Modulating the behaviour of pancreatic tumour cells

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Chapter 2: The oncogenic potential of HOX genes in pancreatic cancer cell dissemination and tumour-induced angiogenesis

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Abstract

HOX genes belong to the conserved super family of homeodomain-containing transcription factors and play fundamental roles in morphogenesis, organogenesis and differentiation. In vertebrates, increasing evidence suggests that HOX genes play a role in cancer progression. The oncogenic potential of HOX genes in certain human cancers has been described, but their role in most cancer types is still elusive. In this study, the contribution of the human HOX genes to metastatic behaviour and tumour-cell induced angiogenesis of four human pancreatic cancer cell lines (PaTu 8988t, PaTu 8988s, AsPC-1 and Panc-1) was assessed.

Initially, expression of the 39 human HOX genes in the studied cell lines was confirmed. Subsequently, their roles in the four pancreatic cancer cell lines were compared using using siRNAs for targeted gene silencing. The gene-specific siRNAs were added to the cells for specific genetic silencing and subsequent knock down effects were screened for altered migration *in vitro* and metastatic behaviour and tumour cell-induced angiogenesis *in vivo*. We show here that HOX genes of the paralogue groups 9 and 10 have a significant effect on pancreatic cancer cell behaviour. Furthermore HOXA2, HOXA5, HOXA7 and HOXB8 are shown here to have profound effects on the malignant behaviour of the pancreatic cancer cell lines studied.

Introduction

Tumourigenesis and tumour-progression involve interactions of cancer cells with each other, neighbouring cells and their microenvironments (Wang W. et al, 2007; Hannahan D. and Weinberg RA., 2000; Murdoch C. et al., 2008). Cancer cells often induce surrounding endothelial cells to build new vessels as a response to VEGF signalling by the vasculature in order to balance the blood supply with the demands of a growing tumour (Goel HL. and Mercurio AM., 2013). The vascular network is further employed by metastasizing cancer cells for their long and short distance traveling (Cao Y., 2005). The complex mechanisms that regulate these "nomadic" cancer cells include intravasation and extravasation. To successfully form a micro-metastasis, cancer cells need to use the vasculature for their transport (Fiedler IJ., 2003). The importance to understand and prevent metastasis formation is underlined by the fact that over 90% of all cancer related deaths are due to secondary tumours arising from metastasizing cancer cells (Jemal A., 2007). This is especially true for pancreatic cancer types which have a poor prognosis in 85-90% of the patients due to metastatic complications (Ganeh P. et al, 2007). The 5-year survival is estimated at 5% (WHO, 2014) and pancreatic cancer is responsible for 6% of cancer deaths each year (Jemal A., 2007).

Links between HOX gene expression and malignant transformation have been investigated based on the rationale that Hox expression in cancer cells is considered to reflect re-emergence of embryonic cell behaviour (Shah N., and Sukumar S., 2010). It is widely accepted that the processes of normal embryogenesis and neoplasia share many of the same pathways, and that tumour development is an aberrant form of organogenesis (Htuchin ME. et al., 2005; Hendrix MJC. et al., 2007).

The oncogenic potential of HOX genes has clearly been implicated in several human cancer types (Shah N. and Sukumar S., 2010). For example, HOX genes were shown to play a role in regulating lineage determination and maturation in hematopoietic tissues (Mahdipour E. and Mace KA., 2011; Magli MC., 1997) and a linkage to increased HOX gene expression was shown in acute leukaemia's (Kongsuwan K. et al., 1988; Alharbi RA., 2013).

The zebrafish has become an *in vivo* cancer model that allows us to study both tumour-induced angiogenesis (Nicoli S. and Presta M., 2007; Nicoli S. and Ribatti D., 2007) and the formation of micro-metastases of xeno-transplanted cancer cells (Haldi M., 2006) and tumour fragments (Marques I, 2009). In the transparent zebrafish embryos, invasion and migration of tumour cells, their circulation in the vascular system, as well as the formation of micro-metastases and the tumour-induced angiogenesis can be followed with high resolution in real-time (Marques I, 2009). Importantly, these zebrafish models allow to quantitate both metastatic behaviour of transplanted tumour cells and tumour-cell induced angiogenesis (Nicoli S. and Presta M, protocols 2007; Marques I., 2009). In this

study, two *in vivo* zebrafish models were used to characterize and compare the involvement of the human HOX genes in tumourigenesis of human pancreatic cancer cells. HOX genes are members of the Homeobox gene family. These genes code for transcription factors controlling morphogenesis, organogenesis, differentiation and regulate specification of positional identity in vertebrates. HOX genes are homeobox-containing genes. They code for transcription factors that help regulate morphogenesis, organogenesis, differentiation and positional specification in metazoans (Krumlauf R. and McGinnis W., 1992; Pearson JC. et al., 2005).

In humans, HOX genes are located in four clusters (A-D) which consist of a total of 39 genes, assigned to 13 paralogue groups based on their sequence similarity. Clusters A-D are located on different chromosomes, 7p15, 17p21, 12q13, and 2q31, respectively (Bayley WJ., 1997). Temporal and spatial control of HOX gene expression is essential for correct developmental patterning in many animals (Durston AJ. et al., 2012; Duboule D. 1994). Regulation of embryonic segmentation and the formation of the antero-posterior axis involve cell motility, morphogenesis, division, differentiation and apoptosis. These processes have prominent roles in tumourigenesis and –progression (Hannahan D. and Weinberg R.A., 2000).

Because of the redundancy of genes that belong to the same paralogue groups, all 39 genes of the four clusters were screened for metastatic behaviour and tumour induced angiogenesis. Expression of HOX RNA was assessed using PCR in the four cell lines. Then, HOX genes were knocked down using siRNA and these cells were subsequently used in an *in vitro* migration assay, an *in vivo* metastasis assay and in an *in vivo* angiogenesis assay. Using the results obtained, an estimation can be made about the set of HOX genes that might be involved in pancreatic cancer.

Materials and Methods

Animal care and handling

Tg(fli1:eGFP) zebrafish were kept at 28° C in tanks with day/night light cycles of 10 hours dark alternated with 14 hours light. The zebrafish were handled in compliance with Dutch animal care regulations and standard operating procedures. Zebrafish eggs were harvested at two hours post fertilization (hpf), followed by incubation at 28° C in egg water (sea salt, 60µg/ml in tap water). The developing embryos were kept in incubators at constant temperatures dependent on experimental requirements.

Cell culture

All cell lines (PaTu 8988t, PaTu 8988s, PANC-1 and AsPC-1) were cultured at a temperature of 37^0 C and a CO_2 pressure of 5%.The PaTu 8988t cell line and the PaTu 8988s cell line were isolated from liver metastases over a decade ago (Elsässer HP., 1992). Their behaviour in an *in vitro* wound healing assay and in an *in vivo* zebrafish xenotransplantation assay were described in detail before (Marques I., 2009). PaTu 8988t and PaTu 8988s cells were cultured in DMEM (Gibco) high glucose supplemented with 10% FCS (Gibco). AsPC-1 cells (ATCC® (American Tissue and Culture Collection) CRL-1682™), first cultured 30 years ago, derived from the metastatic site (ascites) of a pancreatic adenocarcinoma. (Chen WH. et al., 1988) were cultured in RPMI (Gibco) with 10% FCS. PANC-1 (ATCC® CRL-1469™), first described in 1975, originated from an epithelioid carcinoma of the pancreatic duct. (Lieber MJ., 1975) were cultured in RPMI with 15% FCS.

PCR and gene silencing using siRNA

SiRNA polynucleotides for silencing the HOX genes and the corresponding primer pairs, were purchased from Santa Cruz Biotechnology (HOXA1 siRNA (h) sc-35583, HOXA2 siRNA (h) sc-38673, HOXA3 siRNA (h) sc-38675, HOXA4 siRNA (h) sc-75277, HOXA5 siRNA (h) sc-38678, HOXA6 siRNA (h) sc-89444, HOXA7 siRNA (h) sc-38680, HOXA9 siRNA (h) sc-38682, HOXA10 siRNA (h) sc-38684, HOXA11 siRNA (h) sc-60802, HOXA13 siRNA (h) sc-45666, HOXB1 siRNA (h) sc-38686, HOXB2 siRNA (h) sc-38688, HOXB3 siRNA (h) sc-38690, HOXB4 siRNA (h) sc-38692, HOXB5 siRNA (h) sc-75279, HOXB6 siRNA (h) sc-38694, HOXB7 siRNA (h) sc-45835, HOXB8 siRNA (h) sc-75281, HOXB9 siRNA (h) sc-45669, HOXB13 siRNA (h) sc-43851, HOXC4 siRNA (h) sc-60804, HOXC5 siRNA (h) sc-75287, HOXC6 siRNA (h) sc-45673, HOXC8 siRNA (h) sc-60806, HOXC9 siRNA (h) sc-75289, HOXC10 siRNA (h) sc-44810, HOXC11 siRNA (h) sc-75283, HOXC12 siRNA (h) sc-95877, HOXC12 siRNA (m) sc-146071, D1 siRNA (h) sc-38696, HOXD3 siRNA (h) sc-38698, HOXD4 siRNA (h) sc-75295 HOXD8 siRNA (h) sc-94725, HOXD9 siRNA (h) sc-35585, HOXD10 siRNA (h) sc-44814, HOXD11 siRNA (h) sc75291, HOXD12

siRNA (h) sc-75293, HOXD13 siRNA (h) sc-45656)). SiRNA and control siRNA were used in a final concentration of 50 nM and subsequently delivered into the pancreatic cancer cell lines using EndoPorter™ (Gene Tools, LLC). The control siRNA contains a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA. The efficiencies of the HOX siRNAs and HOX expression in the used cells were tested by RT-PCR, which was described previously including total RNA preparation (van der Meer DLM., 2006). The annealing temperature was set to 55° C and the extension temperature to 72° C.

In vitro *migration assay*

The *in vitro* migration assay was carried out as was previously described (Liang CC., 2007). Cells were grown to confluence in 6-well plates (Greiner). Then, the monolayer was scraped along a straight line with a 200 µl pipette tip. Pictures of the scratch were taken under a Leica stereomicroscope at 0h, 24h and 48h and the gap was measured and gap closure was calculated as a percentage of the initial gap (scratch).

Cell staining

Cells were plated in 24-well plates (Greiner) at a seeding density of 4,0 $*10^4$ cells and incubated for 48 hours at 37^0 C and 5% CO_2. SiRNA and EndoPorter® (Gene Tools, LLC) were added according to manufacturers' protocol, and the cells were incubated again at 37^0 C and 5% CO_2 for 24 hours. After detaching the cells with trypsin (Gibco), two washing steps were done with DPBS (Dulbecco's PBS, Gibco), after which the cells were transferred to 1.5 ml Eppendorf tubes and centrifuged 5 min, at 1500 rpm. Then, the cells were stained according to manufacturer's protocol after which the cells were suspended in 25 µl DPBS for injection into the embryos.

Cell transplantation into zebrafish embryos

After dechorionation, 2 dpf zebrafish embryos were anesthetised with Tricaine methanesulfonate (TMS, MS-222, Sigma). Using a manual injector (Eppendorf, Celltram), the cell suspension was loaded into an injection needle (15 µm internal- and 18 µm external-diameter (Oxford Apparatus) and cells were injected in 48 hpf Tg(fli1:eGFP) zebrafish embryos. After injection, the embryos were incubated at 35^o C and checked for cell presence at 2 hours post transplantation (hpt). Embryos with fluorescent cells outside the implantation area at 24 hpt were excluded from further analysis. All other embryos were incubated at 35° C for analysis during the following days. Two independent experiments are combined for each treatment and cell type (Table 2). Every experiment consisted of 50 injected and approved embryos. For statistical analysis, a student t-test was performed which showed that all changes observed were significant (with p-values <0.01).

Tumour cell-induced angiogenesis

The tumour-cell induced angiogenesis assay was in general performed as previously described (Nicoli S., Presta M., 2007; Nicoli S., Ribatti D., 2007).The 48 hpf zebrafish embryos were dechorionated and anesthetised using 0.042 mg/ mL Tricaine (Sigma). Then, the embryos were positioned on a 1. 8% agarose dish for injections. Cells were stained as described above, then suspended in 20 µl 12. 0 mg / ml Matrigel® solution (Cultrex R Basement Membrane Extract, R&D systems, Minneapolis, USA) and kept on ice for a short period until injection. The CellTram Oil injector (Eppendorf, Germany) was loaded with an injection capillary (Harvard apparatus) which was filled with 5 µl of the cell suspension in Matrigel®, and embryos were injected as described in more detail in previous studies (Nicoli S., Protocols 2007; Nicoli S., Cancer Res 2007; Vlecken DH. and Bagowski CP., 2009). Every experiment consisted of 50 injected and approved embryos. For statistical analysis, a student t-test was performed which showed that all changes observed were significant (with p-values <0.01).

Imaging

After injection, embryos were incubated in egg-water at 35°C for 24 hours before screening. Embryos were photographed using a Bio-rad confocal microscope equipped with a 1024ES lens (Zeiss) and a Krypton/Argon laser. For light-microscopy images of pancreatic cells, an MZ16FA stereomicroscope (Leica) and a DFC 420C camera (Leica) were used. The data obtained were processed using ImageJ software and Photoshop (Adobe).

Results

Human HOX genes are expressed in pancreatic cancer cells

The four pancreatic cancer cell lines were subjected to PCR to obtain information regarding HOX gene expression (Table 1 and Supplemental Table 1). From this data set it is clear that most HOX genes are expressed in the pancreatic cancer cell lines; HOXD4, HOXD8, HOXD9 and HOXD13 were not detected in any of the cell lines. As an internal assay control, GAPDH expression was analysed by PCR of the same RNA samples.

Table 1

Overview of HOX gene silencing scores in all experiments

Not expressed in any cell line	Expressed but no effects	Effect in PaTu 8988t: migration	Effect patut 8988t: dissemination	Effect patu 8988s: dissemination	Effect: AsPC-1: dissemination	Effect Panc-1: dissemination	Effect in PaTu 8988t: angiogenesis
	A6, A13	A1, A2,	A1, A2, A7, *A9* (induced)	[A4]	A2, A5, A7, A9	A2, A5, A7, A9	A1, *A2*, (induction), A3 and A5
	B6, B13	B2, B8, B9	B2, B8, B9	[B1, B3]	B9, B5 (induced)	B1, B3 (induced), B2, B8, B9	B1,B3, B7, B9, B8,
	C6, C8, C11, C12	C9, C10	C9, *C10* (induced)	[C5, C13]	*C10, C13* (both induction)		*C4, C10* (both induced)
D4,D8, D11, D13		D10	D10	[D9, D11]			D10

HOX genes shown in italic were observed to induce a malignant feature. The genes that reduced malignant processes are shown in normal text.

HOX genes affect pancreatic cancer cell migration in vitro

Despite their well-documented role in regulation of morphogenesis and development in vertebrate and non-vertebrate species limited information is available on the function of HOX genes in cell migration and invasion.

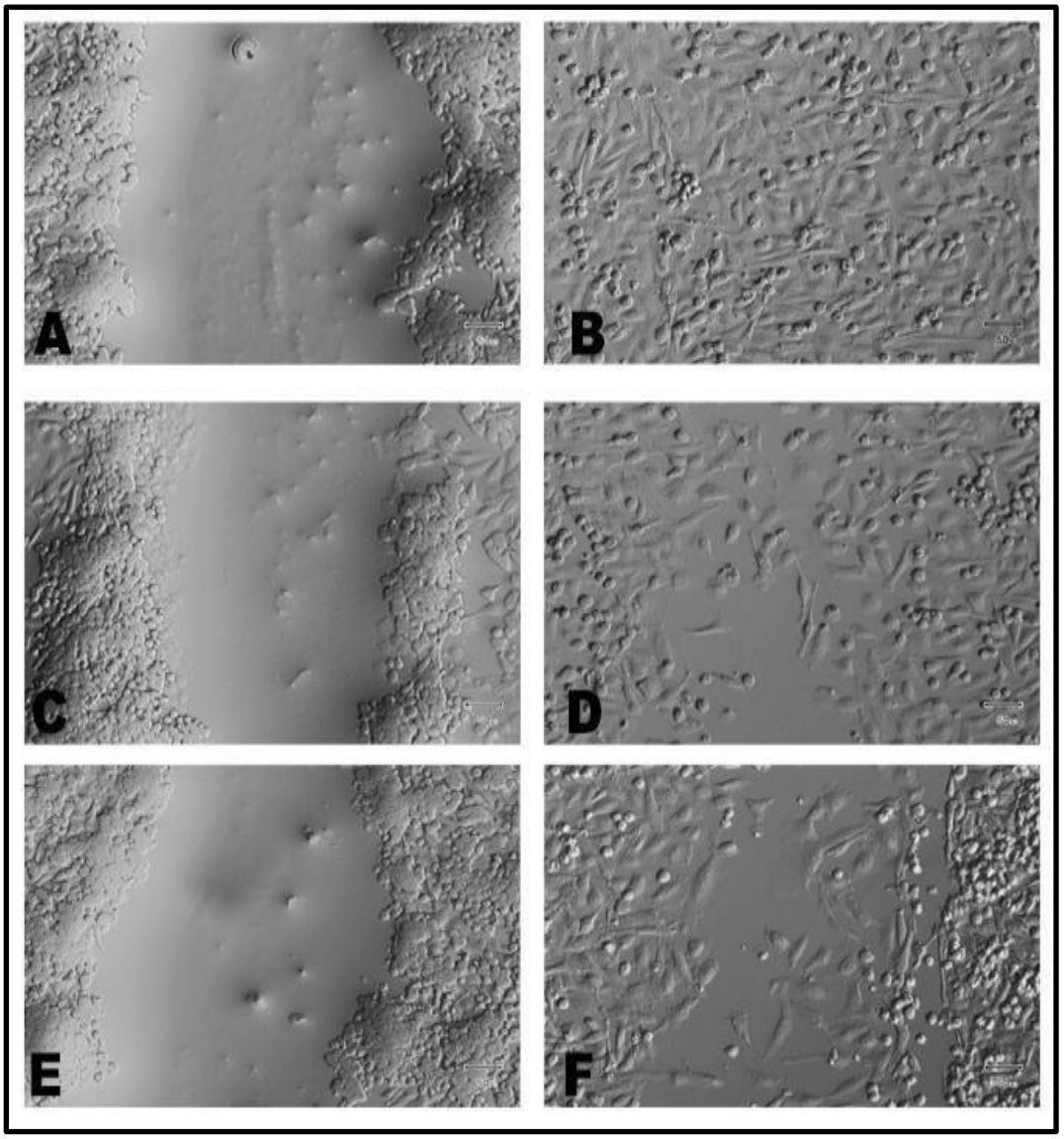

Figure 1a: An *in vitro* migration assay ('scratch assay') shows differences in migration of PaTu 8988t cells treated with control siRNA (A,B), HOXC9 siRNA (C,D), HOXB9 siRNA (E,F). The effects shown in these pictures

were representative for the other HOX genes that show an effect. In pictures A, C and E, the gap is photographed immediately after application of the scratch. In pictures B, D and F, the same area is photographed after 48 hours.

Gene specific siRNAs were used to individually silence all 39 human HOX genes in PaTu 8988t cell line. The results of the *in vitro* migration assay using PaTu 8988t cells showed that knock down of HOXA1, HOXA2, HOXA10, HOXB2, HOXB8, HOXB9, HOXC9, HOXC10, HOXD8, HOXD9 and HOXD10 had a negative effect on *in vitro* migration and led to a decrease in gap closure (Figure 1). These observations suggest a role for these HOX genes in invasion or migration of pancreatic cancer cells.

HOX genes are involved in metastatic behaviour of pancreatic cancer cells in vivo

To study the role of the human HOX genes in metastatic behaviour, four pancreatic tumour cell lines were labelled with a carbocyanine dye (CM-DiI) and ectopically transplanted in the yolk sac of 2 dpf zebrafish embryos of the transgenic zebrafish line, Tg(fli1:eGFP), which express GFP under the fli1 promotor (an early endothelial marker) and therefore exhibit a green fluorescent vasculature (Lawson N.D. and Weinstein B.M., 2002). Gene specific and control siRNAs were used to individually silence all 39 HOX genes and the effects in the zebrafish xenotransplantation model were observed.

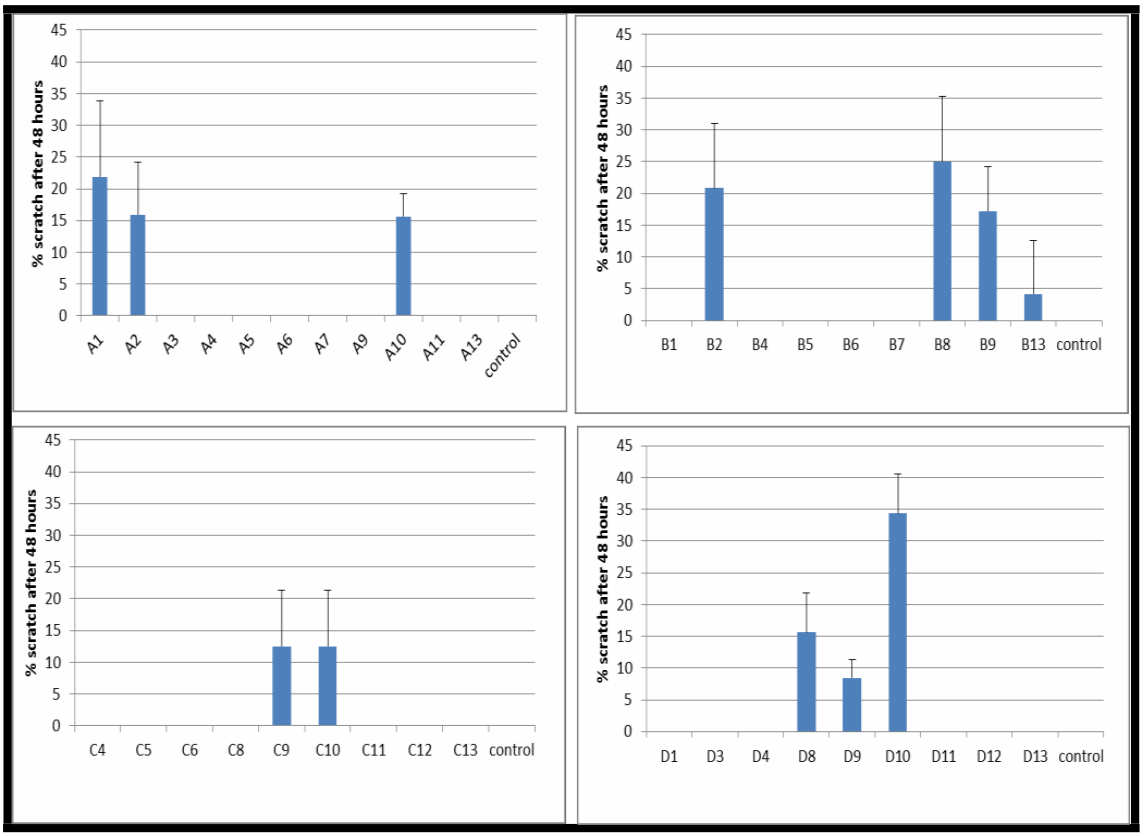

Figure 1b: Gap closure in the migration assay. Gap closure (gap width) over time is shown in percentages compared to the 0h time point (set to 100%). Error bars show standard deviations between three independent experiments.

The results (Table 1 and Figure 2a and b) showed that several HOX genes play important roles in metastatic behaviour either positive or negative. HOXA2, HOXA7, HOXA9, HOXB9, HOXC9 knock downs led to a decrease of metastatic behaviour in all three aggressive cell lines whereas gene silencing of HOXA4, HOXB1, HOXB3, HOXC5 and HOXC13 had a positive effect and led to an increase of metastatic behaviour in PaTu 8988s cells, AsPc-1 showed similar effects for HOXB1,HOXB3, HOXC10 and HOXC13.

The results differed to those obtained with the *in vitro* migration assay using PaTu 8990t cells (Figure 1). However, in contrast to the *in vitro* migration assay where many knock downs had no additional inhibitory effect, metastatic behaviour in the zebrafish *in vivo* model (Figure 2) was completely abolished by an additional set of siRNA knock downs. As observed in an earlier study, the findings indicated significant qualitative and quantitative differences between the two assays and they demonstrate an important role for several additional HOX genes in metastatic behaviour of pancreatic cancer cells.

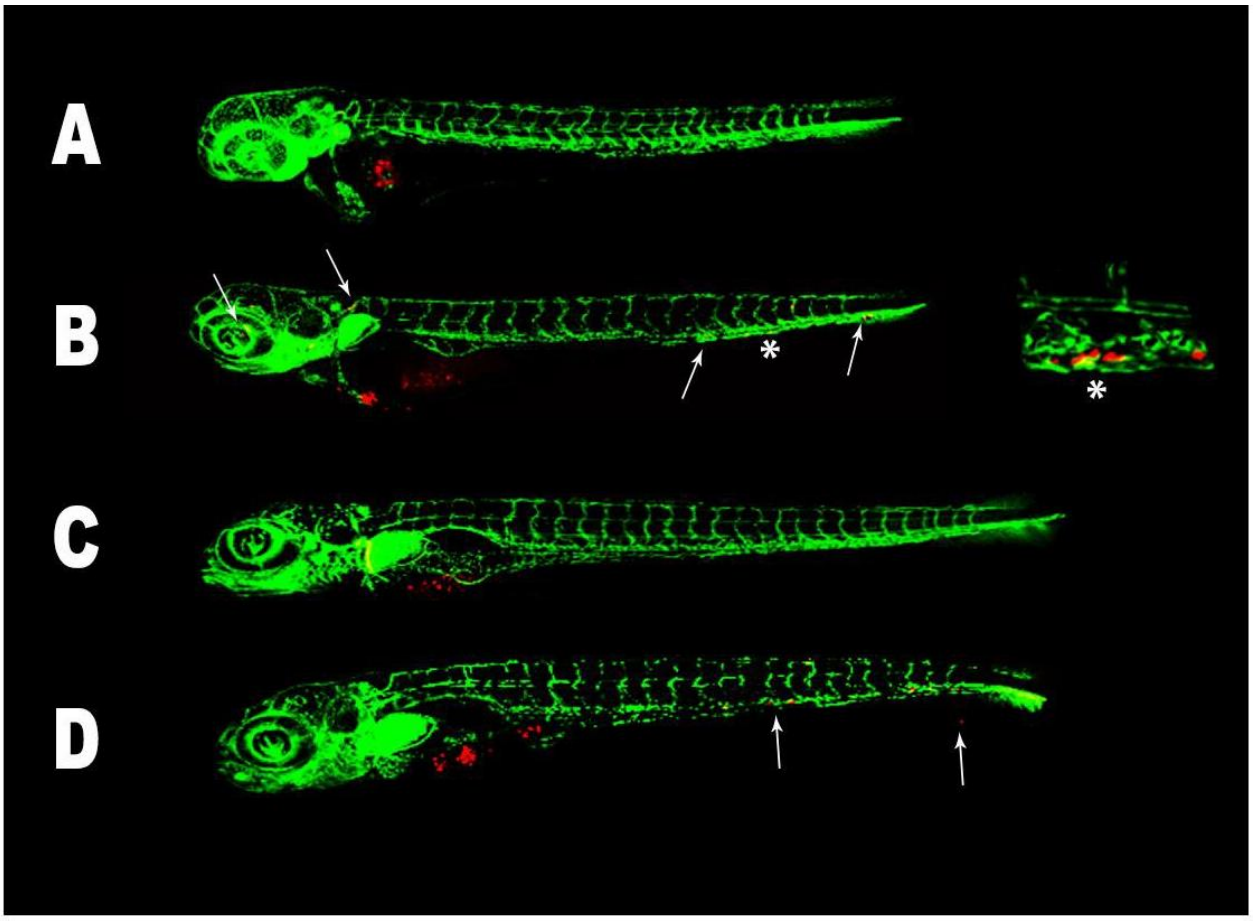

Figure 2: The zebrafish tumour xenograft metastasis assay. Shown are representative pictures taken by confocal microscopy of xeno transplanted. PaTu 8988t cells either treated with the control siRNA (A and C) or treated with the HOX siRNAs (A7 and B7). The pictures show zebrafish embryos 24 hours after transplantation (3 dpf, A, B) and 48 hours post transplantation (5dpf, C,D). In the controls, cancer cells have invaded the embryo and migrated to distant site. This behaviour is blocked by the HOX siRNAs. The area showing many cells with migration behaviour an enlargement is shown (fish B) and here, cancer cells are visible in the vasculature. The arrows show cancer cells which are located in the vasculature. (n=50 embryos per tested siRNA).

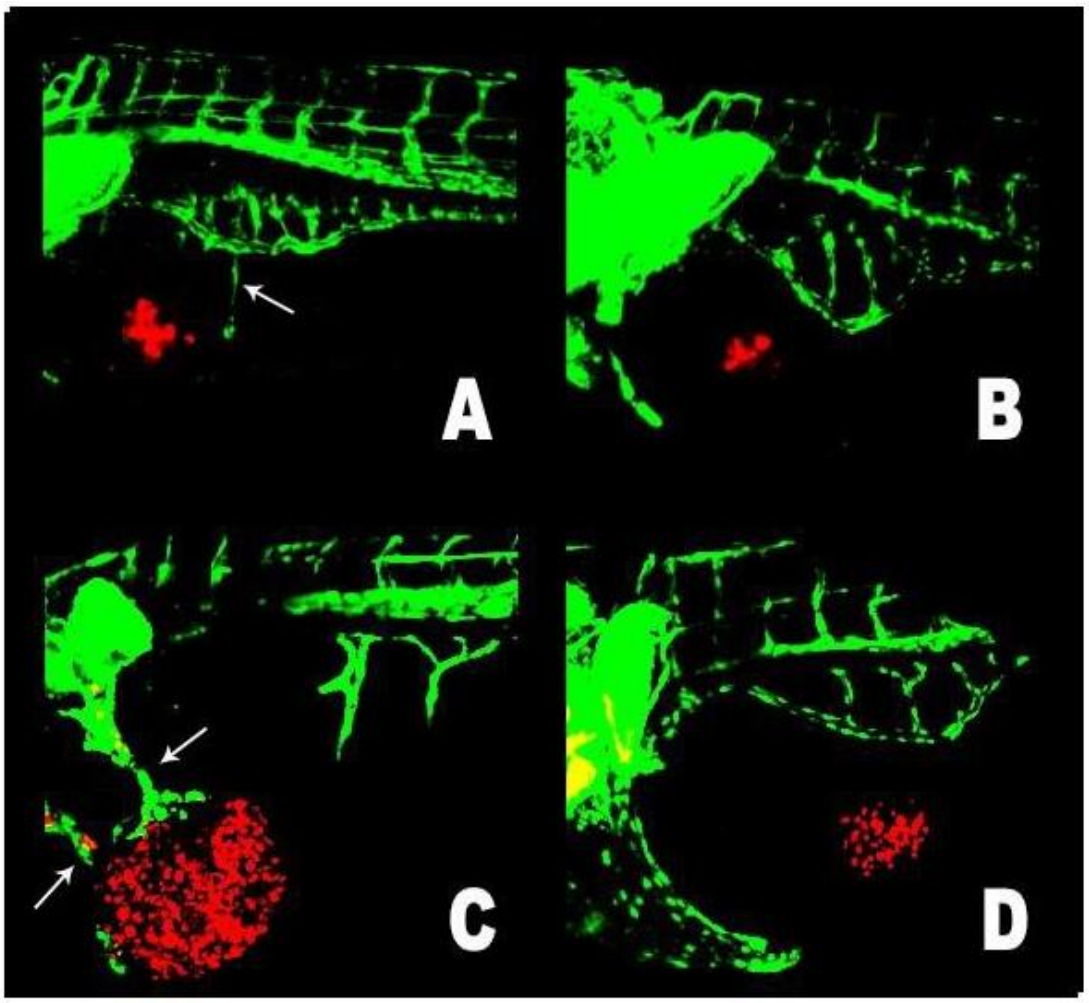

Figure 3: Effects of HOX A2 on angiogenesis in response to tumour cells.

All pictures show PaTu 8990t cells implanted in Tg(:Fli) *Danio Rerio* 36hpf embryos. In Figures A and B similar numbers of cells were injected. Untreated control cells (A and C) cause vessel formation towards the implant. Cells treated with HOXA2 siRNA (and cells treated with siRNAs against other HOX genes showing similar effects listed in Table 1) show inhibition of vessel attraction when implanted in zebrafish embryos. In photos C and D, the amount of cells injected is larger than the amount of cells injected in A and B; Pictures A and B were made at one day post injection. Pictures B and C were taken two days post injection. The images shown are examples taken by confocal microscopy of PaTu 8998t cells in which the cells were injected in the perivitelline space of zebrafish embryos. The induction of a new vessel induced by the tumour cells leads from the SIV to the PaTu 8998t cell cluster. Two independent experiments are combined for each treatment and cell type and the number of zebrafish used for each individual treatment was 50.

Knock down of HOX genes influences tumour cell-induced angiogenesis

To study tumour angiogenesis *in vivo*, a method is available that is based on the injection of pro-angiogenic mammalian tumour cells into the perivitelline space of zebrafish embryos (Nicoli, S., Presta M., 2007). PaTu 8988t and PaTu 8988s cells, AsPC-1 cells and Panc-1 cells were injected into the perivitelline space of zebrafish embryos at 48h post-fertilization. These pro-angiogenic tumour cell grafts induced an angiogenic response originating from the developing sub intestinal-vessels. In all four pancreatic cancer cell lines, the effects of control siRNA to gene specific siRNA for all 39 human HOX genes were compared.

The results indicate that HOXA1, HOXA2, HOXA5, HOXB1, HOXB3, HOXB7, HOXB9, HOXB8 and HOXD10 knock downs resulted in inhibition of angiogenesis. However, knock downs of HOXA3, HOXC4 and HOXC10 was observed to propagate angiogenesis (Table 2).

Discussion

HOX genes regulate cell movement during gastrulation in the developing embryo. Mesoderm cells undergo an EMT as ingression (or involution) takes place. These processes are highly regulated and reflect collinearity: the 3' to 5' sequence of the Hox genes in a Hox cluster matches the sequence in which they act along body axes (HOX1 genes allow involution/ingression earliest). Each Hox gene has a space-time identity on the main body axis of the early embryo. The spatial position of expression and action of these genes is also determined by the developmental age at which they are expressed. This means that HOX1 genes are the first to be expressed and act at the cranial side of the embryo, whereas HOX10 genes determine the difference between thorax and abdomen on the body axis (HOX9 genes work a bit more anteriorly)(Durston AJ. et al., 2011; Durston AJ. et al., 2012).

In the developing embryonic pancreas HOX1 genes, including HOXA1 and HOXD1 are expressed, together with genes from paralogue group 4 (HOXA4, HOXB4, HOXD4) (Slack JM., 1995). These genes seem not to be the most important genes whose signalling affects progression in pancreatic cancer. However, HOXA4 and HOXC4 induced metastasis and angiogenesis respectively (Miller GJ. et al., 2003).

HOX gene expression varies in different pancreatic cancer cell lines

Expression of the human HOX complement was examined using PCR on tumour tissue. The results show that not all HOX genes are expressed and from the ones that are expressed, only a part exerts an effect in the performed assays. The last event could be due to redundancy between HOX genes (Meyers PZ., 2007).

HOX genes of the paralogue groups 1 through 9 were previously shown to have the ability of blocking apoptosis (Morgan R. et al., 2014) and it was proposed that up-regulated expression of these genes resulted in a poor prognosis whereas gain of HOX genes of the paralogue groups 10-13 was suggested to result in a higher survival rate (Kachgal S et al., 2012). This is in accordance with our results regarding application of siRNAs against the paralogue group HOX10 and HOXC13 which induced malignant effects in the PaTu 8988s and AsPC-1 cell lines.

Silencing of HOX genes results in altered migration

Previously, the behaviour of PaTu 8988t cells in an *in vitro* migration assay was described (Marques I., 2009; Vlecken DH. and Bagowski CP., 2009). The effects of the knock down of HOX genes in PaTu 8988t cells was investigated in an *in vitro* migration assay (Liang CC., 2007).

HOXB2 was shown to be expressed in non-resectable tumours and is associated with a poor prognosis (Alharbi RA., et al., 2013; Gray S. et al., 2011) and HOXB2 was previously shown to be involved in metastasis in lung cancer (Boimel PJ., et al., 2007). The data presented here shows that involvement of HOXB2 was merely evident in migration assays performed on PaTu 8988t cells (*in vitro* and *in vivo*).

HOXB5 and HOXB6 were shown to be over expressed in pancreatic tumour tissue (Mizusawa N., et al., 2004) and HOXB6 was shown to be capable of induction of acute myeloid leukaemia as it is able to immortalizing cells (Magli MC., et al, 1997). Additionally, HOXB7 was previously shown to mediate EMT by inducing FGF (fibroblast growth factor) (Wu X., et al., 2006). Also, HOXB7 was shown to be over expressed in pancreatic cancer (AsPC-1 and Panc-1) (Gray S. et al., 2011) and was implicated in metastasis of lung cancer (Chen H., et al., 2008). Our results indicate that HOXB7 is only involved in angiogenesis in PaTu 8988t cells whereas none of the genes of the paralogue group 6 show any reactions to the siRNAs. However, another group of HOX genes was shown to be down-regulated in previous studies of which one is HOXD13, generally involved in development of the digestive and urogenital tract in embryos. HOXD13 (and also HOXB1 and HOXB3) were implied in suppression of metastasis (Gray S., et al., 2011) and therefore loss of HOXD13 is associated with a lower survival rate. Furthermore, it was shown that HOXB1 and HOXB3 are suppressed by miR-10a, which is present in significantly higher concentrations in metastatic pancreatic cancer (Marques I., et al., 2011). This finding confirms our data regarding these two genes on the non-metastable PaTu 8988s cells which disseminate in the absence of RNA of both genes.

The results obtained with the *in vivo* assays differed to those obtained with the *in vitro* migration assay using PaTu 8990t cells (Figure 1). Metastatic behaviour in the zebrafish *in vivo* model (Figure 2) was abolished by an additional set of siRNA knock downs. As observed in an earlier study (Vlecken and Bagowski, 2009), the findings indicated significant qualitative and quantitative differences between the two assays and they demonstrate an important role for several additional HOX genes in metastatic behaviour of pancreatic cancer cells. The differences observed could be a result of embryonic factors that are not present in the media of cultured cells.

HOX genes play a role in tumour induced angiogenesis

Previous research showed that HOXA3 increases endothelial cell migration and induces angiogenesis *in vivo* (Mahdipour E., et al., 2011) instead of inhibiting the vessel formation. This is consistent with our results, because the effect that we observed by knocking HOXA3 out was inhibited vessel formation in PaTu 8988t cells. HOXB7 and HOXB9 were also indicated as involved in tumour angiogenesis caused by malignant mammary cells (Hayashida T., et al.,2010; Caré A., et al., 1996),

similar to our results in PaTu 8988t cells where silencing of these genes inhibited vessel formation in the zebrafish embryos.

The pancreas is located in the upper abdomen, near the junction with the thorax. HOX9 and HOX10 are involved in defining this junction during embryonic development. Therefore it can be hypothesized that influence of these genes during carcinogenic processes in pancreatic cancer are location related rather than tumour type specific. Additionally, HOX9 and HOX10 genes are expressed at one of the later stages during embryonic development, due to the space-time identity of HOX genes on the body axes (Durston AJ. et al., 2011 and 2012) and this is in concordance with the age of the pancreatic cancer cells that were used for the experiments.

The pancreatic cancer cells that were used form a representative selection; The PaTu 8988t and PaTu 8988s cell lines used in this study originated from the same primary tumour (Elsässer HP., 1992). PaTu 8988t was observed to disseminate when transplanted into zebrafish embryos while PaTu 8988s cells did not. Using these cell lines, both induction and reduction of malignant processes can be studied and compared. To expand the dataset, the AsPC-1 cell line and the Panc-1 cell line were added because they originate from different types of tumours of the human pancreas. Panc-1 originates from an epithelioid tumour whereas AsPC-1 cells were derived from an adenocarcinoma (Chen WH., et al., 1982).

Focussing on the effects that were similar for all cell lines, some pronounced features emerge. A number of HOX genes were not expressed according to PCR (Tables 1 and 2), while several other HOX genes are expressed but do not seem to have an effect in any of the assays described (Table 2). These were HOXA13, HOXB13, HOXC8, HOXC11, HOXC12, HOXD4 and HOXD13. Also, HOX genes from the paralogue group 6 (HOXA6, HOXB6, HOXC6) do not exert any effects in the assays performed in this study. Unfortunately, no consensus about oncogenecity or tumour suppressive properties was established in the literature.

Whereas the oncogenic potential of certain HOX genes in human cancers has already been defined (Alharbi RA. et al., 2013; Magli MC. et al., 1997; Gray S et al., 2011; Shah N. and Sukumar S., 2010) , their role in neoplasms is still being evaluated. Progress has been hampered by the experimental approach used in many studies in which the expression of small subsets of HOX genes was analysed, and complicated by the functional redundancy implicit in the HOX gene system. Attempts to elucidate the function of HOX genes in malignant transformation will be enhanced by a better understanding of their upstream regulators and downstream target genes. Secondary tumours arising from cancer cell metastasis are the major cause of death in cancer patients, and currently no anti-metastatic drugs are available for therapy. Moreover, the mechanisms that regulate metastatic

behaviour remain so far only partially understood and current models envision metastasis as a highly dynamic multi-step process.

The zebrafish xenograft metastasis model used in this study provides the means to quantitatively study these dynamic processes in high-resolution in real time. Moreover, the zebrafish xenograft angiogenesis model allows further the investigation of tumour cell-induced angiogenesis in living embryos (Marques I., et al., 2009, Nicoli S., 2007 and 2008). It is important to note, that substantial evidence is accumulating that demonstrates functional conservation of human and zebrafish vascular biology and many pro-angiogenic genes have been described in both fish and men (Bussmann J., 2008; Isogai S., 2003). Two zebrafish xenograft assays were used as powerful tools to address and compare the role of the 39 human HOX genes in metastatic behaviour and tumour cell-induced angiogenesis of human pancreatic cancer cells. The zebrafish xenograft metastasis and angiogenesis assays are well suited to help in delineating (e.g. by reverse genetics using siRNAs) the responsible mechanisms and will assist in discovering novel anti-metastatic drugs in the future.

Conclusion

Whereas the oncogenic potential of certain HOX genes in human cancers has already been reported, the role of these genes in neoplasms is still being evaluated. Progress has been hampered by the experimental approach used in many studies in which the expression of small subsets of HOX genes was analysed, and complicated by the functional redundancy in the HOX gene system. Attempts to elucidate the function of HOX genes in malignant transformation will be enhanced by a better understanding of their upstream regulators and downstream target genes. Secondary tumours arising from cancer cell metastasis are the major cause of death in cancer patients, and currently no anti-metastatic drugs are available for therapy. Moreover, the mechanisms that regulate metastatic behaviour remain so far only partially understood and current models envision metastasis as a highly dynamic multi-step process. We have shown that several HOX genes are implicated in the process of tumourigenesis in pancreatic cancer cells. Future work will be needed to elucidate the molecular and cellular mechanisms involved.

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Supplemental Tables

Supplemental Table 1

RT-PCR of pancreatic cancer cell lines

HOX gene	PaTu 8988t	PaTu 8988s	AsPC-1	Panc-1
A1	Expressed	Expressed	Expressed	Expressed
A2	Expressed	Expressed	Expressed	Expressed
A3	Expressed	Expressed	Expressed	Expressed
A4	Expressed	Expressed	Expressed	No expression
A5	Expressed	Expressed	Expressed	Expressed
A6	Expressed	Expressed	Expressed	Expressed
A7	Expressed	Expressed	Expressed	Expressed
A9	Expressed	Expressed	Expressed	Expressed
A10	No expression	No expression	Expressed	No expression
A11	Expressed	Expressed	Expressed	Expressed
A13	Expressed	Expressed	Expressed	Expressed
B1	Expressed	Expressed	Expressed	Expressed
B2	Expressed	Expressed	Expressed	Expressed
B3	Expressed	Expressed	Expressed	Expressed
B4	Expressed	Expressed	Expressed	Expressed
B5	Expressed	Expressed	Expressed	Expressed
B6	Expressed	Expressed	Expressed	Expressed
B7	Expressed	Expressed	Expressed	Expressed
B8	Expressed	Expressed	Expressed	Expressed
B9	Expressed	Expressed	Expressed	Expressed
B13	No expression	No expression	Expressed	Expressed
C4	Expressed	Expressed	Expressed	Expressed
C5	Expressed	Expressed	No expression	Expressed
C6	Expressed	Expressed	Expressed	Expressed
C8	Expressed	Expressed	Expressed	Expressed
C9	Expressed	Expressed	Expressed	Expressed
C10	Expressed	Expressed	Expressed	Expressed
C11	Expressed	Expressed	Expressed	Expressed
C12	Expressed	Expressed	Expressed	Expressed
C13	Expressed	Expressed	Expressed	Expressed
D1	Expressed	Expressed	Expressed	Expressed
D3	No expression	No expression	No expression	Expressed
D4	No expression	No expression	No expression	No expression
D8	No expression	No expression	No expression	No expression
D9	No expression	No expression	No expression	No expression
D10	Expressed	No expression	No expression	Expressed
D11	Expressed	No expression	Expressed	No expression
D12	Expressed	Expressed	Expressed	Expressed
D13	No expression	No expression	No expression	No expression

Supplemental Table 2

Overview of HOX gene silencing scores in all experiments

Not expressed in any cell line	Expressed but no effects	Effect in PaTu 8988t: migration	Effect patut 8988t: dissemination	Effect patu 8988s: dissemination	Effect: AsPC-1: dissemination	Effect Panc-1: dissemination	Effect in PaTu 8988t: angiogenesis
	A6, A13	A1, A2,	A1, A2, A7, *A9* (induced)	[A4]	A2, A5, A7, A9	A2, A5, A7, A9	A1, *A2*, (induction), A3 and A5
	B6, B13	B2, B8, B9	B2, B8, B9	[B1, B3]	B9, B5 (induced)	B1, B3 (induced), B2, B8, B9	B1,B3, B7, B9, B8,
	C6, C8, C11, C12	C9, C10	C9, *C10* (induced)	[C5, C13]	*C10, C13* (both induction)		*C4, C10* (both induced)
D4,D8, D11, D13		D10	D10	[D9, D11]			D10

HOX genes shown in italic were observed to cause an effect in the tested cells when knocked down. The genes that reduced malignant processes are shown in normal text.

Supplemental Table: Scratch assay results

	absolute distance gap							abs<Jute distance gap											
	day1							day2											
	m1	m2	m3	m4	avg	sd	**gap	ml	m2	m1	m4	avg	sd	**gap day 2				avg%	sd
A1	21,875	400	400	400	400	400	0	100	50	50	ISO	100	87,5	47,87136	12,5	12,5	37,5	25	21,875	11,96784
A.	15,9375	250	300	400	400	337,5	75	100	50	75	50	25	50	20,41241	20	25	12,5	6,25	15,9375	8,252209
A1	0	400	400	400	400	400	0	100	0	0	0	0	0	0	0	0	0	0	0	0
M	0	400	400	400	250	362,5	75	100	0	0	0	0	0	0	0	0	0	0	0	0
f.5	0	400	300	300	400	350	57,73503	100	0	0	0	0	0	0	0	0	0	0	0	0
f.6	0	250	400	400	300	337,5	75	100	0	0	0	0	0	0	0	0	0	0	0	0
A7	0	400	400	400	400	400	0	100	0	0	0	0	0	0	0	0	0	0	0	0
B9	0	400	275	350	400	356,25	59,0727	100	0	0	0	0	0	0	0	0	0	0	0	0
A10	15625	400	400	400	400	400	0	100	50	75	75	50	m1	1443376	125	1875	1875	125	15625	3608439
A11	0	400	400	300	400	375	50	100	0	0	0	0	0	0	0	0	0	0	0	0
All	0	400	400	400	400	400	0	100	0	0	0	0	0	0	0	0	0	0	0	0
81	0	ISO	350	175	400	268,75	124,7915	100	0	0	0	0	0	0	0	0	0	0	0	0
82	2083333	300	400	400	400	375	50	100	100	100	50	50	75	2886751	33,33333	25	12,5	12,5	20,83333	10,20621
B4	0	270	400	300	400	342,5	67,51543	100	0	0	0	0	0	0	0	0	0	0	0	0
B5	0	175	ISO	175	250	187,5	43,30127	100	0	0	0	0	0	0	0	0	0	0	0	0
86	0	375	400	400	400	393,75	12,5	100	0	0	0	0	0	0	0	0	0	0	0	0
B7	0	400	400	400	400	400	0	100	0	0	0	0	0	0	0	0	0	0	0	0
B8	25	400	400	400	400	400	0	100	50	ISO	100	100	100	40,82483	12,5	37,5	25	25	25	10,20621
B9	17,23485	400	400	275	300	343,75	65,74889	100	50	50	75	50	56,25	12,5	12,5	12,5	27,27273	16,66667	17,23485	6,974224
B11	4,166667	ISO	400	400	400	337,5	125	100	25	0	0	0	6,25	11,5	1666667	0	0	0	4166667	8,333333
C4	0	300	400	400	400	375	50	100	0	0	0	0	0	0	0	0	0	0	0	0
C5	0	300	350	300	300	312,5	25	100	0	0	0	0	0	0	0	0	0	0	0	0
C6	0	400	400	400	400	400	0	100	0	0	0	0	0	0	0	0	0	0	0	0
C8	0	175	ISO	100	100	111,25	37,5	100	0	0	0	0	0	0	0	0	0	0	0	0
C9	12,5	200	200	400	400	300	115,4701	100	50	25	25	25	31,25	12,5	25	12,5	6,25	6,25	12,5	8,838835
C10	12,5	400	400	400	400	400	0	100	100	50	25	25	50	35,3553	25	12,5	6,25	6,25	12,5	8,838835
C11	0	400	400	400	400	400	0	100	0	0	0	0	0	0	0	0	0	0	0	0
C12	0	ISO	200	350	400	275	119,0238	100	0	0	0	0	0	0	0	0	0	0	0	0
C11	0	300	400	400	400	375	50	100	0	0	0	0	0	0	0	0	0	0	0	0
01	0	400	400	400	400	400	0	100	0	0	0	0	0	0	0	0	0	0	0	0
03	0	300	400	200	ISO	262,5	110,8678	100	0	0	0	0	0	0	0	0	0	0	0	0
04	0	400	350	300	400	362,5	47,87136	100	0	0	0	0	0	0	0	0	0	0	0	0
08	15,625	400	400	400	400	400	0	100	50	50	100	50	62,5	25	12,5	12,5	25	12,5	15,625	6,25
09	8,4375	375	300	400	400	368,75	47,32424	100	25	25	50	25	31,25	12,5	6,666667	8,333333	12,5	6,25	8,4375	2,853989
010	34,375	400	400	400	400	400	0	100	ISO	100	ISO	ISO	137,5	25	37,5	25	37,5	37,5	34,375	6,25
011	0	ISO	275	250	400	268,75	102,8247	100	0	0	0	0	0	0	0	0	0	0	0	0