by in situ hybridisation for slug, snail and twist mRNA in skin sections at different ages (from E18 to P19) (Fernandes et al., 2004). At E18, cells expressing slug, snail and twist localised to the hair follicle papillae which were characterized by their expression of alkaline phosphatase; versican, a cell surface proteoglycan; and nexin, a matrix-modifying marker expressed during the anagen growth phase of the hair follicle (Fernandes et al., 2004). Importantly, the expression pattern of these markers does not overlap with the expression of keratin-17, which is a marker for the bulge epidermal stem cell niche and the outer root sheath (Panteleyev et al., 1997). Therefore, the cells expressing SKP markers are distinct from the epidermal stem cells located in the bulge. The expression of snail, slug, twist, nexin and versican were at their highest during the growth phase of the hair follicle (anagen phase), and reached their lowest levels during the telogen rest phase. The expression of alkaline phosphatase remained elevated throughout the cycle. During the late stages of embryonic development, whisker vibrissal papillae express slug, snail, twist, nexin, versican and Wnt5a (Fernandes et al., 2004). If SKPs originate from the papillae of the hair follicle or whisker vibrissal then one would predict that SKPs should express at least some of the papillae markers. Indeed, SKPs are positive for nexin, versican and Wnt5a (Fernandes et al., 2004). To further demonstrate that SKPs reside in the papillae, dissected papillae, were cultured under SKP-generating conditions. Whisker vibrissal papillae cultured under these conditions did generate SKP-like spheres (Fernandes et al., 2004). Similar results were obtained using rat whisker papillae and human facial hair (Hunt et al., 2008). In addition, dissected whisker papillae cultured under conditions conducive to neuronal differentiation generated nestin- and βIII-tubulin-positive cells that adopted the same neuronal morphology as those generated from SKPs (Fernandes et al., 2004; Hunt et al., 2008). They could also be differentiated into Schwann cells (Hunt et al., 2008).
These results strongly suggest that one endogenous niche for SKPs is the hair/whisker follicle papillae.

A final test of the hypothesis that SKPs reside in vivo in the papillae would be to determine whether cultured SKPs can induce the formation of new hair follicles in vivo. This was shown to be the case by Biernaskie and colleagues. They injected SKPs and epidermal cells into the dorsal skin of immunocompromised mice and assessed hair formation several weeks post-transplantation. Analysis of the injected skin revealed that passaged SKPs were capable of inducing the formation of new hair follicles in vivo (J.A.B., M.P. and F.D.M, in preparation). In addition, isolation of the dermal papillae of these newly formed hairs yielded SKP spheres in vitro and these SKPs retained their capacity to induce de novo hair follicles when subsequently injected in vivo (J.A.B., M.P. and F.D.M, in preparation). Taken together, these results demonstrate that the follicle papillae are one of the in vivo niches for SKPs.

In light of this finding, an intriguing question is: where are SKPs located in hairless skin? For example, SKPs can be generated from foreskin, which is hairless. Histology of the human foreskin would be necessary to determined whether immature hair follicle-like structures are present in this type of skin and whether they might contain SKP cells. Another possibility is that in hairless skin, SKPs are associated with other skin structures. Analysis of foreskin for expression of SKP markers will be important in the determination of the location of SKPs in this tissue.

1.6.6 The potential of SKPs in cell therapy

The finding that SKPs can differentiate into functional Schwann cells suggests the possibility that these SKP-SCs might have applications in a therapeutic setting. Multiple Sclerosis and spinal cord injuries are two conditions in which remyelination of axons could be beneficial. Focussing on spinal cord repair, the injection of Schwann cells into the injured spinal cord has been reported to be beneficial (Oudega and Xu, 2006; Xu et al., 1997). Currently, endogenous Schwann cells are harvested by peripheral nerve biopsy, an invasive process associated with the considerable problem of subsequent morbidity at the site of collection. This makes SKPs particularly attractive as an alternate source of Schwann cells. To test whether injection of SKP-
SCs into an injured spinal cord would provide a beneficial outcome, one must test whether SKP-SCs could (1) survive in the injured spinal cord environment, (2) myelinate or induce myelination of spared axons and (3) provide functional locomotor recovery with limited side-effects.

To address this first point, naïve SKPs or SKP-SCs were injected into an adult rat spinal cord injury, resulting from the contusion of the spinal cord, 7 days post-injury. As a comparison for survival, CNS neural stem cells grown as neurospheres, were injected into a subset of these animals. Naïve SKPs and SKP-SCs survived well in the injured spinal cord lesion cavity (Biernaskie et al., 2007), whereas the survival of neurospheres was severely compromised.

While both naïve SKPs and SKP-SCs survived the injury environment, the morphology of the grafts was different. SKP-SC grafts had no sharp demarcations between the graft and the host tissue, the cells had adopted a rostrocaudal alignment and many SKP-SCs integrated into the spinal cord (Biernaskie et al., 2007). Within the graft, transplanted cells were spindle-shaped and densely packed. Also, more spared tissue was observed in SKP-SC transplanted cords than in those injected with medium or neurospheres suggesting that SKP-SC had a neuroprotective effect on the remaining spinal cord. In contrast, lesions injected with naïve SKPs exhibited a sharp demarcation between the graft and host tissue and no predominant orientation of the transplanted cells was observed (Biernaskie et al., 2007). These grafts contained cells with a variety of cell morphologies, potentially indicative of differentiation into multiple cell types, and the cells were packed much more loosely than the SKP-SCs (Biernaskie et al., 2007). These observations suggest that while both naïve SKPs and SKP-SCs may survive well in spinal cord lesions, their effects on the host tissue differ substantially.

One biological response to spinal cord lesion is the formation of reactive gliosis or glial scar. This is predominantly composed of reactive astrocytes that will upregulate their expression of GFAP and secrete extracellular matrix proteoglycans such as chondroitin sulfate proteoglycans (CSPGs), which includes neurocan (Silver and Miller, 2004). These inhibit axonal growth and contribute to the barrier imposed by the astrocytic scar, thereby impeding neural regeneration. To test whether reactive astrocytes were present in the grafts, staining for GFAP and neurocan was performed on sections of the cords. This showed that naïve SKP grafts were surrounded with reactive astrocytes, whereas SKP-SC transplants were not (Biernaskie et al.,
In injured spinal cords injected with CNS-derived neurospheres, where a small amount of grafted cells survived, neurocan levels were also higher adjacent to the graft (Biernaskie et al., 2007). Therefore, of these three types of cells, naïve SKPs, SKP-SCs and neurospheres, it is the SKP-SCs which gave rise to the conditions most conducive to potential neuronal regeneration.

The absence of CSPG upregulation, which normally impedes axonal growth, in SKP-SC grafts, suggests the possibility that axonal regeneration can occur in these injured spinal cords. Analysis 12 weeks post-transplantation revealed that many SKP-SCs associated with βIII-tubulin or NFM positive fibres both within the transplant and surrounding it, and many of the axons present in the graft displayed a rostrocaudal orientation (Biernaskie et al., 2007). To determine whether the presence of these axons in the transplant were due to axonal sparing or axonal sprouting, Biernaskie and colleagues stained the transplanted cords, at different time points following the injection, for specific markers which specifically stain either ascending or descending fibres, such as TH for descending noradrenergic and dopaminergic axons, 5-TH for descending serotonergic axons, and CGRP for ascending sensory fibres. The latter were also labelled with Cholera toxin-B (CTB) which was injected into the sciatic nerve one week prior to sacrificing the animals. In SKP-SC injected cords, TH and 5-TH positive fibres invaded the rostral border between the transplant and the spinal cord. The sprouting was seen as far as 1 mm into the transplant. Similarly, CGRP and CTB positive axons were seen more than 1 mm into the transplant in a rostrocaudal orientation. These TH, 5-TH, CGRP or CTB positive axons found within the transplant site were associated with the SKP-SCs. Interestingly, the number of TH and 5-TH positive axons within the SKP-SC transplants increased over time, while no such increase was observed in animals injected with medium alone (Biernaskie et al., 2007). This demonstrates that SKP-SCs facilitated or induced axonal sprouting.

Similar analyses of spinal cords injected with naïve SKPs yielded substantially different results to those summarised above. Many fewer βIII-tubulin and NFM positive axons were present in the naïve SKP transplants than observed with SKP-SCs. Only a few descending axons penetrated the transplant by more than 0.5 mm and their orientation was somewhat random. On the other hand, ascending axons did not invade the graft and axonal growth occurred only along the host/transplant border. Since SKPs can differentiate into neurons in vitro, an analysis of co-expression of YFP and the neuronal markers βIII, NFM, TH and 5-TH was carried out. It
revealed that no naïve SKPs differentiated into neurons in these transplants (Biernaskie et al., 2007). This is in contrast with a previous study which reported that while 65% of similarly injected naïve SKPs were GFAP positive, 16% were positive for neurofilament, suggesting some neural differentiation (Gorio et al., 2004). However, the combination of techniques used to label the injected cells and to analyse the subsequently stained tissues in this study may not have been sensitive enough to distinguish between a differentiated SKP wrapped around an axon and the axon itself. Interestingly in the Biernaskie study, a number of the transplanted naïve cells differentiated into inappropriate cell types such as cells expressing the mesodermal marker SMA or type-II collagen (a marker for chondrocytes) and some with adipocyte morphology (Biernaskie et al., 2007). This is in contrast with the SKP-SC transplants, in which none of these cell types were observed.

The results from the analyses of neurosphere transplants were in sharp contrast to both the naïve SKP and the SKP-SC data. Descending axonal growth was not observed and very limited ascending axonal sprouting was observed in the vicinity of the transplant. This outcome may be a consequence of the limited survival of neurosphere cells.

In the SKP-SC and naïve SKP transplants, 35% and 15% of the YFP cells, respectively, were positive for the peripheral myelin protein P0. Taken together with the data discussed above, these results demonstrate that transplanted naïve SKPs or SKP-SCs can generate myelinating Schwann cells that contribute to the maintenance of the spared tissue and allow or induce the growth of axons into the transplanted area. This data, therefore, predicts that injection of naïve SKPs or SKP-SCs should result in some functional recovery of transplanted injured animals. This was assessed by monitoring their locomotor recovery and their sensory thresholds for mechanical and heat-induced pain. In most animals injected with SKP-SCs, naïve SKPs or neuropheres, hindlimb stepping was re-established by 7 weeks post-transplantation. Remarkably, almost half of the animals injected with SKP-SCs also displayed at least occasional coordinated forelimb/hindlimb stepping, an important hallmark in spinal cord recovery, whereas only a few of the naïve SKP or neurosphere injected animals displayed this behaviour. In addition to gross motor recovery, examination of specific locomotor components such as paw position, toe clearance, trunk control and tail position, showed that at 7 and 9 weeks post-transplantation, SKP-SC injected animals showed a persistent functional recovery that was not observed in the neurosphere or naïve-SKP injected groups. Furthermore, animals injected with
SKP-SCs were less sensitive to mechanical and heat pain when compared to pre-injury data and to animals injected with either naïve SKPs or neurospheres. In contrast, animals injected with naïve SKPs were more sensitive to heat, compared to pre-injury data, and displayed a transient increase in mechanical pain sensitivity. This suggests that animals injected with naïve SKPs, but not SKP-SCs, had developed an overly sensitive pain response.

Thus it has been convincingly demonstrated that SKPs can be differentiated into Schwann cells that are functional and that can induce functional recovery in vivo in a spinal cord injury model. While these results are very promising, a number of important questions remain. Would SKP-SCs also be capable of inducing functional recovery of spinal cords that have been injured for weeks or months before the transplantation is performed? Would heterologous grafts of SKP-SCs be rejected by the host’s immune system? While this study suggests that the formation of tumours by SKP-SCs is unlikely during the first 12 weeks following injection, it will be important to determine whether tumours may arise over a more extended period.

1.6.7 SKPs differ from MSCs and neurospheres

The capacity of SKPs to self-renew and differentiate into a variety of cell types characterizes them as precursor cells. Other precursor or stem cells that have been identified from various tissues include neurospheres and MSCs. One intriguing question is how SKPs compare to these other precursor cells? Since the same culture conditions are used to isolate SKPs and neurospheres, and both can be differentiated into neuronal cell types, a comparison of SKPs with these CNS neural precursor cells promised to be interesting. SKPs were shown to express the intermediate filament nestin (a marker of neuronal precursors), the extracellular matrix protein fibronectin, and Scal but not p75NTR, while neurospheres expressed nestin and p75NTR, but not fibronectin or Scal (Fernandes et al., 2004). The transcription factors, *slug*, *snail*, *twist*, *Pax3*, *sox9*, *Dermo-1* and *SHOX2*, were analysed by RT-PCR in these two cell types. While *Sox9* is expressed in both cell types, neurospheres express very low or undetectable levels of *slug*, *snail*, *twist*, *Pax3*, *Dermo-1* and *SHOX2*. It is also important to recall that naïve SKPs have a better survival rate than neurospheres when injected into an injured spinal cord (Biernaskie et al., 2007). Therefore, based upon these differences in the expression of protein markers and the in vivo transplant data, it can be concluded that SKPs constitute a precursor cell that is distinct from CNS neurospheres.
On the other hand, work comparing SKPs with mesenchymal stem cells is somewhat limited. In the first report of SKPs by Toma and colleagues, immunofluorescence observations indicated that MSCs were positive for vimentin, fibronectin and occasional cells were positive for cytokeratin but negative for nestin (Toma et al., 2001). In contrast, SKPs were positive for nestin and fibronectin but negative for cytokeratin. SKP cells were also observed to be smaller and less flattened than MSCs. Based on these observations, it was suggested that SKPs and MSCs constitute distinct cell populations (Toma et al., 2001). SKPs are grown in the absence of serum, while MSCs are cultured in its presence. This difference in culture conditions may contribute to the differences in morphology and marker expression. Consistent with this hypothesis, Shih and colleagues reported that dermal cells cultured in serum in the presence of EFG and FGF adopted a less flattened and more fibroblast-like morphology than MSCs, but when grown in the absence of growth factors they became larger, flattened and more extended (Shih et al., 2005). To determine whether the differences observed between SKPs and MSCs is reflective of their culture conditions, MSCs were cultured under SKP conditions. The MSCs were found to survive, but did not proliferate or form floating spheres, while sister cultures grown in the presence of serum exhibited normal growth. These results support the conclusion that SKPs and MSCs are different from one another. A complementary comparison would be that of SKPs and MSCs, both cultured under MSC conditions. While a number of existing reports have attempted to address this question (Hoogduijn et al., 2006; Shih et al., 2005), the presence of serum in the initial isolation protocols, which could result in the selection of a different population of cells than those isolated in absence of serum, precludes a strict comparison. An alternative approach would be to compare the transcriptomes of SKPs and MSCs. Such an analysis is currently being performed by a graduate student in Dr. Miller’s laboratory, Hiroyuki Jino, using gene chip array technology. His initial results support the conclusion that SKPs and MSCs are distinct precursor cells (H.J. and F.D.M., in preparation and personal communication). In summary, all currently available data on MSCs and SKPs suggest that these two cell types are distinct, but possess some overlapping properties.

1.6.8 Embryonic origin of SKPs

Another intriguing question about SKPs concerns their embryonic origin. A number of lines of evidence suggest a neural crest origin for SKPs. First, SKPs express multiple neural crest markers, including slug, snail, and twist. Second, they can differentiate into many cell types that
neural crest cells also generate. Third, the neural crest forms the dermis of the face and the
dermal papillae of the whiskers (Sieber-Blum et al., 2004), both locations in which SKPs are
found. Finally, SKPs can first be isolated from embryonic skin at E15 in mouse, a time point
which is subsequent to the first migration of neural crest cells into the skin. To determine
whether SKPs are truly derived from neural crest cells or coincidentally share similarities with
neural crest cells but are derived from another embryonic tissue, lineage tracing experiments
were conducted. To trace neural crest cells and their progeny, transgenic mice expressing cre
recombinase under the control of the Wnt1 promoter, which is transiently expressed by neural
crest cells (Echelard et al., 1994), were crossed with R26R mice to generate offspring that will
express the LacZ transgene constitutively following the excision of a genetic element by the cre
recombinase (Chai et al., 2000). This lineage tracing strategy allows the labelling of neural crest
cells and all their progeny during development. Using this technique, SKPs derived from whisker
papillae or pelage hair follicle were shown to be derived from neural crest cells (Fernandes et al.,
2004; Hunt et al., 2008; Wong et al., 2006).

On the other hand, the origin of the SKPs found in back skin remains to be determined.
Analysis of the back skin of the Wnt1-cre/R26R mice show a low level of transgene expression
in hair follicle papillae which was attributed to low transgene penetrance (Fernandes et al.,
2004). This conclusion seems unlikely, since another group obtained similar results using two
different compound mice, Wnt1-cre/R26R and human tissue plasminogen activator (Ht-PA)-
cre/R26R mice, in which cre recombinase is expressed in neural crest cells independently from
Wnt1 expression (Wong et al., 2006). This suggests that at least some cells composing the
dermal papillae in the back skin are derived from a different embryonic origin. Interestingly,
other populations of cells associated with the hair follicle were reported to originate from the
neural crest and to form SKP-like spheres in vitro (Wong et al., 2006). However, these cells
were positive for p75NTR and Sox10, two markers that are not expressed in SKPs (Fernandes et
al., 2004; Wong et al., 2006). Therefore, the identity of the cell of origin for back skin-derived
SKPs remained an open question until very recently. Jinno and colleagues performed a lineage
tracing experiment using the Myf5-cre:YFP mouse, in which YFP is expressed in Myf5 positive
cells of the somites and their derivatives, and showed that back skin dermis and follicle papilla
are derived from somites (Jinno, Biernaskie, Miller, unpublished data).
1.6.9 SKPs are related to neural crest stem cells

The characterization of SKPs has provided four lines of evidence that SKPs are related to neural crest cells. First, at the gene expression level, SKPs express genes that are expressed by several populations of neural crest cells. These genes include twist, slug, snail, Pax3, Sox9 SHOX2, and Dermo-1 (Fernandes et al., 2004). Second, the differentiation potential of SKPs and neural crest cells is similar, both can generate neurons, Schwann cells, adipocytes and smooth muscle cells (Fernandes et al., 2004). One interesting observation concerning the neuronal differentiation is the capacity of SKPs to generate TH-positive catecholaminergic neurons (Fernandes et al., 2006). This is a type of neuron that cultured murine and avian neural crest cells can efficiently differentiate into, with up to 100% of colonies generating this neuronal type in vitro (Cohen, 1977; Ito et al., 1988; Sieber-Blum, 1989). Neural crest cells can also generate bone and cartilage, two mesodermal derivatives that have not yet been investigated as potential derivatives of SKPs. Third, neural crest cells give rise to the dermal papillae of the whisker pads (Sieber-Blum et al., 2004), one of the niches from which SKPs can be isolated, thus the facial SKPs are also derived from neural crest cells. Finally, if SKPs are related to neural crest cells, then they should also behave like neural crest cells in vivo. To test this hypothesis, SKPs generated from YFP mice, were injected into the chick neural crest migratory stream in ovo, at a developmental time point when endogenous neural crest cells are migrating to their targets. Analysis of these embryos three days following injection showed that SKPs had migrated to many neural crest targets, including spinal nerves, dorsal root ganglia (DRG), peripheral nerves and the vicinity of the sympathetic ganglia (Fernandes et al., 2004). A population of SKPs found in the DRG and the spinal nerve stained positive for S100β, a marker for Schwann cells (Fernandes et al., 2004). Some YFP positive cells were also present in the dermal layer of the skin. This fourth line of evidence, along with the preceding three, demonstrates that SKPs express neural crest markers, and behave like neural crest cells both in vitro and in vivo. Therefore one may conclude that SKPs are related to neural crest cells.

1.7 Rationale, hypothesis and objectives

Previous work discussed above showed that SKPs are multipotent precursor cells. Their neural differentiation has been well described, but their known mesodermal differentiation potential is limited to their in vitro differentiation into adipocytes and smooth muscle cells. Therefore, one
interesting question related to their differentiation potential remained unanswered. Are SKPs capable of differentiating into skeletal mesodermal cell types?

Previous work on SKPs showed their relatedness to NCSCs. This conclusion was based on these four observations: (1) SKPs differentiate into cell types that are derived from NCSCs; (2) SKPs express neural crest cell markers; (3) SKPs migrate into the same location as neural crest cells when injected in chicken embryos; and (4) SKP derived from the whisker pad are derived from the neural crest. The combination of these similarities between SKP and NCSCs and the observation that neural crest stem cells generate the mesodermal tissues, including the skeletal structures of the face, led me to hypothesize that SKPs have the potential to differentiate into skeletal mesodermal cell types.

This hypothesis was tested using two different strategies. First, the mesodermal differentiation potential of rodent and human SKPs was determined in vitro. Specifically, the osteogenic and chondrogenic differentiation potential of SKPs was determined using culture conditions developed to differentiate ex vivo amplified MSCs into these two cell types. This differentiation capability of SKPs may be restricted to a sub-population of SKPs. To test this possibility, the multipotentiality of SKPs with regard to mesodermal differentiation was assessed. Second, the mesodermal differentiation ability of SKPs was determined in vivo. Specifically, SKPs were transplanted into a bone fracture, which is an environment rich in osteogenic and chondrogenic differentiation signals. Skeletal differentiation of SKPs was assessed by determining the expression of osteogenic markers, their morphology, location and integration into the newly formed bone matrix. The results of these experiments are presented in chapter II.

The combination of these in vivo results with those previously published on the injection of SKPs into peripheral nerve suggests the following hypothesis: the differentiation of SKPs is influenced by their environment. To test this hypothesis, two approaches were used. First, the phenotype of SKPs injected into the bone fracture that have leaked out of the injection site and that were located in skeletal muscles and connective tissue adjacent or in close proximity to the fractures was determined. Second, naïve SKPs were injected into an adipogenic environment, the hypodermis of the skin, and their phenotype was then assessed. The results of experiments testing that second hypothesis are presented in chapter III.
Chapter 2
Skeletal differentiation of Skin-derived precursor cells

2.1 Introduction

One fascinating aspect of stem cells is their ability to differentiate into various cell types. In the embryo, neural crest stem cells originate from the neural crest of the developing neural tube and migrate extensively throughout the embryo generating a variety of cell types. Some examples of these tissues include the bones, cartilage, and dermis of the face, the adipocytes of the salivary gland, and the entire peripheral nervous system (Billon et al., 2007; Le Douarin, 1982). Neural crest stem cells also give rise to the first wave of mesenchymal stem cells (MSCs) in the embryo (Takashima et al., 2007), which can differentiate into osteocytes, chondrocytes, and adipocytes (Pittenger et al., 1999).

In contrast to stem cells, progenitor cells have a more restricted differentiation repertoire and less ability to self-renew. In this regard, if the differentiation and self-renewal potential of a progenitor/stem cell are less well-defined, then the term precursor is frequently used, since this term encompasses both stem cells and progenitors. The laboratory of Dr. F.D. Miller has previously isolated and characterized a population of multipotent precursors from rodent and human skin, that they named SKin-derived Precursors (SKPs) (Toma et al., 2001; Toma et al., 2005). These cells reside in the dermis and one of their niches is the hair follicle (Fernandes et al., 2004). SKPs have been shown to share many properties with neural crest stem cells (Fernandes et al., 2004). These properties include the expression of neural crest cell gene markers (Fernandes et al., 2004); migration along the neural crest migratory pathways and to neural crest derived structures when transplanted into the neural tube of chicken embryos at a developmentally appropriate time (Fernandes et al., 2004); and differentiation into cell types normally derived from neural crest stem cells such as peripheral neural cells, including Schwann cells (Biernaskie et al., 2006; McKenzie et al., 2006) and catecholaminergic neurons (Fernandes et al., 2006), and mesenchymally-derived cell types such as smooth muscle cells and adipocytes (Toma et al., 2001; Toma et al., 2005). However, it is unknown whether SKPs can differentiate into skeletal mesodermal cell types as neural crest cells do in the face.
One potential use of multipotent precursor cells is in cell-based therapies. SKPs present a particularly attractive potential source of cells for such applications as their isolation is not invasive, skin being a readily accessible tissue, and since SKPs are present in neonatal and adult human skin (Toma et al., 2005), they represent a potentially autologous source of cells. The laboratory of Dr. Miller has previously shown that SKPs can generate large numbers of myelinating Schwann cells (McKenzie et al., 2006), and subsequent to their transplantation into a contused spinal cord these cells promoted functional recovery (Biernaskie et al., 2007). These results suggest that, under the right conditions, it may be possible to engineer the differentiation of SKPs into a wide variety of physiologically functional cell types for specific therapeutic applications.

One field in which precursor cells are being studied for their clinical potential is in the treatment of skeletal defects or damage. MSCs are currently the precursors of choice for this work as they can differentiate into osteoblasts and chondrocytes (Pittenger et al., 1999). A series of in vivo studies addressing the potential use of bone marrow-derived MSCs for the treatment or repair of bone and cartilage defects (reviewed in (De Bari and Dell'accio, 2007; Mauney et al., 2005)) suggest that such cell therapy is indeed a promising strategy.

Based upon the ability of SKPs to generate many neural crest derivatives and the fact that neural crest cells generate the bone and cartilage structures of the face, I hypothesized that SKPs can differentiate into osteoblasts and chondrocytes. In this chapter I show that rodent and human SKPs can efficiently generate these skeletogenic derivatives in culture, and that they even participate in bone healing when transplanted into a fractured bone. SKPs, therefore, represent an accessible, potentially autologous source of precursor cells for bone and cartilage repair.

2.2 Author contribution

The bulk of the results presented in this chapter was published in Stem Cells and Development (Lavoie et al., 2008) and constitute figures 2.1 to 2.4, 2.7 to 2.10 and 2.12. Figures 2.5, 2.6, 2.11 and 2.13 are initial data that further support the conclusions of this chapter. My contribution to this article (Lavoie et al., 2008) consisted of the experimental design, generation and analysis of the data, writing of the manuscript and addressing reviewers’ comments. The figures and manuscript of this published work were reorganized in this and the following chapter (Chapters II and III) to facilitate clarity and to allow the insertion of additional supporting data. All
experiments described in this chapter were performed or repeated by me or performed in collaboration with the indicated people (see below). Only a few panels presented here (Figure 2-4B) and in the original article were generated by Dr. Jeffrey Biernaskie.

Figure 2.5 is the result of a collaboration between Hiroyuki Jinno (graduate student in Dr. F.D. Miller’s laboratory) and me. I differentiated the cells, harvested the RNA and made the cDNA. I tested the quantity and quality of the RNA extraction, and determined the quality of the reverse transcriptase reaction by running RT-PCR for GAPDH to detect the presence of any contaminating genomic DNA. I also made the list of primers from the published literature. Hiroyuki revised this list and performed the RT-PCR presented here. In Figure 2.6 the SKPs derived from breast skin were isolated and grown by Dr. Joseph M. Antony (post-doctoral fellow in Dr. F.D. Miller’s laboratory). I performed the differentiation and analyses of the differentiated cells.

2.3 Results:

2.3.1 Rodent SKPs differentiate into mesenchymally-derived cell types in culture

To determine whether rodent SKPs can generate osteoblasts, neonatal murine SKPs were cultured for varying periods of time under conditions shown to promote the osteogenic differentiation of MSCs, that is in the presence of serum, dexamethasone, ascorbic acid and beta-glycerophosphate (Pittenger et al., 1999). Cells were cultured either as monolayers or as micromass cultures. Micromasses were generated by centrifugating dissociated SKPs in microcentrifuge tubes as previously described (Johnstone et al., 1998). For comparison, SKPs were also cultured under basal differentiation conditions, which consisted of serum but no other differentiation factors. Osteogenic differentiation was detected by analyzing these cultures for the typical hallmarks of an osteogenic phenotype: alkaline phosphatase (ALP) activity, expression of type-I collagen, the major bone matrix protein, and mineral deposits. Following six to eight weeks in osteogenic conditions, SKPs cultured as a monolayer formed numerous clusters or nodules of cells that contained ALP-positive cells (Fig. 2.1A), similar to nodules formed when MSCs were differentiated into osteocytes (Pittenger et al., 1999). These nodules were also immunopositive for type-I collagen, (Fig. 2.1B), and were mineralized, as detected by staining with Alizarin red (Fig. 2.1C) or with von Kossa's stain (data not shown). In contrast,
Figure 2.1: Rodent SKPs differentiate into osteogenic cell types in culture. (A-C) Photomicrographs of monolayer cultures of neonatal murine (A,B,C left panels) or rat (C, right panels) SKPs differentiated for 6 weeks under osteogenic conditions (Osteo) and either stained for alkaline phosphatase activity (A, ALP, n = 4 samples), immunostained for type-I collagen (B, Coll-I, green, n = 2 samples) or stained to detect mineralization with Alizarin red (C, n = 4 samples). In all cases, SKPs were differentiated for the same period of time in serum alone as controls (Con). In (A,C), arrows denote nodules of positive cells. (D) Micromass cultures of neonatal murine SKPs differentiated for 7 weeks in osteogenic conditions (Osteo), and then stained with Alizarin red (n = 2 samples). Sister cultures were differentiated in serum alone as controls (Con). In (B), cells were stained with Hoechst 33258 (blue) to show cell nuclei. For panels A, C, and D, scale bars = 100 μm and for panel B scale bars = 50 μm.
Figure 2.2: Rodent SKPs differentiate into chondrogenic cell types in culture. (A,B) Photomicrographs of monolayer cultures of neonatal murine SKPs differentiated for 8 weeks under chondrogenic conditions (Chondro), and either immunostained for type-II collagen (A, Coll-II, green) or stained for chondrogenic proteoglycans using Alcian blue (B). As controls, SKPs were differentiated for the same period of time in serum alone (Con). (C) Micromass cultures of neonatal murine SKPs differentiated for 5 weeks in chondrogenic conditions (Chondro) and immunostained for type-II collagen (Coll-II, green) or stained with Alcian blue. In (A), cells were stained with Hoechst 33258 (blue) to show cell nuclei. For panel A scale bars = 50 μm and for panels B and C, scale bars = 100 μm.
sister cultures grown in serum-only conditions did not form cell aggregates and were negative for ALP activity and mineral deposits, and expressed very low levels of type-I collagen (Fig. 2.1A-C). Osteogenic differentiation of mouse SKPs differentiated in micromass cultures also showed mineralization throughout the micromass, as detected by Alizarin red (Fig. 2.1D). Similar results were obtained with embryonic mouse or neonatal rat SKPs (Fig. 2.1C). Thus, rodent SKPs have osteogenic potential.

To establish whether or not rodent SKPs can also generate chondrocytes, differentiation experiments were carried out under chondrogenic conditions similar to those previously defined for MSCs (Schmitt et al., 2003); neonatal murine SKPs were cultured in either monolayer or micromass cultures in the presence of serum, dexamethasone, BMP-2 and ascorbic acid for four to eight weeks. These cultures were then analyzed for expression of the cartilage-specific type-II collagen, and for chondrocytic proteoglycans with Alcian blue. In all samples tested (n = 4) SKPs differentiated in monolayers for four to eight weeks formed many nodules that immunostained positively for type-II collagen (Fig. 2.2A), and were Alcian blue-positive (Fig. 2.2B). Qualitative analysis showed that virtually all cell nodules in these cultures were positive for these markers. However, the quantitation of the exact efficiency at which cartilage nodules formed per plated cells remains to be performed. Similar results were obtained with micromass cultures (Fig. 2.2C). In contrast, no positively-stained cell aggregates were observed in the serum-only conditions (Fig. 2.2A,B). Thus, rodent SKPs can differentiate into cells of the chondrogenic lineage.

2.3.2 Neonatal human SKPs differentiate into osteocytes and chondrocytes in culture

To ask whether human SKPs can also generate osteoblasts, multiple samples (10 samples, one sample per patient) of neonatal human foreskin SKPs were differentiated as monolayer cultures for three to six weeks under the same conditions defined as osteogenic for rodent SKPs. After three weeks very few cell clusters were observed, but by six weeks more were present. The total number of clusters varied from sample to sample. Analysis of ALP activity revealed that it was higher in human SKPs cultured in osteogenic conditions than in those grown in serum alone (Fig. 2.3A, B). Cells expressing active ALP grew as monolayers or in nodules (Fig. 2.3B). Immunocytochemistry at four weeks showed that the increased ALP activity was accompanied
Figure 2.3: Human SKPs differentiate into osteogenic cell types in culture. (A,B) Photomicrographs of foreskin-derived human SKPs differentiated in monolayer cultures for 3 (A) or 6 (B) weeks under osteogenic conditions (Osteo), and then either stained for ALP activity or for mineral deposits using Von Kossa's stain (A) or Alizarin red (B). Mineralized deposits were found associated with both small clusters of cells (B, bottom left panel, arrow), and with nodules (A, bottom left panel; B, bottom middle panel, arrows). Human SKPs were differentiated for the same period of time in serum alone as controls (Con). (C) Photomicrographs of human SKPs differentiated as monolayers under osteogenic (Osteo) or control (Con) conditions for 4 weeks, and then double-labelled for alkaline phosphatase (ALP) and osteopontin (OP), with nuclei counterstained with Hoechst 33258 in the merged image. The top three panels show the same field, as do the bottom three panels. Arrows denote a cluster of cells positive for both markers. (D) Human SKPs differentiated for 4 weeks as monolayers in osteogenic (Osteo) or control (Con) conditions, and immunostained for either type-I collagen (Coll-I, red) or osteocalcin (OC, red) and counterstained with Hoechst 33258 (blue). Arrows denote clusters of positive cells. Scale bars = 100 μm.
Figure 2.4: Human SKPs differentiate into chondrogenic cell types in culture. (A) Monolayer cultures of human SKPs differentiated for 4 weeks under chondrogenic (Chondro) or control (Con) conditions, and either stained for chondrocyte proteoglycans using Alcian blue (left panels) or immunostained for type-II collagen (Coll-II, red) and counterstained with Hoechst 33258 (blue; right panels). Arrows denote clusters of positive cells. (B) Micromass cultures of human SKPs differentiated for 4 weeks under chondrogenic conditions, and either stained with Alcian blue or immunostained for type-II collagen (Coll-II). Scale bars = 100 μm.
by increased ALP protein, and that ALP-positive cells also expressed osteopontin (Fig. 2.3C). Moreover, similar patches of cells expressed two additional osteogenic markers, type-I collagen and osteocalcin (Fig. 2.3D). After three weeks both Von Kossa (Fig. 2.3A) and Alizarin red (data not shown) stains demonstrated mineral deposits associated with some of these nodules. These deposits were more abundant by six weeks and localized both to cells in nodules, and to some that grew in monolayers (Fig. 2.3B).

To determine whether human SKPs can generate chondrocytes, they were cultured as monolayers under conditions defined as chondrogenic for rodent SKPs. By four weeks, these cultures contained many Alcian blue-positive nodules (Fig. 2.4A). Immunostaining revealed that cells within these nodules also expressed type-II collagen (Fig. 2.4A). Qualitative analysis showed that virtually all cell nodules were positive for these markers, although the intensity of the signal varied from cluster to cluster of cells. Similar results were obtained with cells in micromass cultures (Fig. 2.4B). In contrast, only occasional small clusters of Alcian blue-positive cells were seen in the serum-alone conditions (data not shown). Thus, human neonatal SKPs can differentiate into both osteocytes and chondrocytes, in culture.

Preliminary data were also generated to confirm the osteogenic and chondrogenic differentiation of neonatal human SKPs at the mRNA level using RT-PCR. These results were generated from one sample and at only one time point per condition. Human SKPs were differentiated for four weeks in chondrogenic, or six weeks in either osteogenic or control, conditions. The expression of the chondrocyte gene encoding aggrecan, and the two osteogenic genes encoding type-I collagen, and ALP were analysed by RT-PCR. The data showed the presence of aggrecan mRNA in human SKPs differentiated in chondrogenic conditions (Fig. 2.5A, RT”+” lanes). The presence of the transcript also in the control conditions is consistent with the observation that a few small cell clusters did form in this condition. Real time RT-PCR will be required to precisely determine the relative increase in aggrecan gene expression resulting from different differentiation times and conditions. Nonetheless, this result supports the conclusion that human SKPs can differentiate into chondrogenic cells.

The same SKPs sample, differentiated under osteogenic and control conditions, was analysed for the expression of the osteogenic markers type-I collagen and ALP. RT-PCR analysis revealed the expression of mRNA for these two markers in cells cultured under
Figure 2.5: Human SKPs express skeletogenic gene markers following mesodermal differentiation. (A-C) RT-PCR analysis for skeletogenic markers on human SKPs differentiated for 4 weeks under chondrogenic (Chondro), or 6 weeks under either osteogenic (Osteo) or serum alone conditions (Con). (A) RT-PCR for the chondrogenic gene marker Aggrecan. (B) RT-PCR for osteogenic gene markers type-I collagen (Coll-I) and alkaline phosphatase (ALP). (C) RT-PCR for GAPDH as a loading control. RT + represents mRNA sample treated with reverse transcriptase and RT – represents the same sample not treated with reverse transcriptase used to detect genomic DNA contamination. H2O represents reactions with no template.
osteogenic conditions (Fig. 2.5B), confirming the immunofluorescence data (Fig. 2.3C,D). The presence of PCR products corresponding to these two markers in cells differentiated under control conditions is consistent with the presence of occasional clusters of mineral deposits in these cultures. The input of equal amounts of cDNA was demonstrated for these reactions by performing RT-PCR for GAPDH on all samples (Fig. 2.5C). To confirm that the RT-PCR results were due to gene transcription and not the presence of genomic DNA, analyses were also performed on the cell lysates used to generate cDNAs but without the addition of the reverse transcriptase. This revealed the absence of genomic DNA from all samples (Fig. 2.5A-C, RT “–“ lanes).

This analysis remains incomplete as more samples must be analysed to impart true significance to the results. However, the initial data described here support the conclusion that human SKPs can differentiate into chondrocytes and osteoblasts in vitro.

2.3.3 Human breast skin derived SKPs differentiate into osteogenic cells in vitro.

To establish whether the osteogenic and chondrogenic differentiation potential of human SKPs is limited to those derived from neonatal foreskin, SKPs derived from one adult breast skin sample from a women undergoing breast reduction surgery, were cultured in osteogenic, chondrogenic or control conditions. Osteogenic differentiation was assessed by determining the level of ALP activity and the presence of mineral deposits. ALP activity was up-regulated in cells cultured under osteogenic conditions compared to those under serum-only conditions (Figure 2.6A). Mineral deposits that stained positive with Alizarin red or Von Kossa were present in cells differentiating in osteogenic medium, but were absent from those cultured in control conditions (Figure 2.6A, center and right panels). These results suggest that SKPs derived from adult skin, and isolated from sources other than foreskin, are also capable of differentiating into osteogenic cells. Since these results were obtained using only one sample, it will be important to repeat this experiment using samples derived from a wide range of skin locations and from patients of different genders and age groups. This would determine whether the osteogenic potential of SKPs is conserved amongst SKPs derived from various sources.
Figure 2.6: Non-foreskin-derived human SKPs differentiate into mesodermal cell types in vitro. (A-B) Breast skin-derived SKPs isolated from one skin sample and cultured in standard SKP conditions were then differentiated in monolayers under osteogenic (osteo) (A), chondrogenic (chondro) (B) or serum alone (Con) (A-B) conditions for 5 weeks. (A) Osteogenic differentiation was determined by staining for ALP activity (left panels) and for mineral deposits using Von Kossa’s stain (center panels) and Alizarin red (right panels). Arrowheads denote ALP-positive cells and arrows denote mineral deposits. (B) Cells from the same culture as in (A) differentiated in chondrogenic conditions and stained with oil-red-O and hematoxylin. Arrows denote adipocytes positive for lipid vesicles. In A, scale bars = 100 μm and in B scale bars = 50 μm.
The same breast skin-derived SKPs sample was also cultured as a monolayer under chondrogenic or control conditions, as described for the human foreskin-derived SKPs. In this experiment no cartilage nodules were formed under either condition. This experiment must be repeated using the micromass culture technique, and with multiple samples to increase its significance. Interestingly, among cells differentiated under chondrogenic conditions, three to five cells per well were observed that stained positive with oil-red-O, suggesting their adipocyte phenotype (Figure 2.6B). This result remains to be confirmed and quantified using adipogenic differentiation conditions, but it suggests that human SKPs derived from skin locations other than foreskin may have an adipogenic potential.

2.3.4 SKPs are multipotent with regard to osteogenic and chondrogenic differentiation

To determine the proportion of individual SKP spheres that are bipotent with regard to the chondrogenic and osteogenic lineages, human SKP spheres (1-3 passages), from low density cultures (50 000 cells/ml) were isolated and individual spheres were differentiated in osteogenic, chondrogenic or serum-only conditions for four to six weeks. Osteogenic differentiation was assessed by staining mineral deposits with Alizarin red (Fig. 2.7A), 78.9 ± 4.0% (mean ± standard error) of the spheres displayed Alizarin red-positive nodules. In serum-only conditions 18.1 ± 10.0% of the spheres produced small clusters of mineralized cells, indicating that some osteogenic differentiation of human SKPs occurs even in serum alone. Following chondrogenic differentiation, cells were stained with Alcian blue (Fig. 2.7B); 66.8 ± 7.2% of human SKP spheres generated Alcian blue-positive nodules while 14.6 ± 5.8% of the serum-only spheres generated small, Alcian blue-positive cell clusters. Since 65-80% of human SKP spheres can differentiate into osteogenic or chondrogenic phenotypes, then one may deduce that at least a subpopulation of SKP spheres must be bipotent.

To establish whether individual SKP cells are also bipotent, clonal cells were generated by plating adult rat skin cells at a very low density (1000 skin cells/ml). Fernandes and colleagues have previously shown that clonal primary SKP spheres are generated at a plating density of 25 000 skin cells/ml (Fernandes et al., 2004). These clonal SKP spheres were then
Figure 2.7: Most human SKPs differentiate into cells of the skeletogenic lineages. (A) Quantification (top panel) of the percentage of human foreskin-derived SKP spheres that, when differentiated under osteogenic conditions (Osteo) for 6 weeks, gave rise to clusters of cells that stained with Alizarin red (bottom panel). Some spheres generated cells of the osteogenic lineage even when differentiated in serum alone (Con). (B) Quantification (top panel) of the percentage of human SKP spheres that, when differentiated under chondrogenic conditions (chondro) for 4 weeks gave rise to nodules that stained with Alcian blue (bottom panel). Some spheres generated cells of the chondrogenic lineage even when differentiated in serum alone (Con). A and B, represent the mean proportion of positive spheres ± SE, n = 3 different samples, 32 spheres were differentiated per sample/condition (statistical analysis was performed using a paired logistic regression, **P<0.01). For A and B, scale bars = 250 μm.
Figure 2.8: Rat SKPs are multipotent with regard to skeletogenic lineages. (A-C) Single rat SKP cells were expanded, and then differentiated under osteogenic (Osteo), chondrogenic (Chondro), or Schwann cell conditions for 4 weeks. Cells differentiated (A) under osteogenic conditions were stained with Alizarin red, (B) under chondrogenic conditions were stained with Alcian blue and (C) under Schwann cell conditions, were immunostained for the Schwann cells markers p75 neurotrophin receptor (p75) and S100β (left panel, red and green, respectively) or for GFAP (red, right panel). The arrow in (C) denotes a cell with Schwann cell morphology that co-expresses p75 and S100β. For A and B, scale bars = 250 μm, for C, scale bars = 20 μm.
expanded individually until large enough for cell differentiation (12-15 passages). All four clones thus generated demonstrated Alizarin red-positive mineral deposits when cultured under osteogenic conditions, but not when cultured in serum-only conditions (Fig. 2.8A). Two of the clones also generated Alcian blue-positive chondrocyte nodules when grown under chondrogenic conditions, but not under serum-only conditions (Fig. 2.8B), and two also generated Schwann cells, as determined by immunostaining for the Schwann cell markers p75NTR, P0, and/or GFAP, when differentiated under appropriate conditions (Fig. 2.8C). Thus, some SKPs are bipotent with regard to osteoblast and chondrocyte potential, and at least some of these SKPs can also generate Schwann cells.

2.3.5 Transplanted SKPs behave like endogenous mesenchymal cells during bone healing

To confirm the osteogenic potential, the ability of SKPs to differentiate into osteocytes was examined in vivo in the context of bone healing. To do so, a bone fracture model, where the tibia of NOD-SCID mice was unilaterally fractured, was used (Chen et al., 2007; Hiltunen et al., 1993). To facilitate the tracking of the injected cells, SKPs were derived from adult transgenic rats that express GFP in all cells (Okabe et al., 1997). An average of 5x10^5 (range 4x10^5 – 7x10^5 depending on cell availability) naïve SKPs were mixed with matrigel, and transplanted by injection directly into the fracture site immediately after the fracture was induced. Bones were analyzed three weeks following the fracture, a time at which ossification is on-going, and at six weeks post-fracture, at which point healing is largely complete (Hiltunen et al., 1993; Le et al., 2001).

To identify tissue structures that contained transplanted cells, bones were demineralised, sectioned, immunocytochemistry was performed for GFP, and then the samples were counterstained with Nissl-Red (for cell bodies) and Hoechst 33258 (for nuclei) to define tissue morphology (Figure 2.9A right panel). Alternatively, sections were washed in PBS, photographs of the GFP fluorescence were acquired, and then the sections were stained with hematoxylin and eosin and photographs of the same field were captured (Figure 2.9A, left and centre panels). At both three and six weeks, many GFP-positive transplanted cells were observed within the bone
A

B

C

D

E

F

Naïve

Prediff

# of cells (x 10^3)

3wks

6wks
**Figure 2.9:** *SKPs integrate into the callus and newly-formed bone when transplanted into a bone fracture.* (A) Longitudinal sections through the tibia at the level of a bone fracture 3 weeks post-injury showing the location and integration of surviving, naïve (left and middle panels) or pre-differentiated (right panel) GFP-positive adult rat SKPs. For all panels the bold hatched line denotes the fracture site while the small hatched line represents the boundary between the callus and the adjacent muscle. The left panel is a low magnification photomicrograph showing the tissue histology stained using hematoxylin and eosin. The boxed region is shown at higher magnification in the centre panels, with the top panel showing the GFP-positive cells within the bone callus (green, arrows), and the bottom showing both the fluorescent green transplanted cells (arrows) and the tissue morphology as stained with hematoxylin and eosin. The right panel is a higher magnification image of a section of transplanted bone immunostained for GFP (green) and counterstained with a fluorescent red Nissl to show tissue morphology. (B-E) Longitudinal sections through the tibia at 3 (B,C) and 6 (D,E) weeks post-fracture showing the region of the newly-healed bone. (B) Photomicrograph of a section immunostained for GFP to detect transplanted cells (brown) and counterstained with Alcian blue to detect cartilage proteoglycans (blue) and nuclear fast red to show cell nuclei (red). The hatched line delineates the newly-formed bone, C = callus outside the newly-formed bone, and Bm = bone marrow. Arrows denote transplanted cells that have integrated into the newly-formed bone and arrowheads denote transplanted cells immediately adjacent to the newly-formed bone that have osteoblast morphology. (C) Photomicrographs of a section immunostained for GFP (green, both panels) and counterstained with fluorescent Nissl red to show cells (red, right panel). The micrographs are labelled as for (B). (D) Photomicrograph of a section that was immunostained for GFP (brown) and counterstained with methyl green. Labels are as in (B). (E) Photomicrographs of two different sections immunostained for GFP (green) and counterstained with Nissl red (red) and Hoechst 33258 (blue) to show cell nuclei. The right panel shows a 13 μm thick optical section on the confocal microscope. Labels are as in (B). (F) Quantification of the mean number of transplanted cells surviving within the callus at 3 and 6 weeks post-fracture. Both naïve and pre-differentiated (Prediff) SKP transplants were quantified (3 bones per condition represented as mean number of cell per callus ± SE). In (A), scale bare = 500 μm (left panel) and 250 μm (right panel). For (B-E) scale bars = 50 μm.
callus and the surrounding tissues (Fig. 2.9A). Within the callus, many of the transplanted cells had an elongated, fibroblast-like morphology, similar to the endogenous mesenchymal cells (Fig. 2.9B,C). At three weeks some transplanted cells localized immediately adjacent to the newly-formed bone matrix and had a cuboid morphology similar to endogenous osteoblasts, consistent with an osteoblast phenotype (Fig. 2.9B,C). A few were also present within the newly-formed bone matrix (Fig. 2.9B,C). By six weeks, transplanted cells within the bone matrix had a clearly identifiable osteocyte morphology (Fig. 2.9D,E). The GFP-positive processes of these cells could be seen extending through the surrounding bone matrix (Fig. 2.9E). To quantify cell survival, bones were sectioned throughout the extent of the fracture; immunofluorescence for GFP on every sixth section was performed; and all positive cells within these sections were counted. In some experiments a similar analysis was performed by counting every third section and very similar results were obtained when the total number of cell per callus was calculated (data not shown). At three weeks, approximately 27 000 GFP-positive cells were present within the bone callus (Fig 2.9F), while by six weeks, approximately 6 300 cells remained within the callus (Fig. 2.9F). Taken together, these data demonstrate that transplanted SKP-derived cells survived within the fractured bone, and that many were morphologically similar to the endogenous, non-GFP-positive cells, suggesting that they were differentiating into the reparative cell types present in the healing bone.

To determine the phenotype of the transplanted cells and to confirm the histological analysis, sections were triple-labelled for GFP, to follow transplanted cells, and for ALP and osteopontin to detect the osteogenic phenotype (Fig. 2.10A,B); ALP is an early marker for osteogenesis, while osteopontin is a mid-differentiation marker. Confocal microscopy demonstrated that the endogenous cells fell into four categories; cells that expressed neither ALP nor osteopontin, those that expressed one or the other, and cells that expressed both. Analysis of the GFP-positive, transplanted cells demonstrated that they too fell into these four categories, and that the transplanted ALP- and/or osteopontin-positive cells were morphologically similar to the corresponding endogenous cells (Fig. 2.10A,B). In contrast, skeletal muscle cells adjacent to the bone were negative for these markers (data not shown).

Confocal microscopy was then used to quantify the percentage of transplanted versus endogenous cells that expressed ALP and/or osteopontin (Fig. 2.10C). After three weeks,
Figure 2.10: SKPs differentiate down the osteogenic lineage when transplanted into the injured bone environment. (A) Confocal micrographs of a section through the fracture region 3 weeks post-injury, triple-labelled for GFP to detect transplanted cells (green), and for the osteoblast markers ALP (red) and osteopontin (OP, blue; the bottom right panel is a merge of the three coloured images). The long arrow denotes a transplanted cell that expresses both ALP and osteopontin, and the short arrow a transplanted cell that only expresses ALP. B = newly formed bone, C = callus outside of the newly formed bone, Bm = bone marrow. (B) Confocal photomicrographs of a section through the fracture region 6 weeks post-injury, double-labelled for GFP (green, bottom panel) and alkaline phosphatase (ALP, red, both panels) and counterstained with Hoechst 33258 (blue, both panels). The arrowhead denotes a transplanted, ALP-positive cell, and the arrow a transplanted, ALP-negative cell that has integrated into the bone. (C) Quantification of the percentage of both endogenous (Endo) and transplanted (SKPs) cells that co-express ALP and/or osteopontin at 3 and 6 weeks post-injury, in sections similar to those shown in (A,B). Represented as the mean percentage of cells per bone ± SE, n = 3 bones. (D) Confocal photomicrographs of a 6 μm thick optical section through the newly formed bone 6 weeks post-fracture, double-labelled for GFP (green) and osteocalcin (OC, red), and counterstained for Hoechst 33258 (blue, right panel only). The arrow denotes an osteocalcin-positive transplanted cell at the edge of the newly formed bone. The asterisks denote transplanted cells that have integrated into the newly formed bone and have the morphology of osteocytes. (E) Quantification of the number of transplanted naïve and pre-differentiated (Prediff) SKPs that have integrated into the newly-formed bone, as shown in (D), at 3 and 6 weeks post-injury (n = 3 bones per group, mean ± SE, linear regression adjusted for the variable week, *p<0.05). For A, B and D, scale bars = 20 μm.
approximately 30% of endogenous, and 44% of the GFP-positive transplanted cells expressed the early osteogenic marker ALP, but not osteopontin. An additional 17% of the endogenous and 24% of the transplanted cells expressed both ALP and osteopontin. Very few cells in either group expressed osteopontin alone, and 50% of the endogenous and 30% of the transplanted cells expressed neither marker. Thus, after three weeks, approximately 47% of endogenous and 68% of transplanted cells expressed markers indicative of osteogenic differentiation.

By six weeks, an increased percentage of both endogenous and transplanted cells had differentiated along the osteogenic lineage (Fig. 2.10C); approximately 48% of the endogenous and 36% of the transplanted cells expressed ALP alone, while 28% of the endogenous and 47% of the transplanted cells expressed both ALP and osteopontin. Virtually none of the cells expressed osteopontin alone at this later time-point, and only 17% of the transplanted cells and 24% of the endogenous cells expressed neither marker. Thus, by six weeks post-fracture, approximately 76% of the endogenous cells and 84% of the remaining transplanted cells expressed an osteogenic phenotype. Thus, naïve SKPs differentiate along an osteogenic pathway when transplanted into the injured bone environment, and behave very much like the endogenous mesenchymal precursors that participate in bone healing.

The localization of GFP-positive cells within the bone matrix (Fig. 2.10B) indicated that at least some of the transplanted cells became mature osteocytes. To confirm this conclusion, immunofluorescence was performed for the late osteogenic marker, osteocalcin. This analysis revealed that a small number of transplanted cells were localized adjacent to, but not integrated into, newly formed bone, and that they were positive for this late marker (Fig. 2.10D). Consistent with the interpretation that some, but not many, of the cells in the callus matured into osteocytes, quantification demonstrated that approximately 100 of the 6000 surviving transplanted cells had integrated into the bone matrix after six weeks (Fig. 2.10E).

Mouse SKPs were used for a few experiments. These cells were derived from transgenic mice constitutively expressing the enhanced yellow fluorescent protein (YFP). In these initial experiments less YFP-positive cells were observed at three and six weeks post-injection than in animals injected with rat SKPs (qualitative observations, not quantified). It is possible that mouse SKPs may be more adversely affected by the fracture environment in terms of survival and/or proliferation, than rat SKPs. Since the apparent survival rate of the rat-derived SKPs was
**Figure 2.11: Mouse SKPs differentiate into osteogenic cells in vivo.** (A-C) Sections of mouse tibia injected with YFP-mouse SKPs at the site of tibia fracture at 3 (A-B) or 6 weeks (C) post-fracture. Sections were immunostained for GFP and the osteoblast marker ALP (A, red), or osteocalcin (B, red). (A-B) Arrows indicate cells co-expressing the markers, (A) GFP and ALP or (B) GFP and osteocalcin (OC). Dashed lines represent the border between the bone matrix and the cellular part of the callus. (C) Photomicrographs of a section of 6-week old callus immunostained for GFP (green) and counter stained with Hoechst 33258 (blue) to visualise the nuclei (left panel), and of the same section stained with hematoxylin and eosin (middle panel). The right panel is a merged image of the immunofluorescence and histological stains. An arrow denotes a GFP-positive cell integrated within the newly formed bone. An asterisk indicates a GFP-positive osteoblast lining the bone. Bm = bone marrow.
greater, all subsequent experiments were performed using these cells. However, some of the bones injected with mouse YFP-SKPs were analysed for the expression of the two osteogenic markers, ALP and osteocalcin by fluorescence microscopy. The analysis indicated that after three weeks, many YFP-SKPs located within the callus expressed the osteoblast maker ALP (Figure 2.11A) and low levels of osteocalcin (Figure 2.11B). These data suggest that at least some of these cells could differentiate into osteoblasts. To test this possibility, YFP-cells within the bone matrix were identified by taking photomicrographs of sections immunostained for YFP and counterstained for nuclei. The same sections were subsequently stained with haematoxylin and eosin to visualise the tissue structure and photomicrographs of the same field of view were captured. The overlay of the photomicrographs of the fluorescence and histological stains revealed that at six weeks post-injection occasional YFP-positive osteocytes were present in the newly formed bone (Figure 2.11C, arrow, n = 2 bones). Occasional YFP-positive osteoblasts were also observed lining the bone matrix (Figure 2.11C, asterisk, n = 1 bone). These experiments must be repeated, and the results quantified and analysed with confocal microscopy in order to extract their full significance, but these qualitative analyses support the idea that, like rat SKPs, mouse SKPs could differentiate \textit{in vivo} into cells of the osteoblast lineage.

Experiments were performed to determine whether pre-differentiation along the osteogenic lineage in culture would enhance the survival and/or differentiation of transplanted SKPs \textit{in vivo}. GFP-positive rat SKPs were differentiated for 7 days in osteogenic conditions, and then an average of $5.0\times10^5$ cells were transplanted into the fractured, stabilized tibia. Quantification revealed that approximately 25,000 and 6,100 transplanted GFP-positive cells were present within the bone callus three and six weeks later, respectively, numbers very similar to those seen with undifferentiated SKPs (Fig. 2.9F). However, the number of such cells within the bone matrix was significantly (linear regression adjusted for the variable week, p <0.05) decreased relative to the undifferentiated cells (Fig. 2.10E). Thus, the fracture environment is itself sufficient to instruct SKPs to differentiate along the osteogenic lineage, and pre-differentiation does not facilitate this process.
**Figure 2.12:** SKPs generate chondrocytes when transplanted into a chondrogenic environment. (A,B) Sections through the bone callus at 3 weeks post-fracture in a region containing cells with the morphology of chondrocytes. (A) The section was immunostained for GFP (green), and counterstained with Nissl red (red) (n = 3 bones). (B) The section was immunostained for GFP (black) and counterstained with Alcian blue (blue) to detect chondrocyte proteoglycans (n = 1 bone). Arrows denote GFP-positive cells with chondrocytic morphology (A,B). (C) Deconvoluted confocal micrographs of a section through the bone callus at 3 weeks post-transplant triple-labelled for GFP (green), osteopontin (OP, red), and ALP (blue; the right panel is a merged image of the individual immunofluorescences). Arrows denote triple-labelled cells with the morphology of hypertrophic chondrocytes, an asterix denotes an endogenous, ALP-positive, osteopontin-positive cell with similar morphology (n = 3 bones). For A and B scale bars = 50 μm, in C scale bar = 40 μm.
Figure 2.13: SKPs differentiate into hypertrophic chondrocytes when injected into the site of bone fracture. Photomicrographs of a section from a 3 week old callus immunostained for GFP (Green), type-X collagen (Red) and counter stained with Hoechst 33258 for nuclei (Blue). An arrow indicates a GFP-positive type-X collagen-positive cell and the arrowhead denotes endogenous cells positively stained for type-X collagen. N = 1 bone, 4 sections were analysed. Scale bar = 50 μm.
2.3.6 SKPs generate other mesenchymally-derived cell types in vivo

While these data indicate that the majority of SKPs differentiated into an osteogenic lineage when transplanted into the fractured bone, 15-30% of the GFP-positive cells in these experiments did not express either ALP or osteopontin, even at six weeks post-transplantation. Perhaps these cells may have adopted other fates. To test this possibility, immunofluorescence analysis for βIII-tubulin, a marker for developing neurons, and GFAP, a marker for glial cells, demonstrated that none of the transplanted cells adopted a neural fate (data not shown). SKPs can also differentiate into chondrocytes, a cell type normally seen during bone healing. Qualitative analysis of the fractured bone sections revealed the presence of occasional GFP-positive cells with a chondrogenic morphology at three weeks post-transplantation (Fig. 2.12A,B, n = 3). These cells were located within nests of endogenous chondrocytes, and stained positively for Alcian blue (Fig. 2.12A,B). Moreover, some of these potential chondrocytes co-expressed ALP and osteopontin (Fig. 2.12C, n = 3), suggesting that they might be hypertrophic chondrocytes, consistent with their presence during bone healing. This was supported by staining some sections for the hypertrophic chondrocyte marker, type-X collagen, GFP to identify the transplanted cells, and counterstained with Hoechst 33258 for nuclei. Using conventional fluorescence microscopy occasional co-localisation of GFP with type-X collagen was observed (Figure 2.13). This experiment must be repeated to achieve a significant sample size, optimised to minimise degradation of the GFP signal during the process of immunostaining for type-X collagen, which requires proteolytic digestion, and quantified. Nonetheless, this result supports the conclusion that SKPs can differentiate into hypertrophic chondrocytes in vivo in the context of a bone fracture. It is important to note that at six weeks post-fracture, a time when endochondral ossification is complete and the injured bone enters a phase of bone remodelling, GFP-positive chondrocytes were not observed.

2.4 Conclusion

Taken together these results support the hypothesis that skin derived precursor cells can differentiate into skeletal mesodermal cell types both in vitro and in vivo.
Chapter 3
SKPs differentiate into multiple cell types in vivo depending on their environment

3.1 Introduction:

One set of interesting observations from the data described in Chapter II is that naïve SKPs injected into a bone fracture environment differentiated into cell types that are normally present at the site of injection. This suggests that the local environment can “instruct” the differentiation of injected naïve SKPs. A similar phenomenon, though not explicitly reported, was also seen in other studies in which SKPs were injected into either the peripheral or central nervous systems (Biernaskie et al., 2007; McKenzie et al., 2006). Based on these observations, I hypothesized that the differentiation fate of transplanted SKPs is dictated by their environment. To test this hypothesis the phenotype of SKPs injected into different tissues, or localising to various areas in the vicinity of the transplantation site, was characterized. Two transplant models were used, the stabilized bone fracture and the hypodermis injection. The bone fracture model is described in Chapter II. One advantage of this model is that many different tissues are damaged during the surgical procedure, including the knee ligament, knee cartilage and skeletal muscle. Damage to the knee tissues results from the insertion, through the knee, of the intramedullary pin that stabilises the fractured bone. The skeletal muscles, juxtaposed to the crest and shaft of the tibia, are also damaged due to their displacement to allow the insertion of the surgical scissors used to fracture the bone. In this model new tissues are also formed, for example neovascularisation is induced to allow endochondral ossification and the periosteum is extended to cover the callus. Therefore, flooding the fracture site with SKPs can be expected to result in some of the transplanted cells coming into contact with these injured or newly forming tissues.

The hypodermis is the innermost part of the skin and is rich in adipocytes. To test whether SKPs can differentiate into cell types normally present in this environment, SKPs were injected into the hypodermis of back skin and the resulting phenotype of the injected cells analysed. This chapter describes published and initial data obtained by using these two models, and demonstrates that some SKPs can differentiate into a range of cell types depending on their
location; SKP-derived adipocytes were seen in the hypodermis of the skin; SKP-derived perivascular cells were observed localising to areas surrounding and within the callus; SKP-derived neural cells were present in areas of the skeletal muscle where endogenous neural cells are also found; and some of the SKPs integrated into the newly forming periosteum and damaged ligament. These results, presented below, support the hypothesis that the fate of SKPs differentiation is dictated by their environment.

### 3.2 Author’s contribution:

This section contains published data from the Stem Cell and Development paper (Lavoie et al., 2008) (Figures 3.1, 3.2 and 3.6) and some further unpublished data (Figures 3.3, 3.4 and 3.5) presented here to support the conclusions of this chapter. The results presented in this chapter were either generated or reproduced by me. Figure 3.6 is the result of a collaboration with Dr. Jeffrey Biernaskie, (post-doctoral fellow in Dr. F.D. Miller’s laboratory). The injections for figure 3.6A were performed by Dr. Biernaskie, and I stained and analysed the sections. The results in panel 3.6B were entirely generated by Dr. J. Biernaskie. However, I did successfully reproduce this result by analysing ear skin injected with naïve SKPs and skin overlaying the site of a bone fracture experiment (data not shown).

### 3.3 Results:

#### 3.3.1 SKPs colonise the periosteum

A callus is normally formed following the incidence of a bone fracture in the attempt to restore bone continuity. The forming callus is eventually covered by the periosteum, a cellular membrane that covers the outside surface of the bone tissue. The periosteum is an important structure as it is one site where osteogenic precursors reside (reviewed in (Augustin et al., 2007; Wlodarski, 1989)). The tibia of immunocompromised mice was fractured subsequent to the insertion of an intramedullary pin that stabilized the fracture site, as described in Chapter II. Naïve SKPs were injected at the time of the fracture and animals were allowed to recover for three to six weeks prior the analysis of callus that formed. A number of the injected SKPs were
Figure 3.1: SKPs integrate into the periosteum when transplanted in bone fracture. Photomicrographs of bone callus sections at the level of the periosteum (p) 6 weeks post-transplant immunostained for GFP (green in left and center panels; brown in right panel) and counterstained for fluorescent Nissl red (center) or methyl green (right) to visualized the tissue structure. The left and center panels show the same field. The periosteum is delineated by hatched lines, and arrows denote transplanted cells that have integrated into the periosteum (p). Scale bar = 40 μm.
A

low mag

B

cross section

3D-reconstruction

GFP

CD31

SMA

Merge

GFP/SMA

BV

BV

BV

BV

BV

BV

BV

BV

BV

BV

BV

BV

BV

BV
Figure 3.2: Transplanted Rat-SKPs generate perivascular cells when localizing in the vicinity of a bone fracture. (A-B) Confocal micrographs of a field adjacent to newly-healed bone at six weeks post-transplant: (A) double labelled for GFP (green) and smooth muscle/pericyte marker SMA (red); (B) triple-labelled for GFP (green), the endothelial cell marker CD31 (blue) and SMA (red). (A) An asterisk indicates an endogenous blood vessel associated with SMA-positive cells that are GFP-negative, arrowheads highlight GFP-positive transplanted cells that are not associated with a blood vessel and express very low levels of SMA, and (A-B) arrows denote transplanted, CD31-negative, SMA-positive cells associated with the exterior of CD31-positive vessels (n = 3 bones). BV = blood vessel. A and B scale bar = 16μm.
observed to localise to the exterior surface of the forming callus, a site where the periosteum will form to recover the exposed bone surface (Fig 3.1). An interesting possibility is that transplanted SKPs localizing to this site might integrate into the forming periosteum and populate this osteogenic precursor niche, thus potentially providing a long-lasting source of mesenchymal precursors. To test whether the transplanted SKPs had integrated into the periosteum, immunostaining for GFP was performed on sections derived from three and six weeks old callus. In all seven animals examined, GFP-positive transplanted cells were observed scattered within the periosteum close to the fracture site, displaying a spindle-like morphology similar to the endogenous mesenchymal precursors (Fig. 3.1). This suggests that SKPs can integrate into the periosteum, but it remains to be determined whether they can subsequently give rise to osteoblasts that will generate new bone matrix.

3.3.2 Transplanted rat SKPs differentiate into perivascular cells when localizing in proximity of the fracture.

New blood vessels are formed during the process of bone repair and some of these enter the callus. Qualitative analysis at three and six weeks post-fracture demonstrated the presence of these new blood vessels both within and in very close proximity to the bone in all five bones examined. Some GFP-positive cells were observed to co-localize with the new blood vessels (Fig. 3.2A). To determine the phenotype of these cells, deconvolution confocal microscopy of sections immunostained for smooth muscle actin (SMA), a marker for perivascular smooth muscle cells and pericytes (reviewed in (Armulik et al., 2005)) and CD31, a marker for endothelial cells, revealed that the GFP-positive cells expressed SMA, but not CD31 (Fig. 3.2B). This was seen in the three bones analysed (at least four sections per bone were analysed). The levels of SMA expression in GFP-positive cells that closely wrapped the endothelial tubes were similar to those seen in the endogenous perivascular smooth muscle and/or pericytes. In contrast, GFP-positive cells that were not associated with blood vessels expressed very low or undetectable levels of SMA (Fig. 3.2A,B). In some cases several hundred micrometers of blood vessel were wrapped by the GFP-positive perivascular cells (Figure 3.3A). Among the sections analysed, two types of cell morphologies were observed. Qualitative analysis showed that most of them displayed many finger-like processes wrapping the endothelial cells (Figures 3.3A left
Figure 3.3: Rat-SKPs–derived perivascular cells adopt different morphologies when associated with small or larger blood vessels. (A-C) Photomicrographs of GFP-positive cells associated with blood vessels in a mouse injected with GFP-rat SKPs at six weeks post-injection. (A) Photomicrographs of area outside the bone but in the vicinity of the fracture. Green is the epifluorescence of GFP cells, blue and red are the autofluorescence of the adjacent tissue in the blue and red channels, respectively. (B) One optical slice acquired by confocal microscopy of a section immunostained for GFP (green, left and right panels) or SMA (red, middle and right panels) and counter stained with Hoechst 33258 to visualize the nuclei (blue, right panel). (C) Confocal micrograph of 1.8 μm thick optical slice of section co-immunostained for GFP (green), SMA (red), CD31 (blue, bottom left panel), and counter stained with Hoechst 33258 (blue, bottom right panel). (A, C) CT = connective tissue and BV = Blood Vessel. (B-C) Arrows denote GFP-positive cells co-expressing SMA and asterisks indicate endogenous cells expressing SMA. Scale bar in (A) = 100 μm, in (B) = 40 μm and in (C) = 15 μm.
panel and 3.2B), whereas in other cases the entire cell wrapped the vessel as one large band or ring (Figure 3.3A right panel, and 3.3B, C). As an indication of the blood vessel type, their diameters were measured. This analysis revealed that cells displaying finger-like processes associated with small diameter vessels (average diameter of \(9.1 \, \mu\text{m}\), range= 5.8 to 13 \(\mu\text{m}\), \(n = 9\) blood vessels), a size typical of capillaries. Cells displaying the ring morphology were found associated with larger vessels (average diameter of \(16.3 \, \mu\text{m}\), range= 14.4 to 18.4 \(\mu\text{m}\), \(n = 4\) blood vessels) (Figure 3.3B,C). It is interesting to note that some of these blood vessels were composed of a mixture of transplanted and endogenous cells (Figure 3.3B, asterisk). This suggests that SKPs can contribute, with endogenous cells, to the formation of blood vessels and that depending on the type of vessels in which they participate, they can differentiate into the appropriate types of perivascular cells normally found on that vessel. While the cell morphology supports this conclusion, the immunophenotype of these SKP-derived perivascular cells must be further characterised in order to be able to conclude that SKPs can differentiate into two types of perivascular cells. Nonetheless, these initial results support the conclusion that SKPs can generate blood vessel perivascular cells (smooth muscle cells or pericytes), as do neural crest precursors during development. The data also support the hypothesis that the cell types into which SKPs differentiate is dictated by their local environment, as in this case they differentiated into cells morphologically identical to the endogenous cells composing the blood vessels with which they associated.

3.3.3 Transplanted mouse SKPs that localized to skeletal muscle differentiate into neural cells.

In the fracture model, skeletal muscles immediately adjacent to the tibia are displaced to allow the insertion of the surgical scissors used to generate the fracture. This displacement may result in damage to the muscle fibres or to sites of innervation. This damage would normally heal following the occurrence of a fracture. It is possible that some of the transplanted SKPs could contact these damaged areas. To determine whether these SKPs could contribute to the repair of this tissue, and thereby further testing the hypothesis that the fate of differentiating SKPs is dictated by their environment, the location of the transplanted cells was determined in sections of fractured bones, six weeks following injection with YFP-mouse SKPs. A few YFP-positive cells
Figure 3.4: *Transplanted mouse-SKPs differentiate into neural cells when localizing to skeletal muscle.* Photomicrographs of the leg skeletal muscle adjacent to the fractured tibia injected with YFP-mouse SKPs. The animal was analyzed 6 weeks post-transplantation. The section was immunostained for YFP (green, right panel and white, top left panel) and the neuronal marker βIII-tubulin (red, right panels and white, bottom left panel) and counterstained with Hoechst 33258 (blue, bottom right panel). Scale bar = 100 μm.
were observed to localise to injured muscles. They did not localise to the skeletal muscle fibres, rather they adopted a distinct morphology, characterized by a small cell body and two long processes (Figure 3.4). This cell morphology is reminiscent of neuronal cells or Schwann cells wrapping neuronal axons. To determine whether these SKPs had differentiated into neurons, sections were immunostained for YFP to track the transplanted cells, for βIII tubulin, a neuronal marker, and with Hoechst 33258 to mark the nuclei. Fluorescence microscopic analysis revealed a co-localization of the YFP and βIII tubulin signals, suggesting the neuronal differentiation of these SKPs (Figure 3.4). The known capacity of SKPs to differentiate into Schwann cells suggests that an alternative conclusion is possible; these YFP-cells may have differentiated into Schwann cells that have wrapped themselves around motor neurons. Limitations of the microscopic technique employed preclude the determination of the true explanation from these data. Confocal microscopy must be performed, in combination with immunostaining for markers which allow discrimination between Schwann cells (GFAP, S100β, and P0) and neurons (NFM and βIII tubulin). The high resolution that can be obtained with confocal microscopy should permit the precise determination of which markers colocalize with the YFP cells. In addition, it will be important to repeat these experiments as only a few of these cells were observed and analysed.

3.3.4 Transplanted rat-SKPs that localized to the injured knee integrate into the ligament tissue.

To stabilise the fractured tibia, a non-corrosive pin was inserted into the marrow cavity. This was achieved by puncturing the head of the tibia at the knee joint, which resulted in damage to the anterior knee ligament and knee cartilage. During the analysis of the localisation of the SKPs following their injection into the bone fracture, a few GFP-positive cells were observed in the knee area (in all four animals examined). To visualise the precise location of these GFP-cells, immunohistochemistry experiments were performed (Figure 3.5). This analysis was not carried out for all experimental animals, but of those qualitatively analysed (n = 2), all exhibited a few GFP-cells in the knee ligament. This needs to be quantified, but this observation raises the possibility that SKPs may also contribute to ligament and tendon repair. Further characterisation of these cells is required to confirm their phenotype, the efficiency with which they integrate into
Figure 3.5: *Transplanted rat-SKP localizing to injured knee integrate into ligament.*
Photomicrograph of the anterior knee ligament of a mouse injected with GFP-rat SKPs at the site of tibia bone fracture. GFP-positive cells were detected by immunohistochemistry (brown). The section was counterstained with Alcian blue (Blue) and Nuclear fast red (red/purple) to visualize the tissue structure. Hatched lines delineate the contour of the ligament. Arrows indicate GFP-positive cells. CT = connective tissue. Scale bar = 100 µm.
the injured ligament, and whether their integration contributes to its repair and functional recovery. It is interesting to note that at this location, no SKP-derived cells exhibited adipocyte, chondrocyte or perivascular cell morphologies. This suggests that the knee ligament environment may have prevented the differentiation of SKPs into these cell types and/or induced their differentiation into cells with morphologies similar to those endogenous to this location.

3.3.5 Transplantation of rat-SKPs into the hypodermis induced their differentiation into adipocytes.

The above data suggest that it is the local environment that dictates the phenotype adopted by differentiating SKPs. SKPs can generate adipocytes in culture (Toma et al., 2001; Toma et al., 2005). If the local environment indeed instructs the differentiation pathway of SKPs, it would be predicted that transplantation of naïve SKPs into an adipogenic environment should induce their differentiation into adipocytes. This prediction was tested by injecting naïve SKPs into the hypodermis layer of the skin, a layer rich in adipocytes. GFP-positive SKPs were transplanted into the back skin of NOD/SCID mice that have an impaired immune system, to prevent the rejection of the transplanted cell by the host immune cells. Four weeks post-transplant, animals were sacrificed, and skin was isolated and sectioned. These skin sections were immunostained for GFP to identify the location and morphology of the transplanted cells. Photomicrographs for GFP of these immunostained sections were taken and the same sections were then stained with oil-red-O to identify the lipid vesicles of adipocytes. Photomicrographs of these oil-red-O stained sections were taken at precisely the same location as those taken for the GFP. The overlay of these pictures revealed that many GFP-positive cells exhibited an adipocyte morphology and stained positively for the lipophilic dye oil-red-O (Fig. 3.6A, n = 3). Others sections were immunostained for GFP and the adipocyte marker, fatty acid binding protein (FABP)(n = 2). Photomicrographs of these sections, taken using a confocal microscope, demonstrate the co-localisation of these two markers (Fig. 3.6B). A quantitation of the efficiency of adipogenic differentiation of injected SKPs need to be performed, but it is interesting to note that in these experiments no bone formation was observed at the site of injection and conversely, no adipogenic differentiation of SKPs was observed within the bone callus when SKPs were injected into the bone fracture site. Thus, SKPs can generate osteocytes,
chondrocytes and adipocytes *in vivo*, as they can in culture, and the appropriate differentiation is determined and dictated by the local microenvironment.
Figure 3.6: Rat-SKPs generate adipocytes when transplanted subcutaneously. (A,B) Photomicrographs of sections through the hypodermis of mice into which GFP-positive rat-SKPs were transplanted 4 weeks earlier. (A) Photomicrographs of the same field for epifluorescence of GFP (green, left panel) and stained with oil-red-O (red, right panel), and viewed under fluorescence illumination (left panel) or bright field illumination (right panel). Ep = epidermis, D = dermis, Hy = hypodermis. The hatched lines delineate the hypodermis, and the arrows indicate GFP-positive, oil-red-O-positive adipocytes. Asterisks denote endogenous adipocytes in the same region. (B) Photomicrograph of a section immunostained for GFP (green) and fatty acid binding protein (FABP, red; yellow results from double-labelled cells). For B, scale bars = 50 µm.
Chapter 4
Materials and Methods

4.1 Primary culture

Isolation: Mouse SKPs were isolated as previously described (Fernandes et al., 2004; Toma et al., 2001) from neonatal (P0-P3) mice from strains CD1 and EYFP (The Jackson Laboratory, Bar Harbour, ME). Rat SKPs were derived using the same techniques from newborn Sprague-Dawley rats, or from adult rats (>3 weeks) that expressed GFP under CMV enhanced chicken beta-actin promoter (Okabe et al., 1997). Rodent SKPs isolation protocol was approved by the animal care committee of Hospital for Sick Children, Toronto. Briefly, neonatal skin was dissected from the back of sacrificed animals cleaned by removing the connective tissue attached to the inner surface of the skin using surgical forceps. The skin was then washed in PBS twice, minced and digested in collagenase (1 mg per ml) at 37˚C for 45 minutes. Skin cells were then mechanically dissociated, filtered through a 40 μm cell strainer, re-suspended in 5 millilitres of culture media, composed of DMEM:F12 at a ratio of three to one with no other additives, counted and plated in SKP media at densities varying from 1000 – 50 000 cells per millilitres of SKP proliferation medium [DMEM:F12 (Invitrogen, Carlsbad, CA) at ratio 3:1, supplemented with 2% B27 supplement (Invitrogen), 40 ng/ml FGF-2, 20 ng/ml EGF (both from Collaborative Research, Bedford, MA), and 1% penicillin and streptomycin (Cambrex, East Rutherford, NJ)] supplemented with 40 μg/ml fungizone (Invitrogen). Two to three days after plating, floating cells were transferred to new culture dishes to remove adherent cells. Adult rat skins were process using the same protocol with the following modification. After dissecting out the connective tissue, skin sample was cut in small piece of 0.5 cm² and incubated overnight at 4˚C in collagenase. Epidermis was then peeled and the skin sample was then minced and processed as for neonatal skin. Human SKPs were isolated from neonatal foreskins or breast skin using protocol previously described (Toma et al., 2005) and following ethics approval by the Hospital for Sick Children Research Ethics Board. The procedure is as for the adult rat skin with the following modification, Liberase Blenzyme-1, at a concentration of 0.62 Wunsch U/ml (Roche Molecular Biochemicals, Laval, Québec), was used instead of collagenase.
Feeding and passaging: Both rodent and human SKPs were fed every three to four days with a volume of fresh feeding media [5% B27, 200 ng/ml FGF-2, 20 ng/ml EGF, and 1% penicillin and streptomycin] that corresponds to 20% of the total volume of the culture. Cultures were passaged by dissociating SKP spheres using collagenase at a concentration of 1 mg/ml and the dissociated cells were plated in SKPs proliferation medium containing 30% SKPs conditioned medium. Cultures were used between their first to fifth passages unless otherwise stated.

Clonal cultures: Rat skin cells were plated at density of 1000 cells/ml and 7 to 14 days after plating, single spheres were isolated, dissociated, plated in SKPs medium containing 50% conditioned medium, and passaged and expanded, as for bulk cultures, for 12 to 15 passages.

4.2 Cell differentiation

Osteogenic differentiation: Rodent SKPs are dissociated and plated at a density of 15,500 cells/cm² and human SKPs at 8,000 cells/cm² in 8 well chamber slides (Nalgene NUNC International, Rochester, NY) coated with 1% poly-D-lysine and 2% laminin (DB Bioscience, Mississauga, ON) in osteogenic differentiation medium [DMEM supplemented with 10% FBS (Hyclone, Logan, Utah), 100 nM Dexamethasone, 10 mM β-glycerophosphate (Sigma, St Louis, MO), 250 μM ascorbic acid (EM Science, Gibbstown, NJ), 1% penicillin and 1% streptomycin], as previously defined for MSCs (Pittenger et al., 1999). Differentiation media was changed every two to three days. Cell differentiation was assessed, as described below, after three to six weeks in differentiation conditions.

Chondrogenic differentiation: rodent or human SKPs were plated at 15,500 cells/cm² in 8 well chamber slides coated with 1% poly-D-lysine and 2% laminin in chondrogenic differentiation medium [DMEM:F12 (3:1) supplemented with 10% FBS, 100 nM dexamethasone, 250 μM ascorbic acid, 50 ng/ml BMP-2 (R&D systems, Mineapolis, MN) and 1% penicillin and 1%
For micromass cultures, 100,000-200,000 cells were dissociated, counted, and pelleted prior to differentiation as previously described (Johnstone et al., 1998). In all cases, cells were fed every two to three days by changing 90% of the media. Monolayer cultures were analyzed immediately, and micromass cultures were fixed in 4% paraformaldehyde (EMS, Hatfield, PA), frozen and ultimately cryosectioned.

Schwann cell differentiation: Schwann cell differentiation was performed as described previously (Biernaskie et al., 2006). Briefly, rodent SKPs were dissociated and plated in dishes coated with 1% poly-D-lysine and 2% laminin in Schwann cell media [DMEM:F12 media in a ratio of 3:1 supplemented with 5 μM forskolin (Sigma), 50 ng/ml heregulin-1β (R&D Technology), 2% N2 supplement (Sigma) and 1% penicillin and streptomycin]. Cells were fed every three to four days by replacing 80-90% of the culture media with fresh media. Cells cultured in differentiation conditions for two to four weeks were assessed for Schwann cells differentiation by immunofluorescence techniques, as described below, to detect expression markers and morphology.

Single sphere differentiation: Human foreskin-derived SKP spheres from primary or secondary passage cultures were plated, one at a time, each in a separate well of 8-well chamber slides coated with 1% poly-D-lysine and 2% laminin, and containing either osteogenic, chondrogenic or serum-only media. For each osteogenic or chondrogenic differentiation, a serum-only control condition was plated using cells from the same sample to allow a paired statistical analysis. For each experiment, 32 spheres were plated per condition. Cell differentiation was determined using histological stains: Alizarin Red that stains mineral deposits for osteogenic differentiation or Alcian blue that stains cartilage proteoglycans for chondrogenic differentiation. Wells were considered positive if at least one cluster of cells stained positive for either of the two stains. Reported is the average proportion of positive well with its associated standard error, three independent samples were differentiated per condition.
4.3  *In vivo* transplants

*Bone fracture:* All animal procedures were approved by the animal care committee of Hospital for Sick Children, Toronto. Fracture of the tibial bone of NOD-SCID mice was performed as previously described (Chen et al., 2007) with few modifications. Briefly, animals were anaesthetized with isoflurane. The head of the tibia was punctured with a suture needle to allow the insertion of a non-corrosive metal pin in the marrow cavity of their tibia for stability and their tibia was then fractured. The fracture was performed with surgical scissor after the skeletal muscles, juxtaposed to the tibia, were pulled from the bone to prevent unnecessary damages to this tissue. GFP-positive rat or YFP-positive mouse SKPs dissociated in collagenase, counted and resuspended in matrigel (DB Bioscience, Bedford, MA) were then injected, using a 10 μl Hamilton syringe within the fracture to flood the fracture site and the skin was closed with silk sutures and metal staples. After recovery, an analgesic (ibumorphine) was administered for the first few days. At three or six weeks post-fracture, animals were perfused, and the fractured leg without the skin was fixed in 4% paraformaldehyde (Fisher Scientific, Fair Lawn, NJ) for one hour, decalcified in 0.23M EDTA (Sigma) for four days (by changing the EDTA solution every 12 hours), dehydrated overnight in 30% sucrose (Sigma), cryoprotected in O.C.T., frozen and cryosectioned.

*Hypodermis injections:* Dissociated GFP-positive rat SKPs were injected into the hypodermis of a NOD-SCID mouse, using a 10 μl Hamilton syringe, and four weeks later animals were perfused, and skin was dissected, fixed in 4% paraformaldehyde, cryoprotected, frozen and sectioned.

4.4 Immunofluorescence and immunohistochemistry

Fixed, cryoprotected tissues were sectioned at 20 μm on a Leica cryostat, and sections were collected on 2% gelatin-coated slides. Cells from monolayer differentiations or sections of tissue or micromass were washed with PBS, fixed with 4% paraformaldehyde for five minutes and rinsed with PBS. For immunofluorescence, sections or cells were incubated with either 10%
BSA/0.3% Triton-X100 blocking solution for one hour or with 0.5% NP-40 for five minutes and then with 6% normal goat serum blocking solution for one hour. Sections or cells were then incubated with primary antibodies diluted in blocking solution overnight at 4°C. Slides were then washed in PBS, and incubated with secondary antibody or in combination with Nissl Red (1:200, Invitrogen) for one hour at room temperature. In some cases, mouse antibodies were used with the MOM kit (Vector, Burlingame, CA.) as per the vendor’s protocol. The following antibodies were used for immunofluorescence: Mouse anti-ALP (1:600, Kind gift from Dr. Jane Aubin, University of Toronto), rabbit anti-osteopontin (1:250 [Ab8448] or 1:1000 [Ab14175], Abcam, Cambridge, MA), anti-osteocalcin (1:100 [OC-4], Abcam), chicken anti-GFP (1:1000, Invitrogen, Eugene, Oregon), mouse anti-GFP (1:1000, Molecular Probes), rabbit anti-GFP (1:500, Chemicon), mouse anti-coll-II (1:500, Calbiochem), rabbit anti-coll-I (1:40, Calbiochem), goat anti-FABP (1:100 (C-15), Santa Cruz, ), rabbit anti-p75 (1:500, Promega), mouse anti-S100β (1:1000, Sigma), rabbit anti-GFAP (1:500, Axell), rat anti-CD31 (1:250, Abcam), mouse anti-SMA (1:500, Sigma). Secondary antibodies used were: anti-mouse Alexa 555, anti-rabbit Alexa 555, anti-mouse Alexa 488, anti-rabbit Alexa 488, anti-chicken Alexa 488, anti-mouse Alexa 647, anti-rat Alexa 647 (all of them 1:1000, Invitrogen) and anti-goat-Cy3 (1:400, Jackson ImmunoResearch). As a fluorescent counterstain, slides were incubated with Nissl Red (1:200, Invitrogen) and/or Hoechst 33258 (1 ug/ml, Sigma). Samples were analyzed using a Zeiss upright fluorescence microscope with Northern Eclipse acquisition software (Empix, Mississauga, ON), a Zeiss LSM 5 Pascal confocal microscope, or a spinning disk confocal microscope using Velocity acquisition software (version 4.3.0; Improvision). For 3D reconstruction, stacks of images were taken 0.2 μm apart, images were deconvoluted and reconstructions performed using Velocity software.

Type-X collagen immunofluorescence: Sections were washed, fixed in 4% paraformaldehyde for five minutes, rinsed in PBS, incubated with 0.1% pepsin (Sigma) in 0.5M acetic acid at 37 °C for 20 minutes then washed and incubated with 2 mg/ml hyaluronidase (Sigma) in PBS pH = 5.0 at 37 °C for 20 minutes. Then sections were washed, blocked in 1% BSA, 6% normal goat serum, 0.3% Triton-X100 for one hour at room temperature and then incubate with the primary antibodies anti-GFP and anti-type-X collagen (Quarlett Immunology and Biotec Product, Berlin).
overnight at 4 °C. The remaining steps with secondary antibodies are the same as for those described above.

**Immunohistochemistry:** Frozen sections were processed as for immunofluorescence with the following modifications; sections were blocked in 10% BSA/0.3% Triton-X100 blocking solution for one hour and incubated with rabbit or mouse anti-GFP overnight. Anti-GFP was detected using biotin-conjugated secondary antibody and streptavidin-conjugated horse radish peroxidase for detection (all from Invitrogen). Sections were counterstained with either Alcian blue and nuclear fast red (Sigma, St. Louis, MO) or with methyl green (Vector).

### 4.5 Histological staining

Samples were washed in PBS and fixed in 4% paraformaldehyde for two to five minutes. ALP activity was determined using the 5-bromo-4-chloro-3-indolyl phosphate (BCIP®)/nitro blue tetrazolium (NBT) liquid substrate system (Sigma). Briefly, cells were incubated with this substrate solution for a maximum of five minutes or until color developed, then washed in ddH$_2$O and the slide was mounted.

To visualize mineral deposits, fixed cells or sections were washed in ddH$_2$O and stained with either Alizarin Red (Sigma) solution (2% Alizarin Red in ddH$_2$O, pH 4.2) for five minutes or, for von Kossa staining, with 5% silver nitrate solution for 60 min under a 60 watt light, then washed in ddH$_2$O and incubated for 10 minutes in 5% sodium thiosulfate solution (all from Sigma). Slides were washed in ddH$_2$O, and photographed immediately.

For chondrocyte proteoglycans, cells were washed in PBS, fixed in 4% paraformaldehyde for five minutes, washed in PBS, rinsed in 3% acetic acid for three minutes, incubated in 1% Alcian blue (Sigma) in 3% acetic acid solution for 30 minutes, washed extensively with ddH$_2$O, and photographed.

To detect adipocytes, skin sections were washed in PBS, mounted and GFP epifluorescence was captured. Sections were then stained with oil-Red-O, and brightfield
micrographs of the same fields were captured. To detect adipocytes in monolayer cultures, cells were washed in PBS, fixed in 4% PFA, washed in PBS, incubated in oil-red-O solution, washed in PBS and then mounted and photomicrographed.

4.6 Quantitative in vivo analysis

To quantify SKPs survival in bone fracture, bones were sectioned at 20 μm, every sixth section through the extent of the callus was immunostained for GFP and counterstained with Nissl Red and Hoechst 33258, and all GFP-positive cells within those sections were counted (13-27 sections per bone). Total number of cells in the callus was determined by multiplying this number by six. Three bones per condition per timepoint were counted. For the phenotypic comparison, sections from three bones per condition per timepoint were triple-labelled for GFP, ALP and osteopontin and counterstained with Hoechst 33258. Five representative photomicrographs from each of two to four sections per bone were obtained with a spinning disk confocal microscope, and the number of cells expressing these markers versus total number of cells per microscope field was determined.

To quantify the diameter of blood vessels, the measuring tool of the Velocity software version 4.3.0 was used. Three measurement per vessel were taken and their average was used to calculate the average diameter of the large (n = 4) and small blood vessels (n = 9). Reported is the overall average with its standard error.

4.7 RT-PCR analysis

Messenger RNAs of differentiated cells were harvested according to the Tri-reagent (Sigma) vendor’s protocol. Briefly, cells were washed in PBS, lysed in Tri-reagent, and RNA was isolated by adding chloroform and collecting the aqueous phase. Isopropanol was then added to this aqueous phase to precipitate the RNA and pelleted by centrifugation. RNA was then resuspended in RNase-free water. The concentration and purity of the harvested RNA was determined with a spectrometer. The cDNAs were generated by incubating the RNA with random primers, dNTP (both from Amrad Pharmacia), RNasin (Promega), DTT, reverse
transcriptase buffer and M-MLV reverse transcriptase (Invitrogen) for one hour at 37°C. For the RT “-” condition, similar reactions were performed in which the M-MLV reverse transcriptase was replaced with H2O. RT-PCR reactions were performed using the following protocol: denaturing step: 94°C for two minutes; amplification steps: 30 cycles of 94°C for 15 seconds, gene-specific annealing temperature (TA) for 30 seconds, 72°C for 15 seconds; and the final extending step: 72°C for 60 seconds. List of primers used:

- **ALP-forward**: TGGAGCTTCAGAAGCTCAACCA,
- **ALP-reverse**: ATCTCGTTGTCTGAGTACCAGTCC,
  TA = 58°C;
- **Coll-I-forward**: CTGACCTTCCTGCGCCTGATGTTC,
- **Coll-I-reverse**: GTCTGGGGCACCAACGTCCAA,
  TA = 60°C;
- **Aggrecan-forward**: GCCTTGAGCAGTTCACCTTC,
- **Aggrecan-reverse**: CTCTTCTCGGGGACACGAGCAG,
  TA = 58°C.

### 4.8 Statistical analysis

All statistical analyses were performed using the software SAS version 8.01 (SAS Institute, Cary, NC). Statistical analysis for the number of cells integrated into the bone matrix was performed using a linear regression adjusted for the variable week using the SAS procedure GLM. The analysis for the single sphere assay was performed with a paired logistic regression using the SAS Logistic procedure.
Chapter 5:
Conclusions and Discussion

5.1 Discussion:

The results presented in this thesis support a number of major conclusions. First, the \textit{in vitro} analyses show that rodent and human SKPs can differentiate into cells from the mesodermal lineage, osteocytes and chondrocytes. Second, data from single sphere and clonal SKP experiments indicate that a significant proportion of rat and human SKPs are bipotent with regards to their skeletogenic potential, and a number of these clones retained the capacity to differentiate into neural cells. This confirms and extends previous reports demonstrating that SKPs are multipotential precursor cells (Toma et al., 2001; Toma et al., 2005). Third, data from the bone fracture experiment show that the environment of a bone fracture is sufficient to induce osteogenic and chondrogenic differentiation of naïve SKPs. Fourth, SKPs differentiate into different cell types depending on their local microenvironment; SKPs injected into regions with ongoing angiogenesis differentiate into perivascular cells, while those injected into the adipogenic environment of the hypodermis differentiate into adipocytes. Taken together, the findings presented here confirm two hypotheses, namely (1) SKPs can differentiate into skeletal mesodermal cell types and (2) the phenotype of differentiating SKPs is dictated by their environment. These results expand the known differentiation potential of SKPs and indicate that they represent an accessible, potentially autologous source of precursor cells for bone and cartilage repair.

5.2 In vitro mesodermal differentiation

The capacity of both rodent and human SKPs to differentiate into mesodermal cell types is important because it shows that this potential is present among SKPs derived from different species. Taken with previous reports showing similar differentiation patterns between rodent and human SKPs (Toma et al., 2001; Toma et al., 2005), these results support the conclusion that the
biologic behaviour of human SKPs are very similar to rodent SKPs (Toma et al., 2005). As a consequence, this justifies the use of rodent SKPs as a model to further the understanding of human SKPs.

One important question stemming from the initial SKP characterization studies by Toma et al. (Toma et al., 2005) and Fernandes et al. (Fernandes et al., 2004) is whether SKPs derived from different skin locations share the same differentiation potential. Data from rodent SKPs show that SKPs derived from the back skin have similar differentiation potential to those derived from the whisker pad (Fernandes et al., 2004). These results suggest that human SKPs derived from different skin locations have similar differentiation potentials. Indeed, the work presented in this thesis also supports this conclusion; both foreskin and breast skin-derived SKPs exhibited mesodermal differentiation. However, it remains to be determined whether these SKPs can also differentiate into chondrocytes, as reported for foreskin-derived SKPs. In addition, the differentiation efficiency among SKPs of different locations has yet to be directly compared, as was assessed for MSCs (Wagner et al., 2005). The results of these experiments would be of great importance in the design and selection of the source of skin for optimal SKP-based cells therapies.

One intriguing observation from the in vitro differentiation assays is the formation of clusters of differentiated cells. The mechanism and the biological functions involved in the formation of these aggregates represent future avenues of research. The observations that the first signs of cell differentiation, either mineralisation or positive staining for cartilage matrix proteins, are seen in these clusters (work presented here and unpublished observation by J.-F.L.), suggest that these structures may play a role in facilitating the mesodermal differentiation. These aggregates are somewhat reminiscent to those observed during in vitro mesodermal condensation of chicken limb mesenchymal cells (Widelitz et al., 1993). To determine whether these structures are similar to mesenchymal condensates, the spatiotemporal expression of proteins involved in mesenchymal condensation such as N-CAM, N-Cadherin, Pax-1, Pax-9, Hoxa-2, Prx-1, Prx-2, BMPs, and TGF-β (Hall and Miyake, 2000), should be assessed in these in vitro clusters of SKPs.

In this study, serum was added to the culture medium used to induce chondrogenic differentiation of SKPs. This contrasts with the conditions used to generate chondrocytes from
MSCs, in which the presence of serum has been reported to inhibit differentiation (Johnstone et al., 1998). In the experiments presented here, the presence of serum is essential for the survival of differentiating cells (unpublished observations by J.-F.L.). It is possible that the use of a lower serum concentration may further increase chondrogenic differentiation without compromising the survival of the differentiating cells, but this remains to be tested.

The chondrogenic differentiation of SKPs is an important finding as it suggests their potential use as an alternative source of precursor cells for cartilage repair. Among the different strategies under investigation to repair damaged cartilage is the differentiation of precursor cells into chondrocytes to generate a cartilage matrix that would then be transplanted in affected area of the joint to be treated. This strategy involves in vitro differentiation of the precursor cells. MSCs are currently the main cell type used in this approach. However, their in vitro differentiation has been shown to result in their differentiation into hypertrophic chondrocytes (Barry et al., 2001; Pelttari et al., 2006; Yoo et al., 1998), which are normally absent from joint cartilage. To test whether SKPs can generate hypertrophic chondrocytes in vitro an initial experiment was performed to assess whether mineral depositions occurs in long term chondrogenic cultures of SKPs. This was used as an indicator for the presence of mineralising hypertrophic chondrocytes. In one experiment mouse SKPs were grown as a monolayer under chondrogenic conditions for 56 days and staining of this culture for mineral depositions using Alizarin red and for cartilage proteoglycans with Alcian blue, showed the presence of cartilage formation, but the absence of mineral clusters in this culture, thereby suggesting their differentiation into non-hypertrophic chondrocytes in vitro (unpublished observation by J.-F.L.). Further experiments assessing the absence of expression of type X collagen in these cultures would be necessary to support this initial observation; if demonstrated in human SKPs as well, the results would have potentially important clinical applications.

The osteogenic and chondrogenic phenotypes of differentiated SKPs were first determined by routine histology and immunofluorescence. These techniques provide a good “snapshot” of the phenotype at one point in time, but do not give information on the evolution of the phenotypes as the SKPs differentiate, nor do they indicate the changing gene expression patterns of SKPs as they differentiate. To better address these questions, a time course experiment for the expression of these genes could be measured at various time points during the differentiation period, using a more quantitative method such as real time PCR. The gene
expression profile of differentiating SKPs could be characterized and compared to that of undifferentiated SKPs. One could then determine the time required to generate SKPs committed to either the osteogenic or chondrogenic lineage. This could help determining the optimal pre-differentiation time that would result in the best integration of SKPs when transplanted in vivo.

5.3 Bone fracture model:

The in vitro mesodermal differentiation of SKPs raises two concerns. Is the osteogenic differentiation of SKPs an in vitro artefact? Are the differentiated cells functional? To answer these questions, SKPs were differentiated in vivo, using a bone fracture model. In this mouse model, many cytokines are produced and released that induce the recruitment and differentiation of endogenous precursor cells and thus, the repair of the damaged bone. To test whether these cytokines could direct the differentiation of SKPs toward functional osteoblasts, naïve SKPs were injected into the site of a bone fracture. The results presented in this thesis show that SKPs respond to this osteogenic environment by differentiating into osteoblasts and osteocytes. Interestingly, at three and six weeks post-fracture the proportion of transplanted cells expressing the osteogenic markers ALP and OP was similar to those of the endogenous cells. This finding, along with the morphology of differentiated cells, argues that naïve SKPs can respond to local differentiation factors by differentiating into the appropriate cell types. Since osteoblasts are the precursors of osteocytes (Franz-Odendaal et al., 2006), and a number of SKPs generate osteocytes, these results imply that SKPs can differentiate into functional osteoblasts. However, the number of SKP-derived osteocytes is fairly low compared to the number of injected cells. This may be a consequence of the normal process of bone formation, in which only a fraction of the produced osteoblasts generate osteocytes (Franz-Odendaal et al., 2006). Another reason is that SKPs may differentiate into other cell types normally present in the callus, such as chondrocytes.

Chondrocytes maturing into hypertrophic chondrocytes are important for the process of endochondral ossification and thus bone repair. The data presented here show that three weeks post-fracture, at a time when endogenous chondrocytes have hypertrophied, small numbers of transplanted SKPs differentiated into hypertrophied chondrocytes at the same time and location as endogenous chondrocytes. Similar to the endogenous cells, these SKP-derived chondrocytes expressed markers of hypertrophic chondrocytes, such as ALP, OP and type-X collagen and were
morphologically indistinguishable from the endogenous hypertrophic chondrocytes present at the same location. These observations confirm the chondrogenic capability of SKPs and show that SKPs can differentiate into chondrocytes capable of maturing into hypertrophic chondrocytes \textit{in vivo} and possibly contributing to the process of endochondral ossification. It is possible that if this analysis was performed at earlier time points, at times when chondrogenesis is ongoing, the number of observed SKP-derived chondrocytes would be higher. While these results show the ability of naïve SKPs to differentiate into hypertrophic chondrocytes, it remains to be determined whether they can also generate articular cartilage. This question can be addressed \textit{in vivo}, by injecting SKPs into a joint affected by cartilage damage. Taken together, these results confirm the \textit{in vitro} findings that SKPs can differentiate into skeletal cell types. However, the number of chondrocytes and osteocytes generated by transplanted SKPs is fairly small in comparison to the number of injected cells. Understanding the reasons underlying the low efficiency of cell integration is important for considering SKPs as a potential source of precursor cells for the cell therapies.

5.4 Low number of transplanted cells integrated into the newly formed bone matrix

One explanation for the low number of transplanted SKPs integrating into the newly formed bone is competition between the transplanted and endogenous cells during the repair process. The competition could result from the slower rate at which SKPs differentiate into mature osteoblasts. One of the first steps in bone healing is the recruitment of mesenchymal cells, which represent 80% of the callus area by the fifth day post-fracture (Hiltunen et al., 1993). At this time, some of these cells have already differentiated, as the osteogenic marker type-I collagen, is present from day one post-fracture (Kon et al., 2001); and the cartilage-associated protein type-II collagen is detectable in the callus on day five of the fracture (Le et al., 2001). It is possible that SKPs may not differentiate as quickly as the endogenous cells, leading to fewer differentiated SKPs lining the newly formed bone as osteoblasts, and becoming integrated into the newly formed matrix. The slower rate of SKP differentiation is supported by the \textit{in vitro} observations that it takes 2-3 weeks to observe cartilage nodules, at which time only occasional small foci of mineralisation are observed when sister cultures are grown in osteogenic conditions (Data presented here and unpublished observation by J.-F.L.). Therefore it is possible that \textit{in vivo} the endogenous mesenchymal cells differentiate faster in response to the differentiation cues than
transplanted SKPs. However, this disadvantage of SKPs is likely limited to the initial phase of bone healing as SKPs expressed the osteogenic markers ALP and OP in the similar proportion as the endogenous cells at three and six weeks post-fracture. Looking at osteoblasts- and chondrocyte-specific phenotypic markers at earlier time points may address the possibility of a lag phase in the skeletogenic differentiation of SKPs in vivo, as compared to endogenous cells. These time points may include day one post-fracture, when osteoprogenitor cells are differentiating into osteoblasts(Kon et al., 2001), and day five post-fracture, when they are differentiating into chondrocytes(Hiltunen et al., 1993; Le et al., 2001).

An alternative, non-exclusive, possibility that could explain the low number of transplanted cells integrating into the bone matrix is that only a small proportion of SKPs may be competent to differentiate into mature osteoblasts capable of differentiating into osteocytes. Support for this explanation comes from the in vitro observations that under osteogenic conditions most SKPs up-regulated the osteogenic marker ALP, but not all cells secreted mineral deposits over the six weeks of differentiation (Data presented here and unpublished observations by J.-F.L). Therefore the removal of populations containing less competent cells prior to transplantation, should improve the integration rate of SKPs injected into the fracture. However, the markers needed for positively sorting the competent cells remain to be identified. Surface proteins expressed on mesenchymal cells undergoing mesenchymal condensation prior to their differentiation into chondrocytes or osteoblast could be potential candidates for such a cell sorting strategy. These proteins include N-CAM, N-CAD, the TGF and BMP receptors, fibronectin and tenascin ((Chimal-Monroy and Diaz de Leon, 1999) and Section 1.3.1) and the transplantation of SKPs expressing a combination of these proteins may optimise the efficiency of bone formation by the transplanted naïve SKPs.

An alternative approach to increase the integration of SKPs into the newly formed bone tissue, which was assessed in this thesis, is to pre-differentiate them to “prime” these cells toward the osteogenic lineage prior to their transplantation. The data obtained from this strategy show that pre-differentiation for seven days resulted in lower number of cell integration in the bone matrix compared to naïve SKPS, while not significantly altering their overall survival. It is interesting to note that a short pre-differentiation time of one to four days improved the formation of bone matrix, in vivo, by MSCs and that longer pre-differentiation times decreased the amount of bone formed (Castano-Izquierdo et al., 2007; van den Dolder et al., 2002). The
mechanisms underlying these results were not addressed in those studies. One possible explanation is that long pre-differentiation time preferentially generates mature osteoblasts that have stopped proliferation and started to mineralise their surroundings. These cells, when injected, may fail to proliferate in vivo, which would result in decreased numbers of mineralising cells and a concomitant decrease in bone matrix formation. It is also possible that these more differentiated cells are not as resistant to manipulation and their overall survival may be negatively affected by the transplantation process. Based on the MSCs data (Castano-Izquierdo et al., 2007; van den Dolder et al., 2002) one might predict that a short pre-differentiation time may be more effective at increasing the integration of SKPs into bone. Concomitantly optimizing the pre-differentiation conditions by varying the concentrations of the different growth factors may also result in a higher integration rate.

Another way to increase the yield of SKPs forming bone matrix might be to load naïve or pre-differentiated SKPs on scaffolds with osteoinductive properties and transplant these loaded matrices into the gap of a fracture. This strategy was used with MSCs, using various materials as scaffold, to produce bone matrix that filled large bone gaps (reviewed in (Mauney et al., 2005)). In our model, this technique may improve the efficiency of SKPs to differentiate in vivo and to fill large bone defects.

One question that remains unanswered is whether SKPs can make bone tissue with mechanical properties similar to the endogenous bone. Due to the low number of SKPs that integrated into the newly formed matrix, the properties of the SKP-derived bone tissue could not be assessed in this model. However, this question might be better answered using different models, such as a non-union fracture model (Oetgen et al., 2008) or an osteogenesis imperfecta model (Guillot et al., 2008). In both of these models, repair is inhibited mechanically (as in the first) or genetically (as in the second). The mechanical properties like stiffness, bending load, and torque could be compared between that the SKP-derived bone tissue and host tissue, at several time points following cell transplantation.
5.5 Survival advantage of rat SKPs

The initial bone transplant experiments suggested that the survival of mouse SKPs was lower than rat SKPs following their injection into the bone fracture. This unexpected observation may be the result of the overall survival sensitivity between SKPs isolated from different species. The reason for this decrease in survival is unknown. This cannot be explained by differential host responses, or graft rejection, as NOD/SCID strain used as the recipient is defective in T and B lymphocyte-mediated immune response. The NOD/SCID mice also have decreased activity of natural killer cells, immune cells implicated in graft rejection (Shultz et al., 1995). A more likely explanation is that mouse SKPs are more sensitive to cell manipulation than rat cells. If the handling of the cells is responsible for the decrease in cell survival, fewer cells should survive this process.

It is also possible that mouse SKP survival is compromised by the fracture environment. The initial endogenous response following the onset of bone fracture is the initiation of the inflammatory response. This results in the up-regulation and release of pro-inflammatory cytokines, such as TNF-α, that may alter the survival of the injected SKPs. During normal fracture healing the inflammation response is essential for the proper repair process as the administration of anti-inflammatory drugs from the time of the fracture increases the chances for delayed or non-union (Gerstenfeld et al., 2003b; Giannoudis et al., 2000; Simon et al., 2002; Zhang et al., 2002). In the experiments presented here, the pro-inflammatory response may not have played a critical role on the survival of rat SKP as many cells survived the first three weeks and the number of injected cells at one week was similar to those observed at three weeks (n = 1, unpublished observations by J.-F.L.). However, the role of the inflammatory response can be assessed by looking at survival of SKPs post-injection in the presence or absence of anti-inflammatory drugs. Two other explanations for the lower number of mouse SKPs observed in the injected bone callus include (1) their migration outside the callus, where they could differentiate into other cell types, and (2) their possible in vivo reduced proliferation compared to rat SKPs. These could be tested by analysing the location and proliferation of injected mouse SKPs in leg sections containing the entire leg tissues, ie, the bone and its surrounding muscle and connective tissues, by immunostaining for YFP to visualize the injected cells and the proliferation marker KI67, and counter staining with NissL Red to visualise the tissue
morphology. It is important to address the issue of differential survival/proliferation, since the ultimate goal of these studies is to transplant humans SKPs into immune-competent human hosts.

5.6 Decrease in the number of injected cells over time

The data presented here show an overall decrease over time in the number of SKPs that were transplanted into the bone fracture. The exact mechanism by which these cells become eliminated remains to be determined, but apoptosis may play a role. It is important to note that during the normal course of bone healing the number of mesenchymal cells present in the callus initially increases to reach a maximum on day five post-fracture and then decreases over time (Hiltunen et al., 1993). A similar pattern was observed with the number of injected SKPs. In one initial experiment, a fractured bone injected with rat SKPs was isolated one week post-fracture (unpublished data by J.-F.L.). This bone showed a similar number of cells to those observed in animals injected for three weeks. However, from week-three to six post-fracture, the number of cells decreased significantly. It is interesting to note while the number of cell integrated into the newly formed bone matrix remained constant between weeks three and six, the number of SKP- and endogenously-derived cells showing less differentiation decreased. It can then be hypothesized that the mechanism involved in the removal of injected SKPs over time is the same as the one used to remove the endogenous cells that are in excess. Indirectly, this suggests that SKPs may be a safe source of therapeutic cells, since excess cells or those that fail to differentiate may be removed from the callus.

5.7 Specificity of the in vivo osteogenic differentiation

One concern associated with this bone fracture model is whether cell types other than skeletogenic cells could also integrate into the bone matrix. This has started to be addressed in preliminary experiments using neurospheres and dermal fibroblasts. In one experiment, mouse neurospheres or mouse SKPs were injected into the fractured bone. The analysis of the bone injected with neurosphere for three weeks revealed that only occasional cells survived (less than 10 cells were present in the entire callus, unpublished data by J.-F.L.). In contrast, many more cells were present in animal injected with mouse SKPs. This initial experiment needs to be repeated, but suggests that survival of other precursor cells from neural origin is compromised in a bone fracture environment.
In a separate initial experiment, rat dermal fibroblasts or rat SKPs were injected in the gap of a bone fracture. At three weeks post-fracture, many dermal fibroblasts were observed in the callus. The qualitative analysis revealed that the number of injected fibroblasts at this time point was similar to the number of rat SKPs. However, in contrast to SKP-injected bones, no injected fibroblast cells were observed in the bone matrix (unpublished data by J.-F.L). This initial experiment needs to be repeated and quantitatively analysed by determining the percentage of injected cells that express the different osteogenic differentiation markers as well as the number of these cells that have integrated into the bone matrix. Nonetheless, this initial experiment suggests that SKPs are more efficient than dermal fibroblasts at integrating into the newly formed bone matrix. Taken together these two experiments suggest that only a subset of cell types injected into a bone fracture are capable of surviving in the fracture environment and integrating into the newly formed bone tissue.

5.8 SKPs differentiate into multiple cell types in vivo

In the bone fracture model described above, not all SKPs injected in the fracture stayed at this location. Some of them leaked out and contacted adjacent tissues such as connective tissue and the skeletal muscle. Interestingly, SKPs present at these locations differentiated into cell types normally found in these tissues, namely, perivascular and neural cells.

**Perivascular cells.** Results presented here show that SKP-derived perivascular cells adopt different morphology depending on the size of the blood vessel they are associated with. This suggests that SKPs do not generate only a single type of perivascular cell, but rather differentiate into the perivascular cell type that is normally associated with a specific blood vessel. Based on the differentiated cell morphology and the diameter of the blood vessels they associate with, it can hypothesized that SKPs differentiate into pericytes in capillaries and into perivascular smooth muscle cells in larger vessels. The next experiments that would address this hypothesis would be to determine the immuno-phenotype of these cells. One would expect SKPs with pericytic differentiation to be positive for pericyte markers, such as NG2, CD146, desmin and PDGFRβ (Crisan et al., 2008), while those with perivascular smooth muscle differentiation to be positive for smooth muscle markers, such as SM22α, h1-calponin, smooth muscle myosin
heavy chain, h-caldesmon, smoothelin, and aortic carboxypeptidase-like protein (Owens et al., 2004). As Crisan and colleagues have suggested that pericytes may be a source of mesenchymal stem cells in a variety of tissues (Crisan et al., 2008), it would then be important to determine whether pericytes generated from SKPs also have the potential to produce MSCs. To test this, the same experimental strategy used by Crisan and colleagues to identify MSCs from perivascular cells could be applied to SKP-derived perivascular-like cells (Crisan et al., 2008). If the results from these experiments show that SKPs can indeed develop into MSCs once transplanted in vivo, it would be tempting to speculate that the beneficial contribution of SKPs transplantation may not be limited to the direct repair of the injured tissue, but also to their integration into the pool of precursor cells, such as MSCs, in the host tissue. This may allow a single transplantation of SKPs to have multiple long lasting beneficial effects.

The mechanisms by which SKPs differentiate into perivascular cells and are recruited in the assembly of blood vessels remain speculative. It can be hypothesised that this differentiation path is induced by the local environment. One line of evidence comes from the results presented here, in which SKP-derived perivascular cells were only detected in locations predicted to have ongoing neovascularisation, such as in the vicinity of and within the callus. The need for the formation of new blood vessels may explain why SKP-derived perivascular cells were not observed following their transplantation in other reports (Biernaskie et al., 2007; McKenzie et al., 2006). In those studies, neovascularisation may not have been sufficient to recruit transplanted SKPs.

The efficiency of SKPs to differentiate into these perivascular cells could have clinical implications, and thus needs to be assessed. One way this could be evaluated is by using an in vivo assay such as the one used by Au and colleagues that analysed the ability of MSCs to differentiate into perivascular cells (Au et al., 2008). In this assay, endothelial cells and SKPs would be mixed, injected into collagen-fibronectin matrices, transplanted in vivo, and then the number of functional blood vessel derived from the assessed cells can be determined. From a clinical perspective, formation of new blood vessels in ischemic tissues can be advantageous. Therefore it would be interesting to determine whether other clinically relevant conditions, such as hypoxia, which is known to induce neovascularisation (Giordano and Johnson, 2001), can lead to recruitment and differentiation of transplanted SKPs into perivascular cells. Moreover, it
would be important to compare the perivascular differentiation efficiency of SKPs and MSCs to determine which cells have the best clinical potential.

Another observation from this work is that SKP-derived perivascular cells were also observed in animals injected with pre-differentiated SKPs. This suggests that the pre-differentiation step did not commit SKPs to differentiate in only one cell type. This is an important observation as it suggests that injected SKPs failing to differentiate into the desired cell types may differentiate into other cell types used by the host to repair damaged tissues.

**Neural differentiation.** SKPs present within skeletal muscle differentiated into cells expressing neuronal markers. These SKP-derived cells localised to the innervated areas of the skeletal muscle, possibly the neuromuscular junction, which may have been inadvertently damaged as a consequence of the bone fracture procedure. The phenotype of these SKP-derived cells remains questionable due to two main reasons. First the sensitivity of the microscopy used to determine the co-localisation of GFP, to identify the transplanted cells, and βIII-tubulin was not sufficient. Co-localization might be better studied using confocal microscopy. Second, a previous report showed that SKPs do not readily differentiate into neurons *in vivo* (Fernandes et al., 2006). This, combined with the fact that skeletal muscles have not been recognised as an active site of neurogenesis suggest that it is unlikely for SKPs to undergo neuronal differentiation in skeletal muscle. However, SKPs can differentiate into Schwann cells *in vivo*, when injected into damaged peripheral nerve (McKenzie et al., 2006) or in the spinal cord (Biernaskie et al., 2007). Since Schwann cells are the cells that myelinate axons of peripheral motor neurons, it can be hypothesized that the transplanted SKPs that migrate to skeletal muscle are in fact differentiating into Schwann cells, not neurons. Such cells would stain for typical Schwann cell markers, such as S100β, GFAP, and P0, and would also show GFP co-localization.

It is also important to note that no SKP cells were found with a striated skeletal muscle phenotype. It is possible that in this model the damage to the skeletal muscle was not extensive enough to induce SKPs to differentiate into skeletal muscle. Alternatively it is possible that SKPs do not have the capacity to efficiently differentiate into skeletal muscles. These two possibilities need to be tested in a different model in which skeletal muscles are damaged sufficiently to induce their repair from endogenous precursor cells. Such model could be an
intramuscular injection of cardiotoxin (Sun et al., 2009) or hind limb ischemia (Aranguren et al., 2008).

5.9 SKPs are multipotent precursors

The data presented here support the conclusion that SKPs are multipotent precursors. At the sphere level, most human SKPs were capable of differentiating into osteoblasts and chondrocytes, suggesting that each human SKP sphere contains either bipotent precursor cells or a mixture of committed cells capable of differentiating into only one cell type. To discriminate between these two possibilities, clonal rat SKPs were generated and differentiated under osteogenic, chondrogenic and Schwann cell conditions. The results of this differentiation experiment showed that SKPs have the potential to differentiate into mesodermal and neural cell types, thus supporting the previous conclusion that SKPs are multipotent precursors (Toma et al., 2001; Toma et al., 2005) and extending the known differentiation potential of SKPs. With the work presented here, the list of cell types that SKPs can differentiate into includes neurons, Schwann cells, adipocytes, osteocytes, chondrocytes, perivascular cells and possibly tendocytes. With such a wide differentiation potential it is intriguing that in the skin, where SKPs are associated with hair follicles, they do not generate any of these cell types. Therefore an important question remaining to be answered is what are the mechanisms involved in determining the phenotype of SKPs in vivo? It is possible that environmental signals may contribute to the determination or the restriction of SKP phenotype.

5.10 SKP differentiation is dictated by the local microenvironment

The combination of the results presented here and those previously published suggest that SKP differentiation is dictated and/or restricted by the local microenvironment. Multiple lines of evidence support this hypothesis. First, ectopic injection of SKPs in the bone fracture resulted in their differentiation into osteoblasts, osteocytes and chondrocytes, cells normally present in the callus. Some of the SKPs also differentiated into perivascular cells in area of neovascularisation. No neuronal, glial or adipogenic differentiation of injected SKPs were observed in the bone tissue, in spite of their known ability to generate these cells types (Fernandes et al., 2006;
McKenzie et al., 2006; Toma et al., 2001; Toma et al., 2005). Second, transplantation of SKPs back into the skin caused their differentiation into adipocytes in the hypodermis (Chapter III) and into dermal fibroblast in the dermal compartment of the skin (Biernaskie and colleagues, in preparation). No evidence of osteogenic or chondrogenic differentiation was observed in the skin. Third, injection of SKPs in peripheral nerve resulted in their differentiation into Schwann cells (McKenzie et al., 2006). In the nerve tissue, the transplanted cells did not differentiate into osteoblast, chondrocytes or adipocytes, cell types normally absent in this tissue. Taken together, these results support the hypothesis that the local environment instructs the appropriate differentiation program by either its induction or by preventing the differentiation into inappropriate cell types. This also implies that SKPs have the ability to detect and properly respond to the differentiation factors present in their environment.

A prediction of this hypothesis is that in the absence of an instructive environment SKPs should either fail to differentiate or differentiate into either random or default cell types. In support of this prediction, in vitro differentiation of SKPs in presence of serum only, resulted in their differentiation into many different cell types with occasional cells differentiating into osteoblasts or chondrocytes (Chapter II) and others differentiating into neurons, glial cells, smooth muscles or adipocytes (Toma et al., 2001).

This prediction can also be extended in vivo. In tissues that fail to repair themselves, the regenerative cues could be missing or factors could be present preventing the coordinated regeneration. One such tissue is the spinal cord. If this prediction is correct, transplantation of SKPs in injured spinal cords should result in either their failure to differentiate or their differentiation into a variety of cell types including some that are not normally present in this tissue. In support of this idea, naïve SKPs injected into injured spinal cord differentiated into a variety of cell types including cells that are normally absent in this tissue, such as smooth muscles, adipocytes and possibly chondrocytes (Biernaskie et al., 2007). These observations also support the hypothesis that SKP differentiation is dictated by their local environment.

To further test this hypothesis, the following experiment is proposed. Clonal SKPs should be injected at different locations along the forming neural tube of embryos at the time neural crest cells migrate to their target tissue. Based on the report by Fernandes and colleagues (Fernandes et al., 2004), SKPs should migrate to the same target tissues as neural crest cells. The
phenotype of these injected SKPs would be determined once they have reached their final target. If it is the environment that dictates the phenotype of differentiating SKPs, the injected clonal SKPs should differentiate into only proper cell types that are normally found at each of the locations they have migrated to. Alternatively clonal SKPs could be injected in different tissues of a developing embryo and then their phenotype could be determined at different time intervals following their transplantation.

These findings also raise questions about the mechanism that control the differentiation potential of endogenous SKPs within the dermis. Hair follicles were previously shown to be one niche for SKPs (Fernandes et al., 2004), and recent work from our laboratory indicates that in hair-bearing skin they reside within both the dermal papilla and dermal sheath of hair follicles (unpublished observations by J.A.B. and F.D.M.). Consistent with the findings of this thesis, cultured hair follicle dermal cells have been previously shown to generate cells with characteristics of osteocytes (Jahoda et al., 2003). Thus, a key question is whether or not the endogenous follicle-associated precursors have the capacity to generate osteocytes or chondrocytes, and, if so, how the dermal environment restricts genesis of these inappropriate cell types. Interestingly, several independent reports suggest that endogenous dermal cells may have skeletogenic capacity. First, in rare cases, osteosarcomas arise directly within the dermis (Massi et al., 2007). Second, vertebrates such as alligators develop a dermal skeleton, where the bone is directly generated from the dermal connective tissue (Vickaryous and Hall, 2008). Finally, when SKPs are transplanted into the dermis, they only generate appropriate dermal cell types (unpublished observations by J.A.B and F.D.M.), arguing that it is the dermal environment that limits differentiation of the endogenous SKPs. To further confirm this conclusion, mammalian SKPs could be injected into the dermis of reptile such as alligators to determine whether SKPs, in this new dermal environment, would contribute to the dermal skeleton.

5.11 Relationships between SKPs, MSCs and NCSCs

The previous work by Fernandes and colleagues led to the conclusion that SKPs are precursor cells related to neural crest cells (Fernandes et al., 2008). The work presented here also supports this conclusion. It was found that SKPs differentiated into the mesodermal cell types osteoblasts, osteocytes, chondrocytes, perivascular cells and adipocytes in vivo, which are cell types derived
from a subset of neural crest cells. This expands the list of neural crest-derived cell types that can be generated by SKPs.

Previous characterisation of SKPs showed that SKPs are related to neural crest stem cells (Fernandes et al., 2004) and that facial SKPs are derived from neural crest cells. Neural crest stem cells derived from different regions along the rostro-caudal axis contribute to the formation of different tissues (See Chapter I). One important question remaining to be answered is whether SKPs are more related to a specific subset of neural crest stem cells or whether they are precursor cells that possess the combination of differentiation abilities of all subsets of neural crest stem cells. Answer to this question will help determining the full differentiation potential of SKPs and in the prediction of their therapeutic potential. The combination of the observations that dorsal neural crest stem cells do not generate skeletal derivatives, even when they are ectopically transplanted in cranial regions (Conway et al., 1997; Couly et al., 1998; Creuzet et al., 2005) with the work of Fernandes and colleagues that showed the expression of Dermo-1 and SHOX2 in SKPs (Fernandes et al., 2004), two genes expressed in mesenchymal cells differentiating into skeletal cell types (Gu et al., 2008; Li et al., 1995), suggest that SKPs may be more related to cranial neural crest stem cells than those derived from the dorsal region. However, SKPs differ from cranial neural crest cells in that the latter cell type is capable of differentiating into skeletal cell types in the dermis when they are transplanted into caudal regions (Couly et al., 1998; Le Douarin and Smith, 1988), whereas skeletal cell differentiation in the dermas has never been observed in SKPs. Thus, another possibility is that SKPs share characteristics with both cranial and dorsal neural crest cells. This could be studied by comparing gene expression profiles of the above three precursor types, as well as that of the undifferentiated neuroepithelial cell. SKPs could also be transplanted in cranial or caudal locations of the developing chick neural tube (as was done for cranial and dorsal neural crest cells), to determine which cell types (and their final location) result. If the phenotypes of the transplanted SKPs are appropriate for the tissue to which they have migrated, this would suggest that SKPs are distinct multipotent precursor cells sharing properties of both cranial and dorsal neural crest cells.

Facial SKPs (Fernandes et al., 2004) and the first wave of MSCs (Takashima et al., 2007) are derived from neural crest cells, but during normal development these cell types diverge, in terms of genetic profiles, growth properties and in vivo function. Previous work by Toma and colleagues showed that SKPs have an antigenic profile distinct from MSCs, and that MSCs and
SKPs behaved differently when cultured in SKP culture conditions (Toma et al., 2001). From these data, it was concluded that SKPs and MSCs are different types of precursors. This was further confirmed in our laboratory by Hiroyuki Jino, who analysed the gene expression profiles of SKPs and MSCs (unpublished observations by H.J. and F.D.M). SKPs and MSCs are independent entities, though, as the work presented here shows that SKPs can respond appropriately to mesodermal differentiation conditions used to differentiate MSCs into osteoblasts and chondrocytes. Thus SKPs and MSCs are distinct precursors but share overlapping differentiation potentials.

The exact mechanism by which SKPs differentiate into osteoblasts and chondrocytes remains to be addressed. As discussed earlier, SKPs may directly differentiate into osteoblasts and chondrocytes, or through a transition stage. This transitional stage may involve a process similar to mesenchymal condensation that allows the genetic reprogramming of the differentiating cells. The characterisation of the mechanisms involved in this differentiation process will help improving the differentiation protocols. A second application resulting from this characterisation is the possible use of SKP as an *ex vivo* cell system to study the mechanisms that are perturbed in some genetic defects affecting mesodermal condensation or differentiation during skeletogenesis.

### 5.12 Use of SKPs as therapeutics: potentials and current limitations

The growing body of work, including that presented in this thesis, suggests that SKPs are good candidates for cell therapy. There are four main advantages in the use of SKPs as precursor cells for cell therapies. First, SKP isolation is relatively uninvasive procedure, skin is an easily accessible organ. As SKPs can be isolated from both neonatal and adult human skin with low morbidity, it is possible to use them for autologous transplantation ((Toma et al., 2005) and see Chapter 2 and Appendix 1). Second, SKPs can be extensively expanded *in vitro* (Toma et al., 2005). This allows the generation of sufficient amounts of therapeutic cells from a relatively small tissue sample. A third advantage is that SKPs can differentiate into a wide variety of cell types, including neural and mesenchymal cells. These cells were shown to be functional *in vivo*, previous work by McKenzie and colleagues (McKenzie et al., 2006) and Biernaskie et al.
(Biernaskie et al., 2007), showed that in different in vivo models, such as peripheral nerve crush, spinal cord contusion and shiverer mouse nervous system, SKP-derived Schwann cells formed de novo myelin and contribute to the repair of the injured tissue. Along this line, the work presented in this thesis shows that SKPs can contribute to the repair or de novo formation of mesenchymally-derived tissues, such as bone and blood vessels, and possibly ligaments. Fourth, SKPs differentiation is dictated by their environment, which is supported by previous results (Biernaskie et al., 2007; McKenzie et al., 2006) and those presented here. This suggests that naïve SKPs transplanted in most tissues (the spinal cord excepted), are unlikely to form teratomas or generate aberrant cell types. In the case of the spinal cord transplants, pre-differentiation of SKPs into Schwann cells prevented their differentiation into undesired cell types (Biernaskie et al., 2007). Taken together, these indicate that naïve or pre-differentiated SKPs are potentially safe precursor cells for cell therapy.

However, a number of questions remain to be addressed before the first clinical trials in humans could be conducted. These include the determination of whether human SKPs have the same differentiation potential in vivo as their rodent counterpart; whether transplanted human SKPs can be rejected by heterologous hosts; and how long would the beneficial effects of the transplanted cells last?

To expand on these issues, most of the work on SKPs has focused on rodent cells. However, a few studies suggest that rodent and human SKPs have similar potentials. McKenzie and colleagues showing that transplanted human SKPs can form myelin in the brain of neonatal shiverer mouse as did their rodent counterpart (McKenzie et al., 2006). Furthermore, in vitro data has also showed that human SKPs share the same differentiation potential as rodent SKPs ((Toma et al., 2001; Toma et al., 2005) and this thesis).

A second concern is that SKPs could trigger an immune rejection reaction following heterologous transplantation, resulting in their elimination from the transplanted host. Therefore the immunogenicity of human SKPs in vivo needs to be addressed. MSCs were shown to have the ability to “evade” the host immune system and inhibit the graft versus host immune response (Uccelli et al., 2008). Whether this immunosuppressive property of MSCs is also shared by SKPs remains to be characterized. This could be assessed by transplanting non-syngenic SKPs to immunocompetent animals and determining the survival and integration of the transplanted
cells in the host tissue over time. If SKPs are indeed eliminated by the host due to immune-mediated rejection, the use of SKPs would be limited to autologous grafting unless appropriate anti-rejection therapy is devised.

A third question remaining to be addressed is how long the beneficial effects of transplanted SKPs would last in humans. Results in rodent from experiments presented here show that SKPs were present in injected tissue for at least six weeks in the bone and connective tissue and at least 10 weeks in damaged spinal cord (Biernaskie et al., 2007). Long-term follow-up studies still need to be performed. Data from the transplantation of MSCs for the treatment of osteogenesis imperfecta show that the beneficial effects of the transplanted cells decrease over time (Horwitz et al., 2001). The data showing the integration of SKPs in the periosteum, which houses osteoprogenitor cells involved for bone repair (Augustin et al., 2007; Fan et al., 2008), raise the possibility that transplanted cells could become part of the pool of osteoprogenitor cells and possibly be recruited for the normal turn-over of the bone tissue or subsequent to bone fracture. However, it remains to be determined whether SKPs homing to the periosteum are capable of contributing to osteoblasts generation, and if so, for how long. One way to answer this question would be to graft SKPs into the periosteum of healthy mice and determine (1) the time these SKPs will remain in this niche; (2) whether integrated SKPs would generate a sustained number of osteoblasts that will integrate into bone over time as a result of bone turnover; and (3) whether these periosteum-engrafted SKPs can be recruited to heal the bone following a fracture. The eventual aim would be to use a single graft of SKPs to provide long-term treatment.

For the use of human SKPs in skeletal repair to become a reality, a number of questions remain to be answered. First, the osteogenic differentiation of human SKPs in vivo needs to be assessed and quantified. Second, in many clinical settings in which therapeutic cells could be used, the fractured bone may fail to repair itself, leading to non-union. This contrasts with the bone fracture model used in this study, in which the fractured bone is capable of self-healing without intervention. Therefore it would be clinically relevant to test whether SKPs, especially human SKPs, are able to differentiate into osteogenic and chondrogenic cells that can promote healing in a non-union fracture model. It is possible that pre-differentiation or the use of osteoinductive scaffolds may facilitate their differentiation in these settings.
Another potential use of SKPs is bone reconstruction for the repair of large gaps between bone parts. Current strategies make use of different matrices embedded with MSCs to form scaffolds that facilitate bone formation (Mauney et al., 2005). A similar strategy need to be tested human SKPs.

Other possible indications for therapeutic cells are in systemic bone diseases, either due to genetic conditions such as osteogenic imperfecta, or degenerative conditions such as osteoporosis. In these conditions, systemic introduction of SKPs may be advantageous in these patients, but as systemic use of SKPs has not been closely studied, there are many issues that need to be addressed. For example, can intravenously injected SKPs engraft into specific injured sites in the body to promote healing? What is the safety profile of injected SKPs? Would local or systemic injection be more effective in targeting specific areas needing repair? How long would the SKP transplants last?

For genetic diseases in particular, either SKPs from heterologous sources that express wild-type proteins, or, autologous SKPs that are genetically modified to express wild-type proteins may be transplanted. However, both of these sources of therapeutic cells may trigger an immune response leading to graft rejection. How the host responds to these transplanted cells is an important issue that needs to be studied prior to initiating any human clinical trials.

One interesting observation in this thesis is the ability of SKPs to integrate into the knee ligament. This may suggest another potential use of SKPs in the treatment of injured ligaments and tendons. More characterisation is required to determine whether these SKP-derived cells differentiate into tendocytes. The efficiency of SKPs to integrate this tissue and whether this can result in mechanical benefits remains to be determined. Due to the high frequency of ligament and tendon damage in the general population and among athletes, this is an important potential application of SKPs for cell therapy.

The observations that SKPs could differentiate into chondrocytes in vitro raised the possibility of using SKPs for cartilage repair. It is important to note that joint cartilage contains non-hypertrophic chondrocytes, while bone fracture healing areas contain more mature, or hypertrophic chondrocytes. To date, SKPs injected into bone-fracture gaps have generated occasional chondrocytes expressing markers of hypertrophic chondrocytes, but it is unknown whether SKPs can generate non-hypertrophic chondrocytes in vivo, which would be a more
physiologic cell type in joint cartilage. Thus, the ability of SKPs to differentiate into joint chondrocytes remains to be assessed. This could be tested by transplanting naïve or pre-differentiated SKPs into joint cartilage defects, and assessing the phenotype (hypertrophic versus non-hypertrophic) of the transplanted cells at different time points post-transplantation. Experiments addressing the feasibility of this approach could result in new potential applications for the use of SKPs as therapeutic.

As alluded to above, the use of genetically modified SKPs to correct genetic defects is another potential application of these cells. Results presented in Appendix I show that rodent SKPs can be infected with genetically engineered adenoviruses, allowing transient expression of a therapeutic protein. One such protein is bone morphogenic protein. This family of proteins have been reported to improve bone healing in patients with open or non-union fractures (Eckardt et al., 2005; Friedlaender et al., 2001; Govender et al., 2002). SKPs could be infected with replication deficient adenovirus encoding the BMP-2 gene and then transplanted in the gap of the non-union fracture. Ideally, these SKPs expressing BMP-2 could serve dual roles to form new bone tissue and to locally produce a therapeutic factor. Alternatively to adenoviruses, retroviruses could be used to permanently integrate the gene encoding the therapeutic protein into SKPs, which, once transplanted into the host, would then give rise to reparative cells. These genetically modified SKPs could improve or expend the therapeutic use of SKPs. For example, the mutant type-I collagen gene present in patient affected by osteogenesis imperfecta could be silenced and a wild type copy of that gene be inserted into SKPs derived from the affected patient. These patient-derived modified SKPs could then be transplanted back into the same patient.

5.13 The use of SKPs as tools to understand diseases

The use of SKPs may not be limited to therapeutic applications. The ability of SKPs to differentiate into multiple cell types suggests they can be used as cell systems to understand the mechanisms of human diseases. For example, SKPs isolated from patients affected by a genetic condition can be analysed in terms of cell survival, growth, and differentiation potential. The differentiated cells could also be characterised for the presence of phenotype or cellular
abnormalities. One could therefore pin-point cellular and molecular mechanisms implicated in the studied disease.

Initial results in Appendix 1 provide some support for use of SKPs in this manner. In this project, SKPs were hypothesized to be the cell of origin for the dermal tumours observed in patients affected by the genetic condition, type-1 neurofibromatosis (NF-1). In addition to cutaneous neurofibromas, these patients can experience skeletal defects, mental retardation as well as predisposition to other tumours such as plexiform neurofibromas and optic nerve gliomas (Crawford and Schorry, 2006). This genetic condition is associated with the partial or complete loss of function of neurofibromin protein, a negative regulator of the Ras-GTPase. The implication of SKPs in the formation of the dermal tumours was hypothesized based on the observations that SKPs can give rise to Schwann cells, which is also the predominant cell type in cutaneous neurofibromas. In addition, both SKPs and cutaneous neurofibromas localize to the dermis. The data presented in Appendix I show that SKPs can be isolated from NF-1 patients and appeared to be normal based on sphere morphology and expression of protein markers. Since Schwann cells are the main cell type found in this tumour, one objective of this project was to differentiate SKPs, obtained from human NF-1 patients, into Schwann cells and determine whether they have increased growth factor-independent survival or proliferation, which may correlate with tumorigenicity. The initial experiments for this differentiation were not successful, due to technical reasons.

One sample of SKPs obtained from NF-1 patient was also differentiated under neuronal conditions. The initial data show a tendency of NF-1 SKPs to generate βIII-tubulin positive cells with shorter and thicker processes than those obtained from normal SKPs. Further experiments to quantify the length of these neuronal processes are necessary. It is interesting to note that this initial observation is in agreement with previous reports showing a decrease in the neurite length following the loss of NF-1 in neurons derived from PC12 cells, neurosphere, and rat hippocampus (Hegedus et al., 2007; Yunoue et al., 2003). This initial result suggests that SKPs cultured from affected human patients, under defined conditions, can show phenotypes similar to those obtained from established cell systems. This supports the idea for the use of SKPs as a source of human cells to study mechanisms of human diseases. This strategy can complement the use of small animal models. Another advantage of SKPs is that cell differentiation can be done both in vitro and in vivo, and once differentiated the human SKP-derived cells could be
transplanted in vivo to assess their properties under physiological conditions as was already described for rodent SKPs (Biernaskie et al., 2007; Fernandes et al., 2006; McKenzie et al., 2006). Taken with the results presented in this thesis, this strategy may be used to understand human genetic conditions affecting bone and cartilage.

5.14 Conclusion:

The results presented in this thesis show that SKPs, isolated from rodents or humans, are multipotent precursors that have the potential to differentiate into osteoblasts and chondrocytes, both of the mesodermal lineage, in vitro. Rodent SKPs can also be differentiated into these cells in vivo when transplanted into a bone fracture. The mesodermal differentiation of SKPs is not limited to these skeletal cell types, as some of them also differentiated into perivascular cells and adipocytes in vivo. The type of cell generated depended on their location (perivascular versus hypodermal, respectively). The data presented here also support the hypothesis that differentiation of SKPs is dictated by local environment. Taken together, this work supports the therapeutic potential of SKPs as precursor cells for the treatment of skeletal defects.
References


Sekiya, I., Vuoristo, J. T., Larson, B. L. and Prockop, D. J. (2002). In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis. Proc Natl Acad Sci U S A 99, 4397-402.


Appendix 1
Neurofibromatosis type-1 project

7.1 Introduction:

This appendix describes the hypothesis, aims and data of my initial thesis project, which was prematurely terminated after a few months due to a competitive conflict. The main purpose of this section is to present some of the initial data from this project that support the suggested applications of SKPs that are described in Chapter 5.

Neurofibromatosis type-1 (NF-1) is a genetic condition with a frequency of 1 in 3500 people (Carroll and Ratner, 2008) that results from a germline mutation or deletion of the neurofibromin (Nf1) gene. NF-1 patients are predisposed to develop benign tumours such as neurofibromas and peripheral nerve sheath tumours that can progress into malignant peripheral nerve sheath tumours. Current therapies are ineffective against these malignant forms and a better understanding of the cellular mechanisms underlying the genesis of these tumours could lead to more successful therapies. The major cell type found in these tumours is the Schwann cell, in which the lost of heterozygocity for NF1 gene is common, and sufficient in mice heterozygotes for NF1 to generate tumours similar to those seen in NF-1 patients (Zhu et al., 2002). These observations suggest that transformed Schwann cells are the cells initiating peripheral nerve sheath tumours in patients.

There are presently no primary human cell systems available to study the events that lead to the initiation and progression of NF-1. SKPs, present in the skin of mice and humans, can be isolated and readily differentiated into Schwann cells (McKenzie et al., 2006). Since stem cells or precursor cells have been recently found as tumour-initiating cells or the “cancer stem cells” responsible for the initiation and progression of a number of solid tumors (Pardal et al., 2003), I hypothesized that SKPs are the target cell or cell of origin for cutaneous neurofibroma, and that the loss of NF1 in these cells would result in tumour formation. The first aim of this project is to determine whether the loss of NF-1 could induce the transformation of SKPs derived from either rodent cells in which NF-1 is lost and in human cells derived from NF-1 patients by testing a series of properties of including self-renewal, growth factor dependency for growth and survival,
ability to differentiate into different cell types, especially Schwann cells. The second aim testing this hypothesis is to determine whether the acute loss of NF1 in mouse SKPs is sufficient to cause tumour formation. This could be achieved by culturing mouse SKPs expressing \textit{Nf1} gene flanked with flox sites. The expression of the Cre recombinase in these cells, by infection with replication deficient transgenic adenovirus encoding the Cre cDNA, would result in the excision of the Floxed-NF-1 allele in the infected cells. By infecting SKPs heterozygous or homozygous for the flox-cre allele, cell heterozygous or null for the NF-1 gene can be generated. These cells could then be injected into mice to determine whether they will form tumours \textit{in vivo}. Alternatively, these NF1-flox mice could be crossed with transgenic mice expressing the Cre recombinase under a gene promoter active in SKPs, such as \textit{Slug}, \textit{Wnt1}, or \textit{Snail} promoter and that also require the presence of tetracycline or tamoxifen to be activated. Such promoter needs to be designed and tested but it utilizes a similar strategies used to amplify certain types of transgenic adenoviruses (Lavoie et al., 2005). The skin of these mice could then be treated locally with tamoxifen to induce the expression of Cre only in SKPs. This will allow the excision of \textit{Nf1} gene only in SKPs localizing to the treated regions of skin. The formation of tumours and the isolation and characterization of these SKPs could then be determined. Our prediction is that SKPs with \textit{Nf1} acutely deleted will be transformed and form neurofibroma.

Most of these experiments, suggested in this project, remain to be performed as the project was terminated in its infancy as a result of a competing conflict. The preliminary results generated are described here and show the proof of principle that SKPs can be used not just as a therapeutic tool, but also as a model to further understand mechanisms of disease.

7.2 Author’s contribution:

I performed all primary cultures and generated all the data presented in this appendix. Human samples derived from NF-1 patients and the NF1 mice were obtained from Dr. Abhijit Guha (Hospital for Sick Children and Toronto Western Hospital) and foreskin sample obtained from Dr. Darius Bagli (Hospital for Sick Children).
7.3 Results:

7.3.1 SKPs can be generated from the skin of NF-1 patients.

The first step in determining whether SKPs are affected in patients diagnosed with NF-1, and whether they are the tumour-initiating cells in these patients was to determine whether SKPs can be isolated from the skin of NF-1 patients. The presence of SKPs in these patients was assessed in skin samples derived from eight patients, four females and four males. The samples were obtained from different skin locations (back, thighs and neck) and the age of the patients ranged from 20 to 50 years old. Skin samples were cleaned and most of the hypodermis was removed by dissection, using the same techniques described in Chapter IV. SKPs were then isolated and grown as for the foreskin samples. In NF-1 samples a few small spheres formed within one to two weeks of culture and at three to four weeks they were big enough to be passaged. Seven out of the eight samples formed spheres. The morphology of the spheres from the first passage (Figure A.1A, top left panel) was identical to those obtained from human foreskin (Figure A.1A, bottom left panel). To determine the expression of proteins that mark SKPs, primary spheres from one NF-1 SKP sample were plated on 1% poly-D-lysine 2% laminin coated dishes for 18 hours to allow their attachment to the substratum and then immunostained for the SKP markers nestin and fibronectin (Fn) and visualized by immunofluorescence. The analysis revealed that SKPs from this sample expressed these two markers (Figure A.1A top center and right panels), which was similar to the cytospun human foreskin-derived SKPs (Figure A.1A bottom center and right panels) used as positive control. This characterisation remains to be repeated with more samples and in these future experiments both NF-1 patient-derived SKPs and normal control SKPs need to be processed the same way, either cytospun or plated overnight in coated dishes.

7.3.2 Neuronal differentiation of NF-1 derived SKP is altered.

To further characterise NF-1 patient-derived SKPs, the differentiation capacity of these SKPs and those derived from normal human foreskin were compared on their ability to differentiate into Schwann cells and neurons. The Schwann cells differentiation was not successful among
Figure A.1: Neuronal differentiation of SKPs derived from NF-1 and normal patients. (A) Bright field photomicrographs of SKP spheres derived from NF-1 patient (top left panel) or foreskin (bottom left panel). Photomicrograph of NF-1 patient-derived SKP sphere seated to the dish substratum for 18 hours (top center and right panels) or foreskin-derived SKPs cytospun (bottom center and right panels) and stained for the SKP markers Nestin (green, center panels) and fibronectin (Fn, green, right panels). Scale bar for top left panel = 250 μm and for the other panels: scale bars = 100 μm. (B) Neuronal differentiation of human SKPs derived from NF-1 patient (left panels) or foreskin (right panels) and stained for the neuronal marker βIII-tubulin (red in top panels and green in bottom panels). Cells were differentiated at passage 1 (top panels) or passage 2 (bottom panels). Original magnifications are indicated on the microphotographs. Arrows point toward βIII-positive cells.
the few foreskin-derived SKP samples tested. This is likely due to a technical reason resulting from the lack of optimal differentiation conditions for human SKPs at that time. Therefore due to the lack of comparative control the Schwann cell differentiation of NF-1 patient-derived SKP remains to be assessed.

The neuronal differentiation was then analysed using protocol previously described (Toma et al., 2005). Cell differentiation was determined by immunofluorescence staining for the neuronal marker βIII-tubulin. Foreskin-derived SKPs formed βIII-tubulin positive cells with neuronal morphology when cultured in these conditions (Figure A.1B, right panels). This is in agreement with previous results from Dr. Freda Miller’s laboratory (Toma 2005). SKPs derived from NF-1 patient differentiated under the same conditions also generated cells that were βIII-tubulin positive (Figure A.1B top left panel), but the length of their processes appeared to be shorter and the size of the cell processes were generally thicker than those formed by foreskin-derived SKPs. This difference appeared more pronounced among NF-1 SKPs passaged twice (Figure A.1B, bottom left panel), while foreskin-derived SKPs at the same passage remained capable of forming neuron-like βIII-positive cells (Figure A.1B bottom right panel). These results suggest a defect in SKPs derived from NF-1 patients to differentiate into neuronal cells under these in vitro conditions. To confirm this conclusion, multiple lines of SKPs, each derived from a different NF1 patient, need to be differentiated to assess their neurogenic differentiation. Quantitative analyses of these cultures is also required to determine the efficiency to the neuronal differentiation, the length and thickness of the neuronal processes, and the expression of other neuronal markers from different neuronal differentiation stages including nestin, p75NTR, NFM, MAP2, and GAP-43. Moreover, the NF-1 status in the human SKPs need to be confirmed.

7.3.3 Mouse SKPs and SKP-derived Schwann cells can be infected by adenovirus.

To determine whether the acute loss of Nf1 is sufficient to induce transformation of SKPs or SKP-derived Schwann cells, a transgenic mouse line expressing floxed alleles of the Nf1 genes was generated by the laboratory of Dr. A. Guha (Hospital for Sick Children). Some mouse carried one floxed allele (NF1$^{\text{flox}^+}$) while others carried only floxed alleles (NF1$^{\text{flox}^{\text{flox}}}$).
**Figure A.2: Primary SKPs and SKP-derived Schwann cells can be infected with adenoviruses.** Mouse SKPs expressing wildtype NF-1 allele (NF-1+/+) or expressing one (NF-1\(^{flox/+}\)) or two Floxed alleles (NF-1\(^{flox/flox}\)) were generated. (A) Microphotograph of primary NF-1\(^{flox/flox}\) SKP (left panel) and passage-2 NF-1\(^{flox/+}\) SKPs (right panel). (B) Skin cells from NF-1\(^{flox/flox}\) animal were infected with 50 multiplicity of infection overnight 100 μl of SKP media in microcentrifuge tubes and then transferred into regular dishes in larger volume of SKP media as per standard SKP protocol. To determine the GFP expression at day 7 post-infection, microphotographs of GFP epifluorescence were taken (left panel) and overlayed on microphotographs of the same microscope field taken with bright light (right panel). (C) Bright field photomicrographs of wildtype SKPs differentiated into Schwann cells (left panel). Immunofluorescence on SKP-derived Schwann cells for the Schwann cells markers p75NTR (red) and S100β (green) and counter stained for Hoechst 33258 to visualise the nuclei (blue). (D) SKP-derived Schwann cells were infected with 50 multiplicity of infection of GFP adenovirus for two (left panel) or five days (right panel). Microphotograph of the epifluorescence for GFP was taken (green) and overlay onto photomicrographs of the same microscope field taken with bright field. For A-D, scale bars = 50 μm.
Expression of the Cre recombinase in these mice resulted in the deletion of the floxed copy of the *NF1* gene, which generate cells heterozygous or homozygous for the *Nf1* deletion. The expression of Cre recombinase can be induced by infecting cells grown in culture with recombinant adenovirus carrying the Cre recombinase cDNA. The generation of this adenovirus has been described elsewhere (Wei et al., 2006). The advantage of this strategy is that SKPs can be generated from cells expressing normal levels of NF-1 and then be infected with the Cre adenovirus either prior to their differentiation into Schwann cells or once they are differentiated. To test the feasibility of this technique, SKPs were first generated from *NF1*<sup>+/+</sup>, *NF1*<sup>flox/+</sup> and *NF1*<sup>flox/flox</sup> mice (Figure A.2A). These SKPs could be passaged at least five times. To determine whether SKPs can be infected with adenovirus, skin cells were infected with 50 multiplicity of infection of adenovirus expressing the reporter gene GFP in very low volume of media overnight and then cultured as standard procedure for SKP cultures. At seven days post-infection, most cells forming spheres were GFP positive (Figure A.2B). This experiment shows that primary SKPs can be infected with adenoviruses and suggest that using similar protocol, passaged SKPs could also be infected with these adenoviruses.

To determine whether SKP-derived Schwann cells can also be infected with adenoviruses, wildtype mouse SKPs were differentiated into Schwann cells. Schwann cell differentiation was determined by the morphology of the cells and the expression of two Schwann cell markers, p75NTR and S100β (Figure A.2C). These Schwann cells were then passaged and infected with 50 multiplicity of infection of GFP adenovirus. Two days post-infection the GFP signal was visible in many cells and was further increased at five days post-infection. Taken together these results show that SKP-derived Schwann cells as well as primary SKPs can be infected with adenoviruses. Since the project was stopped at this stage, the next steps is to determine whether the loss of NF-1 in SKPs would alter their, growth factor dependency, growth curve, and Schwann cell differentiation potential; and whether the loss of NF-1 in SKP-derived Schwann cells would alter their growth *in vitro* and *in vivo*, following their injection in a nerve or subcutaneously.
7.4 Conclusion

Taken together, these results support four conclusions. First, SKP cells can be generated from human patients affected by the NF-1 genetic condition. Second, SKPs derived from these patients may have differentiation defects toward the neuronal lineage similar to those previously characterised in other systems. Third, mouse SKPs isolated from mice expressing floxed NF-1 gene can be generated and infected with adenoviruses. Finally, SKP-derived Schwann cells can be infected with adenovirus. These results also support the idea that SKPs can be used to understand the biology of human diseases caused by genetic alterations.