4. Discussion

4. 1. c-Jun function in bone development

In this work, I could document the expression of c-Jun was in primary cultures of wild type or in *fra-1* transgenic osteoblasts as well as in cells isolated from tumors developing in *c-fos* transgenic mice. I confirmed the presence of c-Jun *in vivo* in tumor tissue deriving from c-Fos induced osteosarcoma. These observations suggested that c-Jun could play a role in bone development as well as in contributing to the bone phenotypes induced by the over-expression of c-Fos and Fra-1. My attempt to delete c-Jun *in vivo* by crossing of Runx2-*cre* mice with *c-jun*$^{fl/fl}$ mice failed due to the inefficiency of deletion of *c-jun* in bone tissues. I only could observe a low level of deletion in the growth plate of Runx2-*cre*/*c-jun*$^{fl/fl}$ double mutants. These results were unexpected, as the same Runx2-*cre* line was successfully tested for conditional deletion of two other genes in bone tissues (Jan Tuckermann, personal communication). Since Runx2 is expressed in both hypertrophic chondrocytes and osteoblasts, the pattern of recombination that I observed to be restricted to the growth plate suggested that the deletion would only be efficient in the chondrocytes. No obvious phenotypes were observed in mice positive for the recombination suggesting that c-Jun would not play a major role in the growth plate. A more detail analysis will be needed to confirm this hypothesis. Another bone/cartilage specific Cre strain will be necessary to address the role of c-Jun in bone. Candidate cre lines are the DERMO-Cre mouse that was reported to be highly efficient for gene deletion in bone/cartilage tissues (Yu et al., 2003) or the mouse strain expressing Cre under the control of the Collagen I promoter that was reported to be only active in osteoblasts (Dacquin et al., 2002).

Another explanation would be that chromatin of the *c-jun* locus was not accessible in cells expressing the Cre under Runx2 control. Finally the expression level of Cre could be too low for efficient deletion of *c-jun* in the tissues investigated. This could likely be the case since the Runx2-*cre* line used in my experiments was shown to be expressed only in bone/cartilage tissue at lower level than another Runx2-*cre* line

that displayed stronger Cre expression at the expense of lower tissue specificity (Jan Tuckermann, personal communication). The evaluation of these lines for efficient *c-jun* deletion is in progress.

To further analyze c-Jun function in bone and osteosarcoma development I decided to delete *c-jun in vitro* by means of viral infection. c-Jun expression analysis of Cre infected primary *c-jun*$^{fl/fl}$ osteoblasts proved that high level of deletion can be achieved using this technique. However, since the infected cells needed to be passaged several times in order to select cells carrying the virus, I only obtained cells which were already undergoing crisis and therefore, could not be used to analyze the impact of c-Jun ablation on osteoblast differentiation. One possibility to circumvent this problem would be to first establish cell lines according the 3T3 protocol (Todaro and Green, 1963) and subsequently infecting the cells with Cre encoding virus. This approach would lead to immortalized cell lines that may be used to study their differentiation properties. Such approach was successfully performed to isolate the MC3T3 cells line widely used to study osteoblast differentiation *in vitro*.

4. 2. c-Jun function in c-Fos induced osteosarcoma formation

Retrovirus-mediated c-Jun deletion was also used to generate transformed *c-fos* transgenic cell lines lacking c-Jun *in vitro*. The efficiency of deletion was confirmed by quantitative PCR and western blot analysis. c-Jun was described in the literature to be required for proliferation and transition of G1 to S-phase during cell cycle progression by positively regulating cyclinD1 (Bakiri et al., 2000; Wisdom et al., 1999; Schreiber et al., 1999; Shaulian et al., 2000). I first analyzed the cell cycle profile of *c-fos* tg/*c-jun*$^{\Delta/\Delta}$ tumor cells compared to *c-fos* tg/*c-jun*$^{fl/fl}$ control tumor cells. Transition from G1 to S-phase was strongly impaired in c-Jun lacking tumor cells compared to control which is in agreement with previous studies (Bakiri et al., 2000; Wisdom et al., 1999). Moreover I could also observe an increased number of dead cells following *c-jun* deletion. The role of c-Jun in the control of cell survival is contradictory. Numerous studies have identified c-Jun as negative transcriptional regulator of pro- or anti-apoptotic factors like p53, FAS and p16 (Schreiber et al., 1999; Ivanov et al., 2001; Passegue and Wagner, 2000). However c-Jun exerts also pro-apoptotic

functions by positively regulating BIM and FASL (Whitfield et al., 2001; Kasibhatla et al., 1998). Hepatoblasts are dependent on c-Jun for survival while in neuronal cells c-Jun induces apoptosis (Eferl et al., 1999; Behrens et al., 1999; Raivich et al., 2004). Therefore the decision of c-Jun to protect cells against apoptosis or to induce cell death may be cell type and stimuli dependent. My data indicate that c-Jun is essential for cell survival in osteosarcoma cells. The target genes mediating the survival function of c-Jun in osteosarcoma formation have to be identified.

Resorting the infected cells to obtain pure GFP-Cre positive cells (hence c-Jun lacking cells) and GFP positive control cells revealed a strong growth advantage for c-Jun bearing cells (cells which escaped the first selection by sorting).

In summary, my data suggest that c-Jun is crucial for c-Fos induced osteosarcoma formation and I speculate that the absence of c-Jun *in vivo* would prevent osteosarcoma initiation by c-Fos. This hypothesis is supported by previous studies investigating c-Jun contribution to osteosarcoma development. Mice over-expressing c-Jun alone did not display any phenotype (Grigoriadis et al., 1993). However, c-Jun over-expression was shown to potentiate c-Fos-induced osteosarcomas. Earlier onset of the tumors as well as increased tumor size were reported in *c-fos;c-jun* double transgenic mice (Wang et al., 1995). Conversely, crossing of *c-fos* tg mice with mice harboring a mutant *c-jun* allele that cannot be phosphorylated on serine 63 and serine 73 by the Jun N-terminal kinases (JNKs) resulted in delay in osteosarcoma formation and reduction of tumor size (Behrens et al., 2000). These mutation impaired c-Jun transactivation properties and lead to a strong decreased tumor formation in these double mutants. Combined with my data, I hypothized that, while c-Jun expression would be necessary for c-Fos-induced tumor initiation, c-Jun phosphorylation by JNK would regulate tumor progression.

4. 3. JunD in bone development

Measurement of body weight demonstrated a slight decrease in the size of JunD knock out mice which is in agreement with previously published work (Thepot et al., 2000). This could be indicative of the presence of a bone phenotype. Determination of spleen-, heart- and fat pad to body weight ratios that are also often indicative of bone phenotypes did not reveal any significant change in JunD knock out animals.

However, potential changes in these ratios would only occur as consequence of strong bone development defects. In addition, these parameters are not altered in all bone phenotypes, for instance osteopenic animals would not be clearly diagnosed by these parameters.

I further analyzed JunD mice by measuring the bone mineral density at different ages which could be indicative of the role that JunD could play in developmental and post-developmental bone homeostasis. Bone mineral density in young male animals was not altered but a significant higher bone mineral density was observed in 20 weeks old JunD knock out animals compared to wild type and JunD heterozygote controls. These results demonstrated that JunD, while not essential for bone development, is involved in the induction of age induced bone loss. This effect was no dose dependant since heterozygous JunD mice did not show higher bone mineral density. After reaching peak bone mass at 12 weeks the wild type and heterozygote animals lost bone mass while bone mass in knock out animals was nearly unaltered. This means that the absence of JunD *in vivo* does not lead to a higher bone formation rate in young animals but is rather involved in the maintenance of the peak bone mass. Whether this effect is mediated by preventing osteoclast resorptive activity will have to be verified by further experiments. The ~5 % higher BMD in JunD knock out mice might not appear relevant, but one must consider that this value has dramatic consequences in terms of bone stability. According the World Health Organization, a BMD of 1-2.5 times of standard deviation below the mean BMD from young healthy adults is considered as low bone mass. Osteoposis is defined as BMD values less than 2.5 times of standard deviation below the mean from young healthy adults. Based on this classification, 20 weeks old *junD*$^{-/-}$ mice do not display low bone mass while wild type animals already suffer from low bone mass.

Age dependant loss of bone mass can be caused by decreasing estrogen levels. To check if *junD*$^{-/-}$ mice are protected against estrogen deficiency induced bone loss, mice were subjected to ovariectomy. I found ovariectomized *junD*$^{-/-}$ mice to be protected against the estrogen deficiency induced bone loss. The underlying mechanisms by which JunD influences bone mass remain to be determined. It is widely accepted that increased bone resorption induced by estrogen deficiency is mediated by interleukin-6 (Jilka et al., 1992), Therefore, one can envision that JunD activate Interleukin-6 expression and thereby absence of JunD will result in protection against bone resorption. This needs to be verified by measuring Interleukin-6 levels.

Another explanation for the higher BMD in *junD*$^{-/-}$ mice might be an enhanced bone formation rate counteracting the increased resorptive activity in aged animals. This would mean that JunD negatively regulates genes which foster bone formation. One candidate gene is *sfrp1.*

The *in vivo* observed bone phenotypes in JunD knock out mice raised the question if JunD directly affects osteoblasts or osteoclast. It was previously reported that JunD has no cell autonomous function in osteoclasts (David et al., 2002). Therefore I analysed primary osteoblasts from *junD*$^{+/+}$, *junD*$^{+/-}$ and *junD*$^{-/-}$ mice. No changes in proliferation and apoptosis were observed when comparing the different genotypes but differentiation capacity of *junD*$^{-/-}$ osteoblasts was increased identifying JunD as a negative regulator for osteoblast differentiation. This is in contrast with another Jun family member, namely JunB. JunB was found to be a positive regulator of bone formation (Kenner et al., 2004). Since *junB*- and *fra-1*- deficient mice display an osteopenic phenotype and in addition Fra-1 over-expressing mice are osteosclerotic, these AP-1 members might form heterodimers which regulate genes responsible for bone formation. Therefore, it is conceivable that the equilibrium between Fra-1/JunB and Fra-1/JunD dimmers would be controlling bone maintenance.

4. 4. JunD function in Fra-1 induced osteosclerosis

Since I did not obtain any *fra-1* tg/*junD*$^{-/-}$ double mutant progenies from the crossings, the function of JunD in Fra-1 induced osteosclerosis could not be addressed. This result indicated that the *fra-1* tg/*junD*$^{-/-}$ double mutant are not viable. It was already described that the absence of JunD in *fra-1* transgenic mice leads to dilative cardiomyopathy provoking death (Ricci et al., 2004). The disease was progressively developing after birth with approximately 40 % of mice surviving the first two weeks and still 20 % of animals viable at 4 weeks of age. Since I genotyped all animals (also the dead ones found in the breeding cages) and only a few dead pups were *fra-1* tg/*junD*$^{-/-}$ double mutant mice, the genotypic constellation was already embryonic lethal or very shortly after birth. Very young dead litters are usually eaten by the parent animals and could often not be collected by the animal caretaker for further genotype analysis. So I can not exclude that the *fra-1* tg/*junD*$^{-/-}$ double mutant might be viable for 3-5 days after birth but still, the absence of survivors was not reported previously (Ricci et al., 2004). The most likely explanation for this discrepancy is the

use of different genetic backgrounds of *fra-1* tg and junD$^{-/-}$ mice in the mentioned study compared to my mice. Indeed Ricci et al. were working with mice in a mixed C57/Bl6-129sv background. In contrast the mice I was using were in pure C57/Bl6 background. It was already reported that the genetic background in mice has a profound impact in modification as well as in penetrance of specific phenotypes. For instance bone formation capacity was demonstrated to be largely dependent on the background of the mice analyzed (Marusic et al., 1999). The susceptibility to tumor formation was also shown to be strongly influenced by the genetic background of the mouse model chosen (Backlund et al., 2001). The choice of C57/Bl6 background was driven by the goals of my studies that were to define the functions of JunD and c-Jun in bone development and their contribution to c-Fos induced osteosarcoma formation and Fra-1 induced osteosclerosis. It was necessary to bring the strains in the homogeneous genetic background. To this end I chose to backcross the mixed C57/Bl6-129sv *fra-1* tg and *junD*$^{-/-}$ animals with C57/Bl6 mice for at least 8 times to obtain animals in pure C/57/Bl6 background. A reason for the earlier lethality observed in C57/Bl6 background could be that the dilative cardiomyopathy in *fra-1* tg/*junD*$^{-/-}$ mice is developing earlier and more severely in the pure background. A backcross to another background will be necessary to analyze the role of JunD in Fra1-induced osteosclerosis.

4. 5. JunD function in c-Fos induced osteosarcoma formation

The lack of JunD in *c-fos* tg mice did not interfere with embryonic development since the observed transmission frequencies of mutant alleles were in line with expected mendelian frequencies. Analysis of spleen- and heart to body weight ratios did not indicate an overt bone phenotype. More interestingly, osteosarcoma formation was strongly impaired in *c-fos* tg mice lacking *junD.* This observation was not expected since in contrast to c-Jun, JunD was not reported to be required for tumorigenesis (Johnson et al., 1996). In addition previous studies demonstrated an anti-proliferative function of JunD by reducing CyclinD1 expression in immortalized fibroblasts (Weitzmann et al., 2000). In addition, primary *junD*$^{-/-}$ fibroblasts displayed reduced proliferation rates (Weitzmann et al., 2000). To check if the lack of JunD has an impact in growth properties of c-Fos induced osteosarcoma, I generated cell lines

from the tumors. JunD had no impact on proliferation but apoptosis rates were increased in JunD-lacking cell lines upon serum deprivation. Increased apoptosis rates in JunD-lacking cells were reported when cells were challenged by UV or TNFα treatment (Weitzmann et al., 2000). These results indicate that the lack of JunD sensitizes cells for apoptotic stimuli.

In addition to increased apoptosis rate by serum deprivation, *junD* deficient tumor cells might also die by other mechanisms. Elevated reactive oxygen species (ROS) are frequently observed in transformed cells due accelerated overall metabolism (Trachootham et al., 2006). It was shown that transformed cells are sensitive to increased ROS production due to impaired detoxifying capacity, thereby undergoing apoptosis (Trachootham et al., 2006). Because JunD limit the cellular levels of reactive oxygen species (ROS), lack of JunD leads to increased ROS concentrations (Gerald et al., 2004) and this might result in apoptosis in c-Fos transformed cells.

Tumorigenic *in vivo* properties of the generated cell lines were checked by injecting them into NUDE-mice. The smaller size of the tumors arising from *junD* deficient cell lines suggests that invasion and progression capacities impaired were cell autonomous. This is supported by the expression data of tumorigenic markers in osteosarcoma samples. Expression of p27 was described to be down-regulated in many osteosarcoma cell lines and loss of expression of p27 correlated with dedifferentiation in human osteosarcomas (Thomas et al., 2004). Expression of p27 was increased in *c-fos* tg/*junD*$^{-/-}$ tumors suggesting that c-Fos cooperate with JunD to inhibit p27 expression. Reintroduction of p27 in osteosarcomas cell lines was recently shown to revert the transformed phenotype by resetting the cell differentiation program (Thomas et al., 2004)

The stress responsive gene p8 was reported to be required by transformed fibroblasts for anchorage independent growth and for tumor formation in NUDE-mice (Vasseur et al., 2001). I found this gene up-regulated in a genomic screen comparing *c-fos* tg osteosarcoma samples with wild type bone samples. Therefore I measured p8 expression in *c-fos* tg/*junD*$^{+/+}$ and *c-fos* tg/*junD*$^{-/-}$ osteosarcoma samples. The level of p8 transcripts was reduced in *c-fos* tg/*junD*$^{-/-}$ sample*s* which could also account for the reduction in tumor size.

JunD was implicated in two cellular processes which are known to play a role in tumorigenesis, namely senescence and vascularization. *junD* deficient fibroblasts undergo premature senescence by up-regulating the master regulator of tumor

suppression p53 (Weitzman et al., 2000). Quantification of transcriptional levels of p53 and the senescence marker p15 and Dec1 shown that they were not altered in *junD* deficient tumor samples, indicating that senescence would not contribute to the reduced tumor size in *c-fos* tg/*junD*$^{-/-}$ mice. An inhibiting role for JunD in regulating vascularization was reported. JunD indirectly reduced ROS production which in turn leads to destabilization of Hif1α and to subsequent down-regulation of master regulator for vessel formation Vegfα and Vegfδ (Gerald et al., 2004). This would suggest that lack of JunD might enhance vessel formation in tumors and in consequence should lead to tumor promotion. Expression of Vegfα and Vegfδ was not affected, so altered vascularization could be excluded as an explanation for tumor size reduction in *c-fos* tg/*junD*$^{-/-}$ mice.

Despite the strong impairment in tumor progression, c-*fos* tg/*junD*$^{-/-}$ mice still developed tumors with 100 % penetrance and the sites of tumor formation were identical to *c-fos* tg/*junD*$^{+/+}$ mice. This indicates that tumor initiation is not affected in *c-fos* tg/*junD*$^{-/-}$ mice. This phenotype was very similar to the *c-fos* tg mice lacking the c-Fos kinase RSK2. It was recently shown that this mice display defects in osteosarcoma progression without affecting tumor initiation (David et al., 2005). Therefore I also checked the expression level of Rsk2 in the established *c-fos* tg/*junD*$^{-/-}$ and control cell lines. Since there was no change in Rsk2 expression, the lack of JunD must affect other pathways and its effect on c-Fos induced tumor formation is independent of Rsk2.

Osteosarcoma formation could also be altered as a result of perturbed bone remodeling. Expression analysis of chondrocyte, osteoblast and osteocyte markers did not reveal significant changes. However Sfrp1, a gene described to negatively regulate bone mass (Bodine et al., 2004) by inhibiting the Wnt pathway and in addition shown to inhibit osteoclastogenesis *in vitro* (Häusler et al., 2004) was found to be down-regulated in tumor samples lacking JunD. Thus, Sfrp1 seems to inhibit both, bone formation and resorption. This dual function of Sfrp1 appears paradoxical but might be dependant on the cellular context. In case of osteosarcoma formation with high sustained expression of c-Fos, basal osteoclastogenic differentiation is already favoured (David, unpublished observation). The down-regulation of Sfrp1 which was shown to inhibit osteoclastogenic by direct binding to RANKL (Häusler et al., 2004) results in an increased osteoclastogenesis. Thus the increased bone formation resulting from down-regulation of Sfrp1 may not compensate for the

increased bone loss, and as a consequence the bone mineral content in tumors lacking JunD decreased. This is illustrated by further analysis of osteoclastogenesis in osteosarcoma samples. Expression of several osteoclast markers were increased in *c-fos* tg/*junD*$^{-/-}$ tumors indicating that osteoclast differentiation or recruitment is enhanced in the tumor. The c-Fos target gene NFATc1 essential for osteoclastogenesis was significantly up-regulated in *junD*$^{-/-}$ tumors. Recent work demonstrated that transgenic dominant-negative Jun mice exhibit osteopetrosis due to impaired osteoclastogenesis by titering out c-Jun which is essential for c-Fos and NFATc1 induced osteoclastogenesis (Ikeda et al., 2004). Since JunD is considered to counteract c-Jun by binding to Fos and thereby limiting c-Jun availability to form transcriptionally more active heterodimer, the lack of JunD may be promoting osteoclastogenesis by increasing the availability of c-Jun to bind c-Fos. Indeed increased osteoclastogenesis was also demonstrated by higher numbers of Trap-positive cells in JunD lacking tumor sections. These results suggest that increased bone resorption takes place in the *junD* lacking osteosarcoma which could finally contribute to the decreased tumor size.

How does JunD influence bone mass in a setting with “normal” c-Fos expression? Expression analysis of genes which were shown to be differentially expressed in the tumor samples were not significantly altered in *junD*$^{-/-}$ calvaria samples compared to controls with the exception of Sfrp1. This identifies Sfrp1 as potential JunD target gene independent on c-Fos. Moreover down-regulation of Sfrp1 is most likely explaining the protection of *junD*$^{-/-}$ mice against age induced bone loss. In agreement, *junD* deficient mice are phenocopying mice lacking Sfrp1. Both animals show no changes in bone mineral density at young age but display a progressive increase in BMD with age (Bodine et al., 2004). *Sfrp1*$^{-/-}$ osteoblasts also showed increased differentiation in agreement with my results but additionally displayed reduced apoptosis rates, a feature not found in *junD*$^{-/-}$ osteoblasts.

In summary, this work revealed an unexpected role for JunD as a factor essential for c-Fos induced osteosarcoma progression. This tumor promoting function seemed to be achieved by regulating two distinct processes. First transformation of osteoblasts is fostered by positively regulating p8 and by down-regulation of p27. Regulation of these two factors are dependent on c-Fos and JunD which argue that c-Fos and JunD probably form heterodimer to directly bind in the promoter regions of these

genes either block transcription of p27 or stimulating transcription of p8. Moreover c-Fos induced osteoclastogenesis mediated via NFATc1 transcription is enhanced in the absence of JunD thereby increasing bone resorption in osteosarcomas. This suggests that, under some condition, JunD is inhibiting osteoclastogenesis.

This work also identified JunD as a new player in bone development. JunD negatively regulates bone mass during aging process, a mechanism most likely to involve up-regulation of Sfrp1.