Summary
Chapter 6

Melanoma is an aggressive type of skin cancer resulting from the uncontrolled proliferation of transformed melanocytes. Its etiology is not completely understood. Ultraviolet (UV) light plays an important role in the development of melanoma. The incidence of cutaneous melanoma is rising in fair-complexed populations. Apart from UV radiation other factors contribute to the development of this malignancy, such as cellular growth factors. In this respect, basic Fibroblast Growth Factor (bFGF) might complement the effects of UV radiation.

Treatment of cutaneous melanoma consists of surgery and sentinel lymph node dissection, if required. In case the sentinel node contains malignant cells, regional lymph node dissection will be performed. Once the disease has metastasized to distant areas, prognosis is poor. Chemotherapy and immunotherapy will hardly or not contribute to improvement of survival in advanced disease. The most well-known drug known to induce a low response rate in melanoma is dacarbazine (DTIC). The imidazotetrazine derivative related to DTIC, temozolomide, has shown as good activity in chemotherapy-naive advanced melanoma as the other most active agent currently in use.

Since metastatic melanoma is a tumor type with very low responsiveness to various treatment modalities, extensive research is carried out to change dismal prognosis. One possible treatment strategy that could be improved is melanoma immunotherapy. Another approach is to further enlighten our understanding of molecular pathways as crucial cellular survival targets and chemotherapy resistance mechanisms, which should lead the way to develop better treatment options in the near future.

It has been well documented that melanocytes progressively acquire growth factor independence during tumor development due to the production of autocrine-acting growth factors. Nowadays, bFGF is the best characterized and probably the most important growth factor in the development of a normal melanocyte to a highly metastatic melanoma cell. Transfection of normal melanocytes with a recombinant retrovirus containing a cDNA for bFGF has shown to induce autonomous growth in culture indicating autocrine stimulation of cell growth. These bFGF transformants, however, did not form tumors in nude mice. In contrast to melanocytes, almost all melanomas produce bFGF protein. Furthermore, bFGF can act as a growth stimulator in every sequential step of melanoma tumor progression. The important role of bFGF as an autocrine growth factor has clearly been established in different studies: (i) neutralizing antibodies against bFGF could inhibit the growth of bFGF-producing melanoma cells, (ii) melanoma cell growth was decreased by antisense oligodeoxynucleotides targeted to bFGF or to FGFR-1, (iii) tumor growth of melanoma xenografts could be completely arrested when tumors were injected with antisense bFGF or FGFR-1 cDNAs.

In this thesis, we further defined the role of bFGF in the aggressive behaviour and chemotherapy resistance of melanoma cells in vitro and in vivo. In CHAPTER 2 we characterized five unselected human melanoma cell lines for bFGF mRNA expression and found lowest transcripts in 1F6 and M14 cells and highest in BRO and BLM cells. Protein levels of all bFGF forms of 18, 22, 22.5 and 24 kD were easily detectable in cell lysates of BRO and BLM, while levels were low in 1F6 and M14. All cell lines contained mRNA transcripts of FGFR-1, FGFR-3 and FGFR-4, although at different expression levels. We then exposed 1F6 and M14 to exogenous recombinant human (rh)bFGF and
demonstrated increased proliferation at concentrations of, respectively, 5 and 20 ng/ml. The increased cellular growth rate coincided with increased phosphorylation of ERK1/2 in M14 cells, but high baseline phosphorylated ERK1/2 in 1F6 was not further stimulated by exogenous rhbFGF. In order to study the effect of endogenous bFGF overexpression, 1F6 and M14 were selected for stable transfection with vectors encoding the 18 kDa (18kD) and all (ALL) molecular weight forms of bFGF, while the control pcDNA3 vector was included in all experiments. In addition, BRO cells with high endogenous bFGF served as a control for detection of bFGF mRNA and protein expression. Overexpression was successful, since clones contained increased bFGF mRNA and expressed high levels of 18 kD or all forms of bFGF. bFGF-overexpressing clones also demonstrated increased storage of the protein on the cell membrane, where it is bound to heparin sulphate proteoglycans. Although bFGF lacks a signal peptide, we could detect little bFGF in conditioned medium most likely caused by saturation of binding sites on the membrane. Biological activity of bFGF overexpressed in 1F6 and M14 clones was confirmed, because cell lysates containing 1.25 ng/ml of bFGF led to an increase in proliferation of human umbilical vein endothelial cells to the same extent as that observed after addition of 1.25 ng/ml rhbFGF. Of interest, bFGF-overexpressing 1F6 cells showed significant acceleration of growth in vitro as well as grown as human tumor xenografts in nude mice, which was not the case for bFGF-overexpressing M14 cells. In vitro, 1F6 clonal cells overexpressing bFGF were smaller, more roundly shaped and did not form dendritic protrusions when compared to 1F6 parent cells. Morphology of bFGF-overexpressing M14 clones as well as tumors reflected that of M14 parent cells and tumors. Surprisingly, bFGF-overexpressing 1F6 tumors contained an abundant number of small blood vessels. Microvessel counts confirmed that the number had increased approximately 3-fold when compared to that in 1F6 parent tumors, whereas the mean surface area had reduced at least twice in size. Overall, these experiments demonstrated that bFGF can exert autocrine stimulation of human melanoma cell growth as well as paracrine stimulation of angiogenesis exemplified in 1F6 cells. Angiogenesis stimulation was, however, not a direct effect of bFGF overexpression, but rather the induction of Vascular Endothelial Growth Factor (VEGF) overexpression. Further, overexpression of 18 kD as well as all molecular weight forms of bFGF in 1F6 resulted in a similar degree of growth acceleration and increased angiogenesis. Although exogenous rhbFGF could stimulate M14 cell growth, bFGF overexpression hardly affected its proliferation rate and did not increase angiogenesis. Unlike 1F6 parent cells, however, M14 parent cells grew better in complete culture medium when compared to medium containing 0.5% serum suggesting M14 dependence on other growth factors.

Since 1F6 melanoma xenografts overexpressing the 18 kD or all molecular weight forms of bFGF demonstrated an abundant number of microvessels, we further examined the mechanism how bFGF can induce VEGF as is described in CHAPTER 3. VEGF mRNA expression was upregulated 4- and 5-fold in clones 1F6-18kD and 1F6-ALL, respectively, when compared to that in parent cells. High secretion of VEGF protein was confirmed in conditioned media. With the use of the transcription inhibitor actinomycin D it was shown that VEGF overexpression was the result of bFGF-mediated increased transcription, since mRNA stability was not changed. Indeed, transient transfection of 1F6 cells with a VEGF promoter-luciferase transcript showed increased promoter activity up to 13-fold in
1F6-18kD and up to 7-fold in 1F6-ALL clones. Although it was shown earlier that exogenous rhbFGF could stimulate 1F6 cell proliferation, increased VEGF mRNA or VEGF protein production was not observed suggesting that endogenous bFGF-stimulated VEGF overexpression was rather the result from an intracrine signaling route. In 1F6 cells transfected with bFGF it was then demonstrated that the phosphorylation status of PI-3K-activated Akt, MAPK p38 and MAPK ERK1/2 was increased when compared to basal p-Akt, p-p38 and p-ERK1/2 levels in 1F6 parent cells. Specific inhibitors of these three signaling routes were applied after which VEGF mRNA and VEGF protein secretion were measured. These experiments demonstrated that VEGF upregulation in bFGF-overexpressing clones was mediated mainly through induction of PI-3K and, to a lesser extent, the p38 signaling route. We also determined whether a relation would be present between bFGF and VEGF in the panel of five unselected human melanoma cell lines. Indeed, the correlation was significant on the mRNA level ($p < 0.05; r^2 = 0.974$). Cells with high VEGF mRNA secreted VEGF in high quantities. These cells, BRO and BLM, were previously shown to easily grow as human tumor xenografts and promptly formed lung metastases, which was in contrast with the other melanoma cell lines tested. Overall, it was demonstrated for the first time in a particular human melanoma cell line that overexpression of endogenous bFGF can result in increased VEGF secretion and angiogenesis. This process was independent of 18 kD or all bFGF forms. Unselected melanoma cell lines known for high bFGF expression also secreted high levels of VEGF, suggesting that this phenomenon may be encountered in the clinic. Moreover, rhbFGF did not stimulate VEGF secretion in 1F6 cells, indicating the presence of an intracrine signaling route as a mechanism to explain our findings.

Since bFGF-overexpressing 1F6 clones demonstrated increased proliferation in vitro as well as in vivo and induced new blood vessel formation when grown as xenografts, it was then examined in Chapter 4 if these cells also possessed higher invasive capacity. To that end, it was explored whether expression of CD13/aminopeptidase (CD13) was present on the cell membrane. CD13 expression has been detected on a number of tumor types, such as melanoma, and facilitates invasion by degradation of the extracellular matrix. Indeed, CD13 mRNA expression was increased up to 150-fold in 1F6-18kD as well as in 1F6-ALL clones. Fluorescence-activated cell sorting analysis confirmed high presence of CD13 on the membrane. CD13 had enzymatic activity as demonstrated from release of 7-AMC from alanine-AMC (L-alanine-4-methyl-7-coumarinylamide trifluoroacetate). Bestatin, a non-specific aminopeptidase inhibitor, abrogated the aminopeptidase activity up to 90%, while exposure to the specific CD13-neutralizing antibody WM15 decreased enzymatic activity 3-fold. Increased invasion of bFGF-overexpressing clones was observed through Matrigel, which was reduced by bestatin and WM15. The migratory capacity of bFGF-overexpressing clones as tested in a wound assay was not changed, indicating that CD13 was not involved in this process. Increased CD13 expression was not the result of increased mRNA stability as measured with the transcription inhibitor actinomycin D. Earlier studies have shown that CD13 transcriptional regulation can be mediated by the distal, myeloid promoter, or the proximal, epithelial promoter. 1F6 clones overexpressing 18 kD and ALL molecular weight forms of bFGF contained a high number of CD13 transcripts from the myeloid promoter. When transient transfections were performed, it was found that not only the myeloid promoter was activated, but also the epithelial promoter. Of interest, it
was not possible to increase CD13 levels on 1f6 cells upon exposure to exogenous rhbFGF, which pointed towards an intracrine mechanism accounting for CD13 upregulation by bFGF transfection. We then examined whether in the panel of five unselected melanoma cell lines a relation would be present between bFGF and CD13 mRNA expression, which was indeed the case ($p < 0.05; r^2 = 0.883$). BRO and BLM cell lines, known for aggressive behaviour in vivo, contained high levels of CD13 protein next to high bFGF expression. These data further pointed towards an important role of bFGF in the aggressiveness of melanoma.

There is increasing evidence in cell lines of malignancies other than melanoma that bFGF can modulate sensitivity to various anticancer agents. In CHAPTER 5 it was explored, whether increased endogenous bFGF levels could contribute to poor chemoresponsiveness in melanoma cells. To that end, experiments were carried out in M14 parent cells and bFGF-overexpressing M14 clones, since in this cell line bFGF had not affected cell growth which would possibly compromise treatment results. Four standard cytotoxic agents with a different mechanism of action were selected. While sensitivity to doxorubicin and docetaxel was not affected, resistance against temozolomide was observed in M14-18kD (2.9-fold; $p < 0.05$) and in M14-ALL (6.9-fold; $p < 0.05$) and against cisplatin in M14-ALL (2.7-fold; $p < 0.05$). It is known that temozolomide adducts are almost exclusively repaired by the enzyme $O^6$-alkylguanine-DNA-alkyltransferase (AGT) encoded by the gene $O^6$-methylguanine-DNA-methyltransferase (MGMT). Indeed, we found that bFGF-overexpressing clones contained increased AGT levels. Further, the AGT inhibitor $O^6$-benzylguanine (BG) resulted in the complete restoration of sensitivity to temozolomide in M14-18kD, but partially in M14-ALL, suggesting that additional mechanisms against temozolomide might play a role in the latter clone. It was then tested whether treatment of M14 parent cells with 5-azacytidine, a demethylating agent, would also induce high AGT protein levels. Indeed, levels of AGT increased gradually in time upon exposure of M14 to 5-azacytidine, which coincided with resistance against temozolomide. Temozolomide resistance was promptly abrogated by addition of BG. A methylation specific PCR showed both methylated and unmethylated CpG sites in M14 parent cells, while M14-18kD cells contained the unmethylated allele only. This indicated that bFGF overexpression had resulted in demethylation of the MGMT gene in this clone. In M14-ALL cells, however, despite increased AGT expression there were no differences in the methylation pattern when compared to the pattern in M14 parent cells. We then explored possible mechanisms for cisplatin resistance in M14-ALL cells. Reversal of resistance against cisplatin upon addition of BG was not observed. Since the mismatch repair (MMR) pathway can be critically involved in the development of temozolomide as well as in cisplatin resistance, we compared the protein levels of three important representatives, MLH1, MSH1 and MSH6, between M14 parent cells and bFGF-overexpressing clones, but did not find major differences in quantities. Overall, we concluded that bFGF can also account for resistance against cytotoxic agents affecting DNA integrity. Temozolomide resistance could partially be explained by demethylation of MGMT resulting in high AGT protein levels. Another, as yet unknown, mechanism should be present as an explanation for resistance against temozolomide and cisplatin in M14-ALL clones. Thus, in M14-ALL cells the high molecular weight forms of bFGF might be responsible for activation of an intracrine mechanism that inhibited the cytotoxic effects of these DNA-damaging agents.
The general conclusion of this thesis is, that bFGF can account for increased melanoma cell growth, invasion and induction of angiogenesis. Moreover, bFGF can play a role in resistance against particular cytotoxic agents. Experimental findings should be translated to the clinic of melanoma, since advanced melanoma is known for high metastatic capacity and poor chemoresponsiveness. bFGF should be further explored for its role in these processes as well as a possible target for new treatments to improve the outcome of this malignancy.