human reproduction

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Effects of embryo culture media do not persist after implantation: a histological study in mice

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STUDY QUESTION: Is post-implantation embryonic development after blastocyst transfer affected by exposure to different assisted reproduction technology (ART) culture media?

SUMMARY ANSWER: Fetal development and placental histology of ART embryos cultured *in vitro* in different ART media was not impaired compared with embryos grown *in vivo*.

WHAT IS KNOWN ALREADY: The application of different *in vitro* culture (IVC) media for human ART has an effect on birthweight of newborns. In the mouse model, differences in blastocyst formation were reported after culture in different ART media. Moreover, abnormalities in the liver and heart have been detected as a result of suboptimal IVC conditions.

STUDY DESIGN, SIZE, DURATION: Fertilized oocytes from inbred and outbred breeding schemes were retrieved and either immediately transferred to foster mothers or incubated in control or human ART culture media up to the blastocyst stage prior to transfer. Placental and fetal anatomy and particularly bone development were evaluated.

PARTICIPANTS/MATERIALS, SETTING, METHODS: B6C3F1 female mice were used as oocyte donors after ovulation induction. C57Bl/6 and CD1 males were used for mating and CD1 females as foster mothers for embryo transfer. Fertilized oocytes were recovered from mated females and incubated in sequential human ART media (ISM1/ISM2 and HTF/Multiblast), in control media [KSOM(aa) and Whitten's medium] or grown *in utero* without IVC (zygote control). As *in vivo*, control B6C3F1 females were superovulated and left untreated. Fetuses and placentae were isolated by Caesarean section and analysed at 18.5 days post-coitum (dpc) for placenta composition and at 15.5 dpc for body weight, crown–rump length (CRL), fetal organ development, morphological development, total bone length and extent of bone ossification.

MAIN RESULTS AND THE ROLE OF CHANCE: No major differences in the number of implantation sites or in histological appearance of the placentae were detected. CRL of KSOM(aa) fetuses was higher compared with zygote control and Whitten's medium. Histological analysis of tissue sections revealed no gross morphological differences compared with the *in vitro* groups or *in vivo* controls. Furthermore, no changes in skeletal development and degree of ossification were observed. However, fibula and tibia of ISM1/ISM2 fetuses were longer than the respective ones from *in vivo* fetuses.

LIMITATIONS, REASONS FOR CAUTION: Findings in the mouse embryo and fetus may not be fully transferable to humans. In addition to skeletal development and placentation, there may be other parameters, e.g. on the molecular level which respond to IVC in ART media. Some comparisons have limited statistical power.

WIDER IMPLICATIONS OF THE FINDINGS: Our data suggest that once implantation is achieved, subsequent post-implantation development unfolds normally, resulting in healthy fetuses. With mouse models, we gather information for the safety of human ART culture media. Our mouse study is reassuring for the safety of ART conditions on human embryonic development, given the lack of bold detrimental effects observed in the mouse model.

Introduction

Assisted reproductive technologies (ART) such as IVF and ICSI have been developed to treat infertile couples. Treatments such as hormone administration, gamete retrieval and *in vitro* culture (IVC) have been developed and introduced to facilitate ART. IVF and ICSI are widely applied nowadays and no severe risks for the couples or the offspring have been noted. In consequence, a continuous increase in the number of ART cycles has been observed globally (Ferraretti *et al.*, 2012). However, only a small share of embryos transferred *in utero* develop into an infant (Kovalevsky and Patrizio, 2005). Human embryos are selected for embryo transfer via morphological criteria. In addition, adequate timing of developmental stages is considered to point to the embryo with the highest implantation potential (reviewed by Reijo Pera, 2011). However, even morphologically normal and timely developing ART embryos show high rates of developmental and implantation failure. Factors, which could be responsible for developmental failure, could be heterogeneity of oocytes, pre-existing problems associated with the subfertility of the couple or possibly IVC conditions.

Recent studies indicated that the use of different IVC media for human embryos can affect the birthweight of newborns (Dumoulin *et al.*, 2010) irrespective of whether embryos were cryopreserved or not (Nelissen *et al.*, 2012). Differences became evident at an early gestational stage (Nelissen *et al.*, 2013). However, others did not find differences between naturally conceived or ART children regarding embryo development, birthweight or preterm delivery (Romundstad *et al.*, 2008; Lin *et al.*, 2013).

Evidence exists that human embryos cultured in different media show different kinetics of cleavage, compaction, blastulation and hatching. In addition, higher implantation rates for Day 3 embryos but not for Days 5 and 6 embryos were described (Van Langendonckt *et al.*, 2001). Similar effects are also seen when mouse embryos were cultured in human ART media. Murine blastocysts show differences in blastocyst formation or hatching after culture in various single step or sequential ART culture media (Schiewe *et al.*, 1999; Schwarzer *et al.*, 2012). The mean cell numbers of the inner cell mass (ICM) differed significantly when embryos were exposed to two different culture media. However, the ratio of cells representing the ICM and trophectoderm (TE) cells was similar (Perin *et al.*, 2008).

In the mouse, the impact of IVC is detectable by alterations in gene expression (Lonergan *et al.*, 2003) or altered DNA methylation patterns in 2-cell embryos (Shi and Haaf, 2002). Furthermore, a loss of imprinting was observed when *in vitro* cultured and *in vivo*-derived embryos were compared (Market-Velker *et al.*, 2010).

However, not only preimplantation development may be impaired after IVC, but fetal development may be affected also. Suboptimal IVC during preimplantation development was shown to cause anatomical and functional abnormalities. In mice, increased weight of the heart and hepatic steatosis was observed (Fernandez-Gonzalez *et al.*, 2004). In cattle, a condition known as 'large offspring syndrome' has been described in which epigenetic alterations correlated with larger offspring size when compared with naturally conceived offspring (reviewed in Young *et al.*, 1998; Sinclair *et al.*, 2000). Accountable for this condition are the supplementation of serum to the culture medium and also the IVC as such. Besides the impact of IVC on fetal development, alterations in placental development and morphology have been reported (Mann *et al.*, 2004; Farin *et al.*, 2006). Placentae of IVF mice are larger (Delle Piane *et al.*, 2010) and placental gene expression alterations were detected. Impaired steroid metabolism was seen in mice and *placenta previa* in humans (Feil *et al.*, 2006; Romundstad *et al.*, 2006; Collier *et al.*, 2009; Fauque *et al.*, 2010). The findings of abnormal proteomic profiles of ART placentae argue for the translation of abnormal gene expression profiles into actual phenotypes (Zhang *et al.*, 2008).

Although the data are conflicting, there is consensus that differences in preimplantation development from the zygote to the blastocyst stage may be influenced by different human ART culture media or the addition of agents such as serum components (Khosla *et al.*, 2001a,b). Currently, the only comprehensive assay for testing the quality of ART culture media prior to clinical use is the mouse embryo assay (MEA). In this toxicity assay, 1- or 2-cell embryos are cultured up to the blastocyst stage *in vitro* (Gardner *et al.*, 2005). Culture media pass the test if at least 70–80% of the embryos develop to the blastocyst stage. The test is not standardized as every company uses different mouse strains which differ in their developmental potential (Tucker and Jansen, 2002). In addition, the follow-up of the concepti after implantation with respect to, for instance, health status has not been performed yet.

The aim of our study was to analyse whether suboptimal IVC affects the quality of ART-derived fetuses after implantation. We tested the hypothesis that ART culture media influence the selection of embryos during preimplantation development *in vitro* but do not affect the embryo after implantation.

Materials and Methods

Mice

Animals were housed in the animal facility at the Max Planck Institute for Molecular Biomedicine (MPI) in Münster and used according to the ethical permit issued by the Landesamt für Natur-, Umwelt- und Verbraucherschutz (LANUV) of the state of North Rhine-Westphalia (animal protocol G160/2010, LANUV reference number 87-51.04.2010.A160). We used B6C3F1 females, a strain commonly used for reproduction studies, and C57Bl/6 (inbred cross) as well as CD-1 females as foster mothers and CD-1 males (outbred cross) for mating. Embryos were transferred to CD-1 foster mothers.

Culture media

ISM1/ISM2 (Origio, Berlin, Germany) and HTF/Multiblast (Irvine Scientific, MTG, Bruckberg, Germany) were chosen as in our previous study (Schwarzer et al., 2012). HTF and Multiblast are fully defined media with known composition; ISM1 and ISM2 are proprietary media with little information on the exact composition. Both media types are sequential media but exhibit differences regarding their chemical contents. Multiblast contains additional amino acids, while the energy sources like glucose, lactate or pyruvate are comparable in both media; ISM1 and ISM2 media basically switch the main energy source from high glucose (ISM1) to low glucose/EDTA (ISM2). Whitten's medium was prepared according to Whitten (1970; for recipe, see Gwatkin, 1972 with modifications from Boiani et al., 2005), KSOM(aa) was used as previously described (Summers et al., 2000; Schwarzer et al., 2012) (for exact content, see Supplementary data, Table SI).
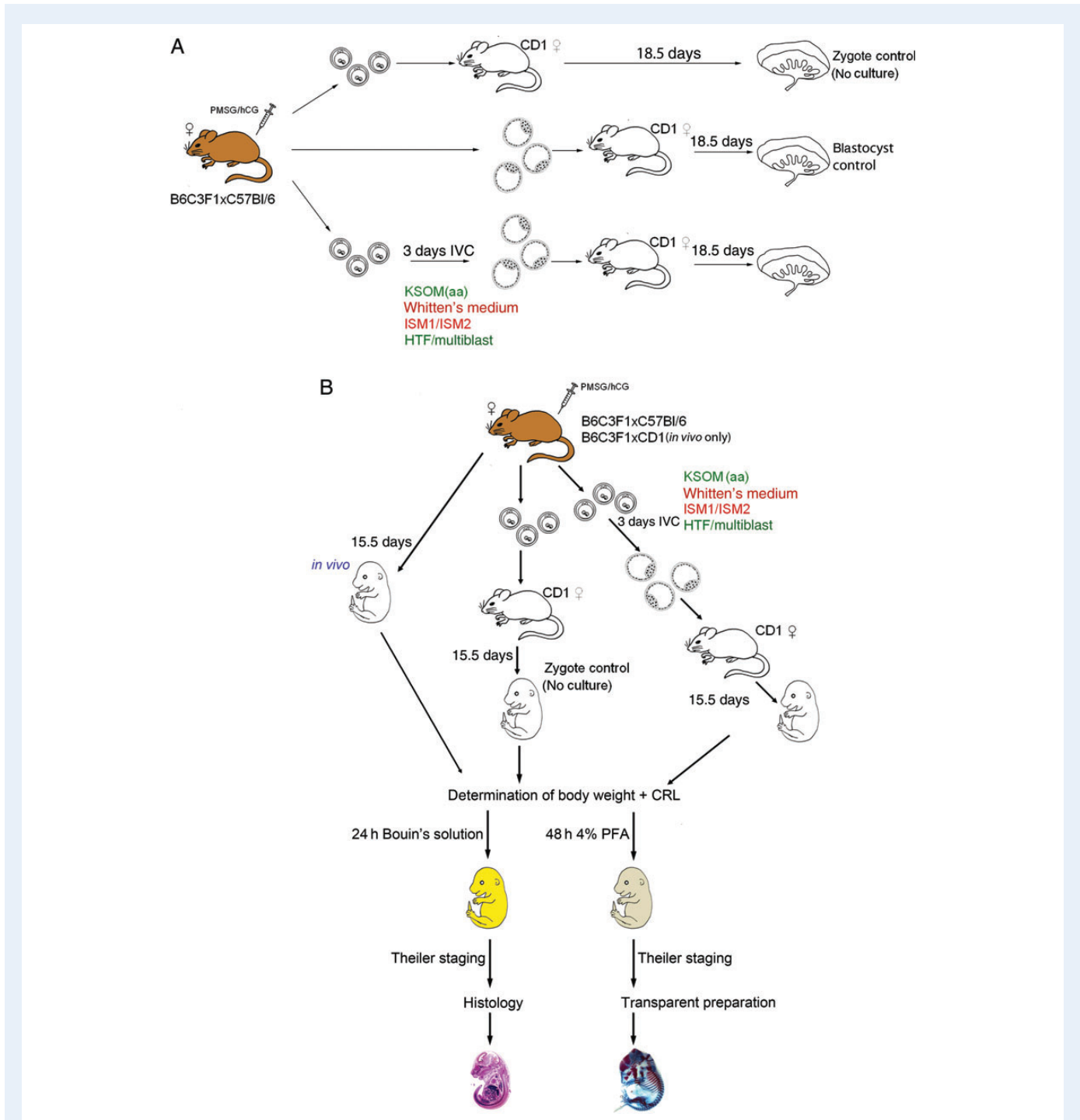

Figure I Experimental design of the project regarding measurements of (**A**) the placenta and (**B**) fetal retrieval and development. For more details, see text. IU, International Units; CRL, crown–rump length; d, days, h, hours).

Embryo collection, embryo culture and embryo transfer

Five- to 6-week-old female B6C3F1 mice were primed with 10 IU pregnant mare's serum gonadotrophin (PMSG, Intergonan 6000, Intervet, Unterschleißheim, Germany) and 10 IU human chorionic gonadotrophin (hCG, Ovogest 300, Unterschleißheim, Germany) in a volume of 300 μl injected intraperitoneally 48 h apart, except in one group, the in vivo control for normal morphological development, where injections were performed 72 h apart from the PMSG injection (for detailed experimental setting, see Fig. 1A and B). This treatment regime is a prerequisite to reduce the negative effect of gonadotropic stimulation on endometrial receptivity (Ertzeid and Storeng, 2001). By allowing more time between PMSG and hCG injections, the negative effects of PMSG on the endometrium are reduced while the hCG injection is still sufficiently close to induce efficient ovulation induction. The oocyte yield declines when hCG injections are performed after 72 h, but this modified hormonal scheme supports efficient implantation into the mouse uterus. B6C3F1 females were mated with C57Bl/6 males overnight to obtain inbred fetuses. An exception was made in the experiment examining paternal influence when females were mated to CD1 males to obtain outbred fetuses (see Fig. 1 for details on the experimental design for retrieval of 15.5 dpc fetuses). Superovulated and mated females which were left untreated until Caesarean section at 15.5 dpc served as in vivo controls. For all other groups, zygotes (0.5 dpc) were flushed from plugged females with Hepes-buffered CZB medium (HCZB; 5.56 mM glucose; 20 mM Hepes; 5 mM sodium bicarbonate; 0.1% PVP) after cervical dislocation (according to the EU-Directive 2010/63/EU). Cumulus cells surrounding the zygote were removed by hyaluronidase treatment (50 IU/mL for 15 min; Calbiochem, Darmstadt, Germany) before embryos were directly transferred (zygote control) or allocated to the different culture media.

Zygotes were cultured in vitro until 3.5 dpc in KSOM(aa), Whitten's medium, ISM1 with a change on 2.5 dpc to ISM2 and HTF with a change on 2.5 dpc to Multiblast. Embryo culture in human ART media was performed according to manufacturer's protocols and using 5.5% CO_2 and 20% oxygen at 37°C. As zygote control, an immediate embryo transfer was performed within 1 h after flushing of the zygotes from the uteri. At 3.5 dpc, only morphologically normal blastocysts with an expanded cavity were selected for embryo transfer into CD1 foster mothers (6–16 weeks old, 25–30 g) that were pseudo-pregnant after mating to vasectomized males. Caesarean sections were performed either at 15.5 dpc to retrieve fetuses or on 18.5 dpc to retrieve placentae. Fetal retrievals were performed at 15.5 dpc since this stage allowed best distinction of bone and skin development. Placentae were retrieved at a later stage to detect changes in the fully mature organ shortly before delivery.

Placental histology

18.5 dpc placentae (B6C3F1 X C57Bl/6 genetic background) retrieved by Caesarean section (see Fig. 1A) were fixed in 4% paraformaldehyde (PFA), processed by a spin tissue processor (Microm STP 120, Thermo Scientific, Dreieich, Germany) [70% (v/v), 90% (v/v), 100% (v/v) ethanol (EtOH), isopropanol (abs.) and xylol (abs.) for 30 min each] and solidified in paraffin. Largest cross-sections (5 μm thick) were rehydrated [100% (v/v), 96% (v/v) and 70% (v/v) isopropanol for 1 min each] and stained with haematoxylin (Sigma-Aldrich, Taufkirchen, Germany) (5 min) and eosin (Sigma-Aldrich) (0.01% (v/v), 3 min). Finally, the tissue slices were dehydrated, cleared [70% (v/v), 96% (v/v) and 100% (v/v) isopropanol, xylol (abs.), for 3 min each] and imaged using a stereomicroscope (Leica WILD M10) at a 20× magnification. For the analysis of acquired images

Table I The implantation rate (total number of implantation sites [sites without fetus (IS) plus sites with fetus (F)]/number of transferred embryos) and fetal rates [number of fetuses born (F)/number of transferred embryos] of the different experimental groups.

Condition	Total embryos transferred (n)/total recipients (n)	Total implants (% transferred embryos)	Implantation rate (IS+F) per female, median	Implantation rate per female, range	No. of fetuses (F) (% transferred embryos)	Fetal rate per female, median	Fetal rate per female, range	P-value Mann–Whitney test (within groups)[a]	Kruskal–Wallis test (all groups)
In vivo		82 (n.d.)	n.d.	n.d.	73 (n.d.)	n.d.	n.d.	n.d.	Implantation rate: P = 0.8028 Fetal rate: P = 0.2762
Zygote control	80/10	37 (46)	0.45	0.00–0.90	25 (31)	0.35	0.00–0.60	0.2861	
KSOM(aa)	53/6	22 (43)	0.45	0.00–0.70	13 (25)	0.25	0.00–0.50	0.3272	
Whitten's	78/8	17 (22)	0.15	0.00–0.63	13 (17)	0.15	0.00–0.40	0.7052	
ISM1/ISM2	132/14.	59 (45)	0.26	0.00–1.00	20 (15)	0.05	0.00–0.43	0.1994	
HTF/Multiblast	53/5	20 (38)	0.60	0.00–0.80	17 (32)	0.40	0.00–0.70	0.8271	

Embryos were collected from B6C3F1 females mated with X C57Bl/6 males or allowed to develop in vivo. Collected embryos were replaced in pseudo-pregnant CD1 females (zygote control) or cultured for 3.5 days in vitro in one of the media before transfer at the blastocyst stage to pseudo-pregnant CD1 females. Implantation was assessed at 15.5 dpc. The Mann–Whitney test was applied to test within a group; Kruskal–Wallis test was applied to test all groups. n.d., not determined.
[a]The MannWhitney test was performed for pairwise comparison of B6C3F1xCD1 and B6C3F1xC57Bl/6 strain specific differences.

of the placentae, spongiotrophoblast and labyrinth layer areas were measured using ImageJ (1.46j).

Determination of fetal morphological developmental stage

Fetuses were retrieved from the uterus by Caesarean section on 15.5 dpc (see Fig. 1B). Weight was determined and pictures were obtained for measurement of the crown−rump length (CRL). Afterwards, fetuses were fixed in Bouin's solution [85% saturated picric acid solution, 10% filtered formaldehyde (37%), 5% acetic acid] overnight or 4% PFA for 48 h. Implantation sites were fixed in Bouin's solution overnight. The numbers of fetuses and implantation sites were counted. The morphological developmental stage of each fetus was determined after fixation and transfer into 70% EtOH. Developmental staging was performed according to the Theiler staging system (Theiler, 1972).

Transparent preparation

Whole-mount transparent preparations with cartilage and bone staining of 15.5 dpc fetuses were performed according to the protocol by Park and Kim (1984) (modified by Ehmcke and Clemen, 2000, Ehmcke and Clemen, 2003). Briefly, after fixation in 4% PFA, the whole fetus was washed in running tap water for at least 9 h before transfer into tubes containing tap water for a maximum of 2 days. Afterwards, the fetuses were dehydrated in EtOH solutions (30%, 50% for 2 h each, 70% overnight). Cartilage was stained with Alcian Blue 8GX (1.5 g/l Alcian Blue in 70% absolute EtOH and 30% acetic acid) for 4−8 h. Excess dye was removed by washing with absolute EtOH overnight until solution remained clear. Afterwards, the fetus was transferred into sodium tetraborate buffer as follows: the object was washed in solutions with decreasing EtOH concentration for 1−2 h (96, 85, 70, 50 and 30%). Subsequently, the fetus was washed twice with 30% sodium tetraborate solution (30% saturated sodium tetraborate solution, 70% ddH$_2$O v/v). Soft tissue was digested with 1% pancreatine in 30% tetraborate buffer (pancreatine solution). Fetuses of 15.5 dpc were digested for 4−7 days at room temperature and pancreatine solution was changed every day. Success of the digest was checked visually. Ossified bone was stained with AlizarinRed Solution (Sigma Aldrich, Steinheim, Germany). This staining solution (0.1 g/100 ml AlizarinRed-S in distilled water, mixed 1:1 with 0.5% KOH directly before use) was incubated overnight before excess dye was removed by washing the whole mount in 87% glycerol/0.5% KOH (1:3), which was then subsequently replaced by 1:1 and 3:1 87% glycerol/0.5% KOH every day. For storage, the stained fetuses were kept in 87% glycerol with added Thymol crystals to prevent fungal infection.

Skeletal morphology was assessed with focus on the presence and condition of the bones and the possible occurrence of bone fusion, especially in the ribs. Furthermore, bones were analysed for the onset and degree of ossification. In particular, the lengths of femur, fibula, tibia, as well as humerus, ulna and radius were determined and the extent of ossification of each bone was evaluated. Measurements were performed using cellSens Standard 1.5 (Olympus Deutschland GmbH, Hamburg, Germany).
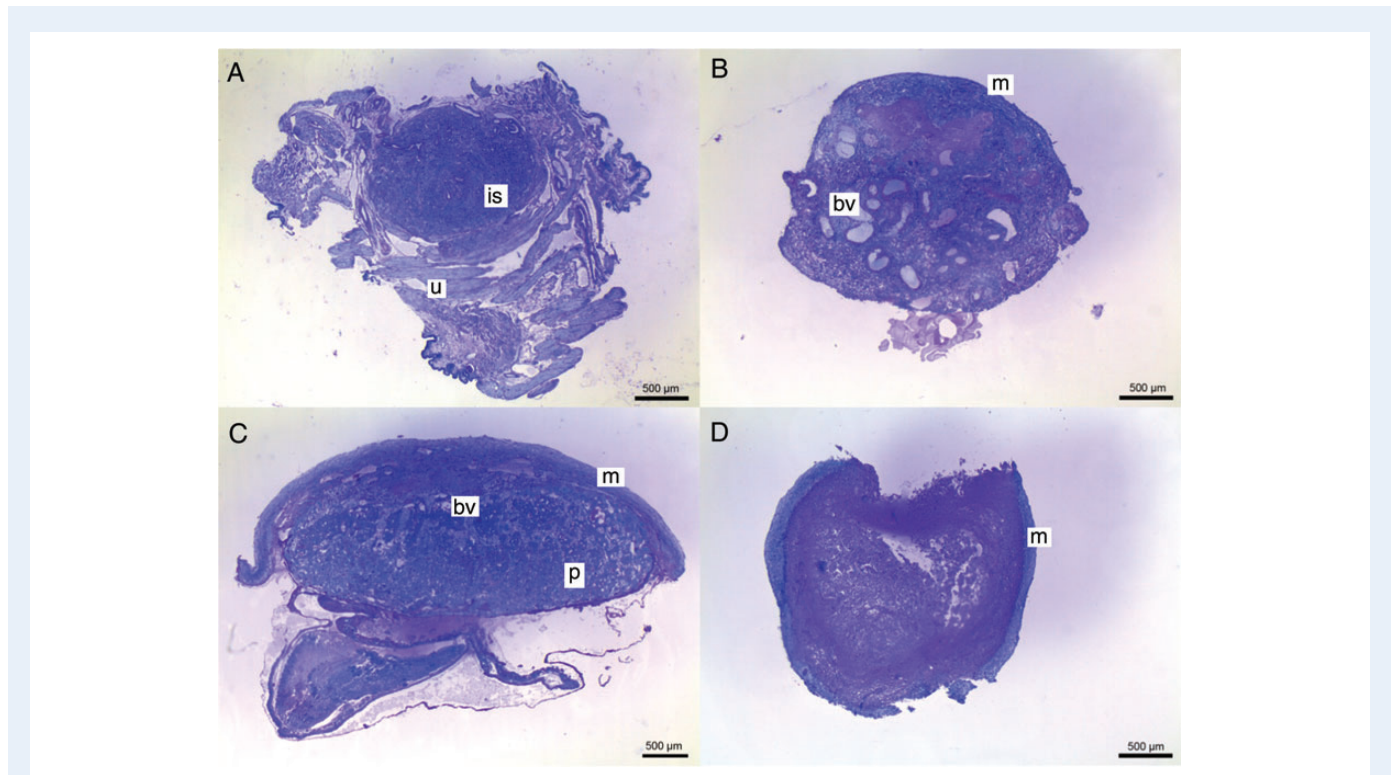
Figure 2 Histological analysis of several implantation sites stained with PAS. (**A**) Image of implantation site (is) with surrounding tissue of the uterus (u); (**B**) implantation site with blood vessels (bv, indicative for placental tissue); (**C**) placenta (p) of an implantation site of an embryo from the HTF/Multiblast group; (**D**) implantation site without blood vessels (=empty residue) surrounded by maternal tissue (m). Embryos were collected from B6C3F1 females mated with X C57Bl/6 males or allowed to develop in vivo. Collected embryos were replaced in pseudo-pregnant CD1 females (zygote control) or cultured for 3.5 days in vitro in one of the media before transfer at the blastocyst stage to pseudo-pregnant CD1 females. Implantation sites were examined at 18.5 dpc.

Table II Analysis of implantation sites for placental blood vessels as an indicator for placenta-like structures within the residua.

Condition	Total implantation sites (% transferred embryos)	Placenta-like structures present, n (%)	Placenta-like structures absent, n (%)
In vivo	9 (n.d.)	5 (56)	4 (44)
zygote control	12 (15)	10 (83)	2 (17)
KSOM(aa)	9 (17)	6 (67)	3 (33)
Whitten's	4 (57)	2 (50)	2 (50)
ISM1/ISM2	39 (30)	32 (82)	7 (18)
HTF/Multiblast	3 (6)	2 (67)	1 (33)

Analysis based on PAS staining. Background B6C3F1 X C57Bl/6. Embryos were collected from B6C3F1 females mated with X C57Bl/6 males or allowed to develop *in vivo*. Collected embryos were replaced in pseudo-pregnant CD1 females (zygote control) or cultured for 3.5 days *in vitro* in one of the media before transfer at the blastocyst stage to pseudo-pregnant CD1 females. Placentae were examined at 18.5 days post coitum. No significant differences were found comparing the experimental groups regarding the number of implantation sites (Kruskal–Wallis $P = 0.427$, $H = 4.909$). n.d., not determined.
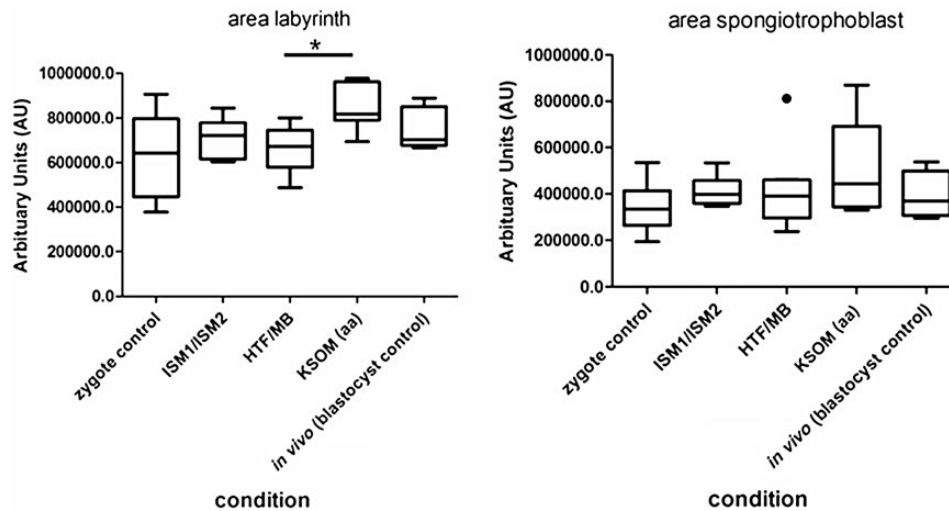

Figure 3 Areas (arbitrary units, AU) of the labyrinth layer and spongiotrophoblast of the placenta show no gross abnormalities between treatment groups. The box depicts the median and quartiles and the whiskers extend for $1.5\times$ the inter-quartile distance, dots indicate outliers. Embryos were collected from B6C3F1 females mated with C57Bl/6 males or allowed to develop *in vivo*. Collected embryos were replaced in pseudo-pregnant CD1 females (zygote control) or cultured for 3.5 days *in vitro* in one of the media before transfer at the blastocyst stage to pseudo-pregnant CD1 females. Implantation sites were examined at 18.5 dpc. Differences were found in the labyrinth layer between HTF/Multiblast and KSOM(aa). (HTF/MB $n = 10$, ISM1/ISM2 $n = 6$, KSOM(aa) $n = 7$, zygote control $n = 6$). No differences were found in the maternal decidua (data not shown). *Statistically significant $P < 0.05$.

Histology

For histological evaluation, 15.5 dpc fetuses and implantation sites were fixed in Bouin's solution, stored in 70% EtOH and embedded in paraffin ($2\times$ 70% EtOH, 80% EtOH, 96% EtOH, 99% EtOH, 100% EtOH for 1.5 h each; 100% EtOH for 2 h; $2\times$ *N*-butylacetate for 1.5 h each; $2\times$ Paraplast 2 and 6 h, respectively; see also Wistuba and Clemen, 1998; Wistuba *et al*., 1999, 2000). Semi-serial 5 μm tissue sections of fetuses were taken using Leica SM 2000R or SM 2010R microtomes. Sections were dried at 38°C before staining. For a histological overview, two staining methods were performed, the periodic acid Schiff (PAS)-staining and the Azan-staining as described by Mulisch and Welsch (2010).

Data analysis and statistics

Images of fetuses and histological sections were adjusted for brightness and contrast using Adobe Photoshop CS 4 (Adobe). Graphs were obtained using GraphPad Prism 5 (GraphPad Software Inc., CA, USA). Boxplots depict the median and whiskers ($1.5\times$ inter-quartile distance according to Tukey). The Kolmogorow–Smirnow test was performed to test for normality distribution of values. Since not all experimental groups were normally distributed, non-parametric statistical analysis was performed. The Mann–Whitney test was performed for pairwise comparison of B6C3F1xCD1 and B6C3F1xC57Bl/6 strain specific differences. The Kruskal–Wallis and the Dunn's *post hoc* tests were performed for multiple-group analysis in all other
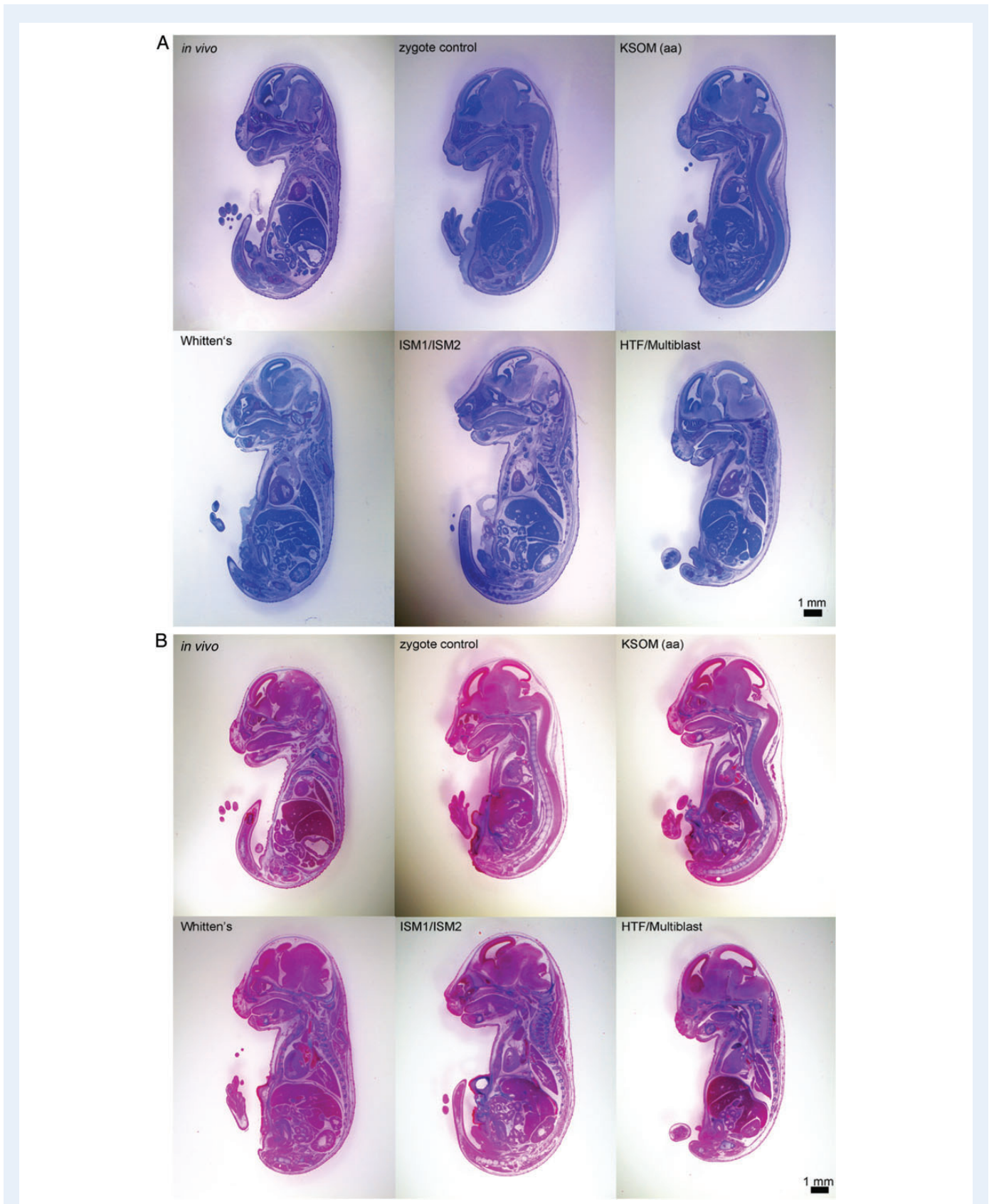
Figure 4 Mid-sections of representative fetuses at 15.5 dpc from the six experimental conditions after PAS (**A**) and Azan staining (**B**). Embryos were collected from B6C3F1 females mated with C57Bl/6 males or allowed to develop *in vivo*. Collected embryos were replaced in pseudo-pregnant CD1 females (zygote control) or cultured for 3.5 days *in vitro* in one of the media before transfer at the blastocyst stage to pseudo-pregnant CD1 females.

experiments. Statistics were calculated using GraphPad Prism 5 (GraphPad Software Inc.). A value of $P < 0.05$ was defined as a trend (*), $P \leq 0.01$ as significant (**) and $P \leq 0.001$ as highly significant (***).

Results

Implantation and fetal rates

Total implantation sites [composed of sites without fetus (IS) and with fetus (F)] in the different culture media groups are presented in Table I. Implantation and fetal rates of the *in vivo* group could not be determined since it was not known how many embryos reached the uterus of the female. Variable litter sizes were not due to differences in the daily performance of embryo transfer (Kruskal–Wallis $P = 0.0607$, $H = 14.92$). Analysis of variance shows that implantation and fetal rates did not differ across the media and the control groups (implantation rate: Kruskal–Wallis, $H = 1.633$, $P = 0.8028$, n.s.; fetal rate: Kruskal–Wallis, $H = 5.110$, $P = 0.2762$, n.s.).

Histological analysis of implantation sites

Implantation sites differed by the presence of placental blood vessels with either low (Fig. 2A) or high (Fig. 2B) frequency of blood vessels (= placenta-like structures) or no vessels (Fig. 2D). The number of implantation sites as well as the percentage containing placental tissue for each experimental group is shown in Table II. Only in the HTF/Multiblast group, we detected one implantation site with a proper placenta (Fig. 2C). No significant differences were found comparing the experimental groups regarding the number of implantation sites (Kruskal–Wallis $P = 0.427$, $H = 4.909$).

Histological analysis of placentae

Placentae retrieved at 18.5 dpc from the different groups were analysed for the presence of gross morphological abnormalities, namely the misallocation of the three main layers that form the placenta: labyrinth layer, spongiotrophoblast (as part of the junctional zone) and maternal decidua (Fig. 3A). There was a significant difference in placental labyrinth area of HTF/Multiblast ($n = 10$) and KSOM(aa) ($n = 7$; Kruskal–Wallis $H = 10.13$, $P = 0.0383$; Dunn's test $P < 0.05$; Fig. 3). All other groups (ISM1/ISM2 $n = 6$, zygote $n = 6$) showed no changes (Dunn's test $P > 0.05$). The spongiotrophoblast area and the ratio of spongiotrophoblast to labyrinth was not different between the tested groups (Kruskal–Wallis $H = 4.269$, $P = 0.3709$; Kruskal–Wallis $H = 2.073$, $P = 0.7223$).

Fetal morphological development of mice after embryo culture

Of 159 fetuses analysed at 15.5 dpc, 157 appeared normal and complied with the exterior morphologic characteristics of Theiler Stage 23. Only one delayed fetus appeared in the *in vivo* group as well as one in the Whitten's group. Both showed features of Theiler Stage 21 in contrast to their littermates. No other major developmental delays were observed in any of the groups. Screening of the histological tissue sections with PAS (Fig. 4A) or Azan staining (Fig. 4B) showed no detectable pathologies in any of the experimental groups. All showed the same morphology and all main organs were present. Further, no major necrotic tissue was visible in any of the fetuses.
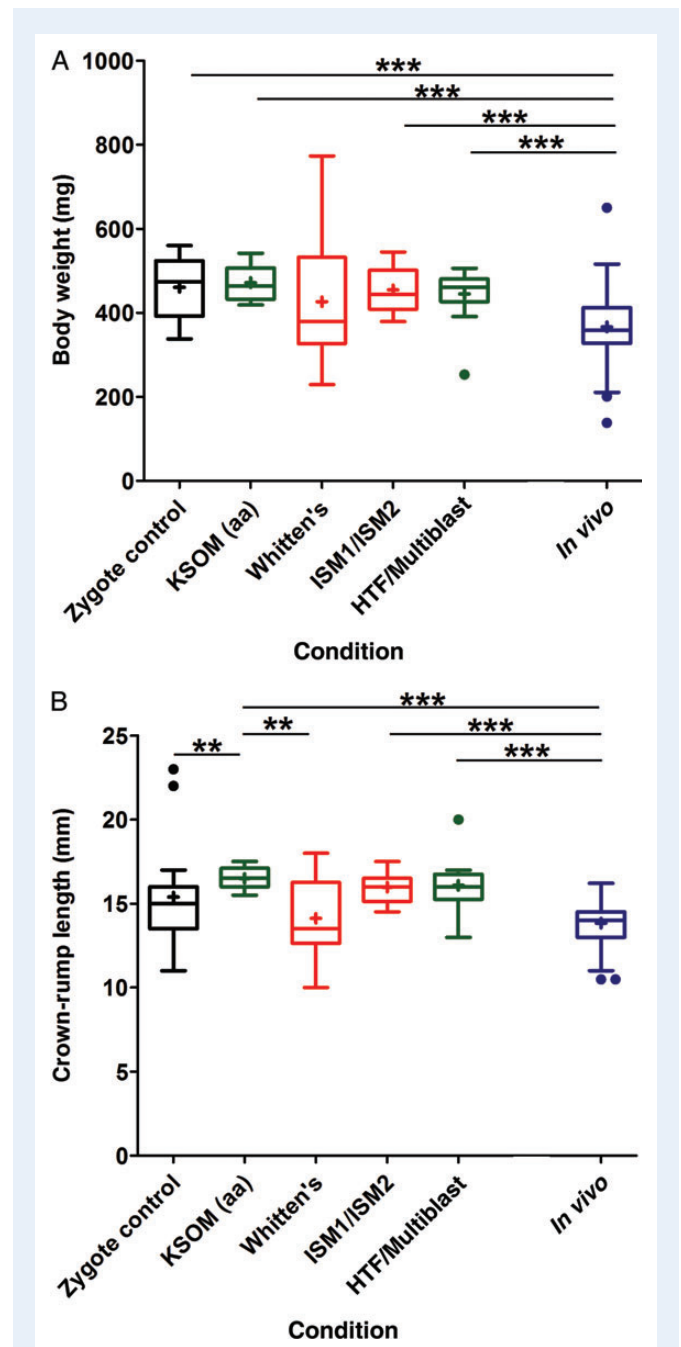

Figure 5 Comparison of body weight (**A**) and crown-rump length (CRL) (**B**) of 15.5 dpc fetuses under the six experimental conditions. The box depicts the median and quartiles and the whiskers extend for $1.5\times$ the inter-quartile distance, dots indicate outliers. Embryos were collected from B6C3F1 females mated with C57Bl/6 males or allowed to develop *in vivo*. Collected embryos were replaced in pseudo-pregnant CD1 females (zygote control) or cultured for 3.5 days *in vitro* in one of the media before transfer at the blastocyst stage to pseudo-pregnant CD1 females. $P \leq 0.01$ significant (**) and $P \leq 0.001$ is highly significant (***); +, mean. (Number of animals in A/B: zygote control $n = 22/18$, KSOM(aa) $n = 13/13$, Whitten's $n = 13/12$, ISM1/2 $n = 20/17$, HTF/Mulitblast $n = 17/17$, *in vivo* control $n = 73/73$.)
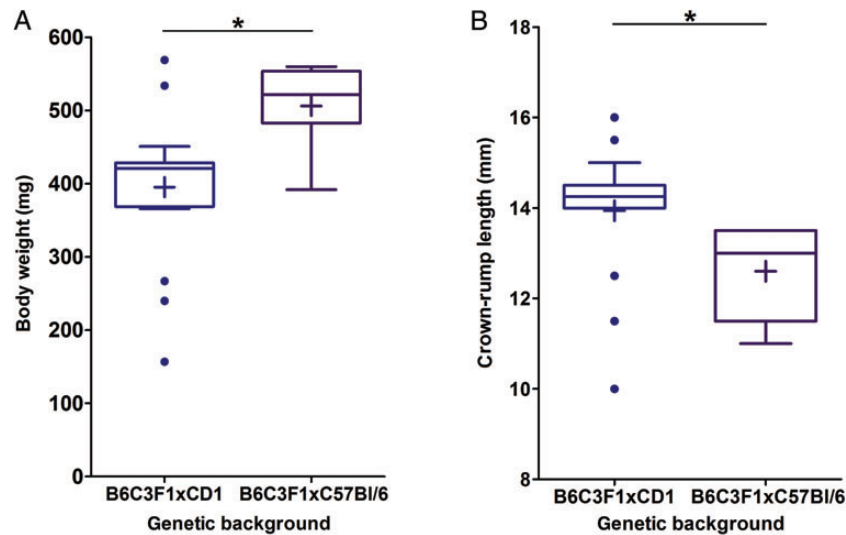
Figure 6 Significant differences of fetal body weight (**A**) and crown-rump length (CRL) (**B**) of non-cultured 15.5 dpc fetuses in two different genetic backgrounds. The box depicts the median and quartiles and the whiskers extend for $1.5 \times$ the inter-quartile distance, dots indicate outliers. Body weight Mann–Whitney test, $P = 0.0110$; CRL Mann–Whitney test, $P = 0.0214$; +, mean. (Number of animals in A/B B6C3F1xCD1 $n = 16/16$, B6C3F1xC57Bl/6 $n = 11/5$.)

Body weight and CRL of 15.5 dpc mouse fetuses from different culture media and effect of paternal genotype

Body weights of inbred fetuses from the in vivo control, the zygote control and the four culture media groups are shown in Fig. 5A. Differences were observed when comparing the fetal weight between the six experimental groups (Kruskal–Wallis test $P < 0.001$). No significant differences in fetal body weight were found between the IVC groups and the zygote control (Dunn's test $P > 0.05$, n.s.). However, fetuses from all in vitro cultured embryos, except the Whitten's group (Dunn's test $P > 0.05$, n.s.), were significantly heavier than in vivo control fetuses (Dunn's test $P \leq 0.001$).

The CRL of inbred fetuses from the in vivo control, the zygote control and the four media groups are shown in Fig. 5B. The zygote control differed significantly from the KSOM(aa) group (Kruskal–Wallis test $P < 0.0001$, Dunn's test $P \leq 0.01$) and KSOM(aa) fetuses differed from Whitten's fetuses (Dunn's test $P \leq 0.01$). All other IVC groups showed no significant differences among each other (Dunn's test $P > 0.05$). However, KSOM(aa), ISM1/ISM2 and HTF/Multiblast groups showed highly significant differences from the in vivo group (Dunn's test $P \leq 0.001$).

Fetuses produced by mating B6C3F1 females with inbred C57Bl/6 males or outbred CD1 males and allowed to develop in vivo exhibited significant differences in fetal body weight as result of paternal influence (Mann–Whitney, $U = 36.00$, $P = 0.0110$) and CRL (Mann–Whitney, $U = 12.00$, $P = 0.055$) at 15.5 dpc (Fig. 6A and B).

Cartilage and bone analysis in 15.5 dpc mouse fetuses after embryo culture

No skeletal abnormalities were observed compared with zygote or in vivo control. No fused ribs were found in any of the fetuses. Normal ossification progression of bones was present in all fetuses.

Ossification of the fingers was according to Theiler Stage 23 in all six conditions (Fig. 7).

The analysis of bone lengths revealed no significant differences between the six study groups for the femur, humerus, ulna and radius (femur: Kruskal–Wallis test, $P = 0.0442$, $H = 11.39$; but Dunn's test $P > 0.05$; humerus: Kruskal–Wallis test, $P = 0.0767$, $H = 9.947$; ulna: Kruskal–Wallis test, $P = 0.1286$, $H = 8.546$; radius: Kruskal–Wallis test, $P = 0.3532$, $H = 5.543$). In contrast to this finding, the in vivo control group differed significantly from the ISM1/ISM2 group in the length of fibula and tibia (fibula: Kruskal–Wallis test, $P = 0.0025$, $H = 18.35$, Dunn's test $P < 0.05$; tibia: Kruskal–Wallis test, $P = 0.0099$, $H = 15.10$, Dunn's test $P < 0.05$). All other groups showed no significant differences regarding the fibula and tibia (Dunn's test $P > 0.05$; Fig. 8).

Analysing the length of ossified bone, no significant differences were found in any of the bones when comparing the six conditions (femur: Kruskal–Wallis test, $P = 0.9584$, $H = 1.050$; fibula: Kruskal–Wallis test, $P = 0.5467$, $H = 4.019$; tibia: Kruskal–Wallis test, $P = 0.9813$, $H = 0.7303$; humerus: Kruskal–Wallis test, $P = 0.5569$, $H = 3.948$; ulna: Kruskal–Wallis test, $P = 0.9692$, $H = 0.9140$; radius: Kruskal–Wallis test, $P = 0.9657$, $H = 0.9606$).

Discussion

Many biological studies pointing at issues of clinical importance are in need of appropriate and valid animal models which can be used to perform experiments under highly standardized conditions. Data derived from human ART clinics provide valuable information on the impact of ART procedures on, e.g. CRL and birthweight, but a thorough histological assessment of fetuses or newborns cannot be performed. Surprisingly, no detailed study has yet been devoted to placentae from live births of ART children. In our present mouse study, we are able to see implantation failure by detection of implantation sites with or
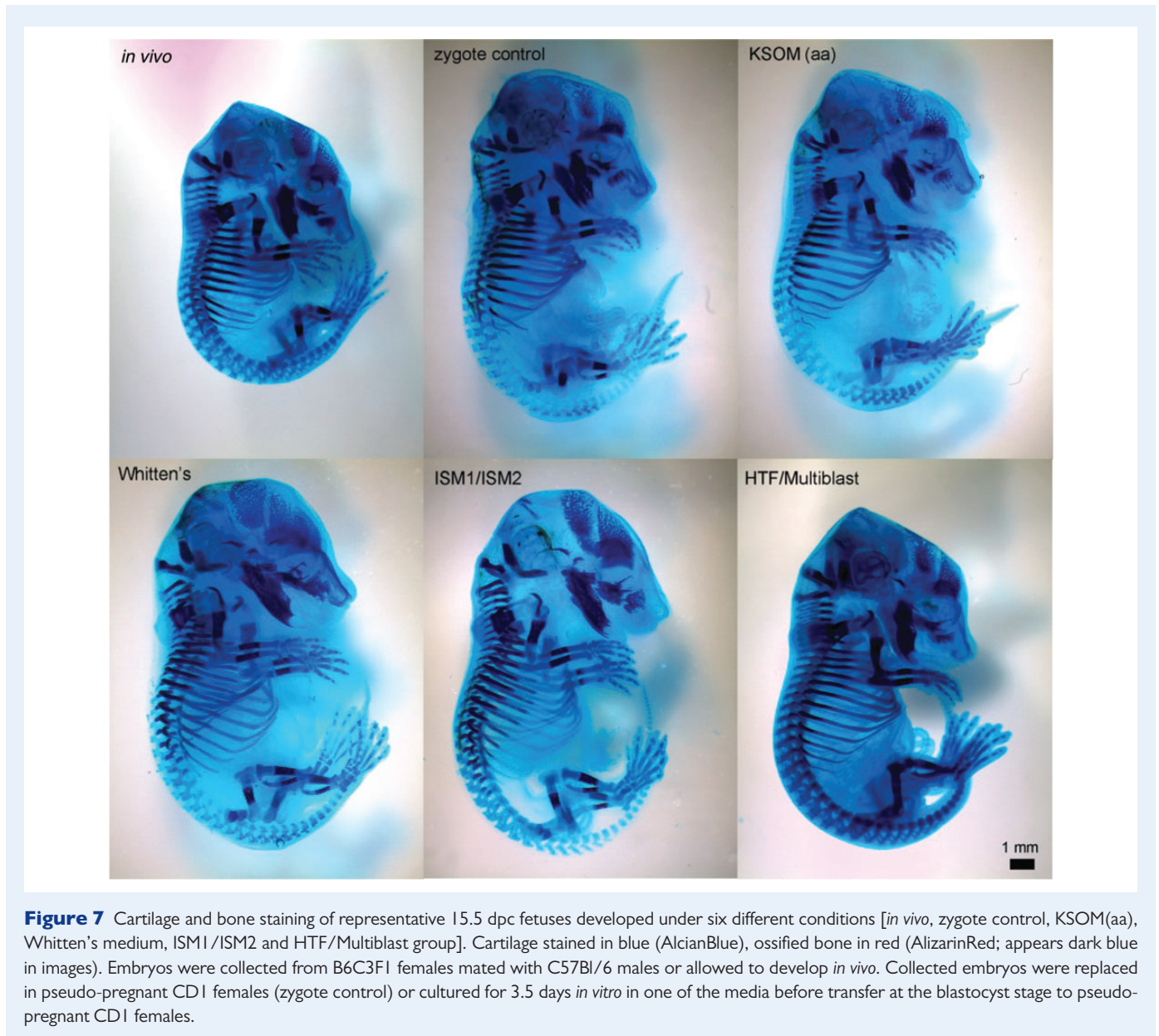
Figure 7 Cartilage and bone staining of representative 15.5 dpc fetuses developed under six different conditions [*in vivo*, zygote control, KSOM(aa), Whitten's medium, ISM1/ISM2 and HTF/Multiblast group]. Cartilage stained in blue (AlcianBlue), ossified bone in red (AlizarinRed; appears dark blue in images). Embryos were collected from B6C3F1 females mated with C57Bl/6 males or allowed to develop *in vivo*. Collected embryos were replaced in pseudo-pregnant CD1 females (zygote control) or cultured for 3.5 days *in vitro* in one of the media before transfer at the blastocyst stage to pseudo-pregnant CD1 females.

without placenta-like structures. In the human, these findings are also not available as material from early ART miscarriages is usually not preserved or carefully screened. Only a standardized and systematic approach in an animal experiment can address all relevant end-points. Prior to clinical use, all clinical IVF media are tested using the MEA which is considered a standardized animal experiment. We propose here an extended version of the MEA to detect the impact of ART media beyond blastocyst development.

The implantation rate is a valuable tool to assess the quality of different embryo culture media (Gardner *et al*., 1999; Van Langendonckt *et al*., 2001). In this study, we found no significant changes in the implantation and fetal rates using different human culture media in contrast to our previous findings (Schwarzer *et al*., 2012). This is intriguing as in both studies only the best blastocysts at 3.5 dpc were selected for embryo transfers. Different numbers of embryo transfers might explain the discrepancy or

we used different quality standards, especially in the ISM1/ISM2 group having in mind that this group generated lower fetal rates in our first study.

A reason for the change in implantation rates may be the ability of embryos to differentially adapt to conditions in their microenvironment. In consequence, IVC may affect the developmental probability of blastocysts by influencing the response to defined check points. In the human, ART blastocysts showing abnormal development are not considered for embryo transfer. It is believed that these embryos might not implant *in vivo* as they will be selected out by nature prior to reaching the uterus. In ART cycles, poor embryos are commonly transferred as higher-grade embryos are not always available. Under those artificial conditions, these embryos have the chance to implant and generate a viable pregnancy. The *in vitro* conditions promoted their probability to survive, although these were rather incompetent preimplantation embryos. However, following implantation, other mechanisms of selection may be activated and
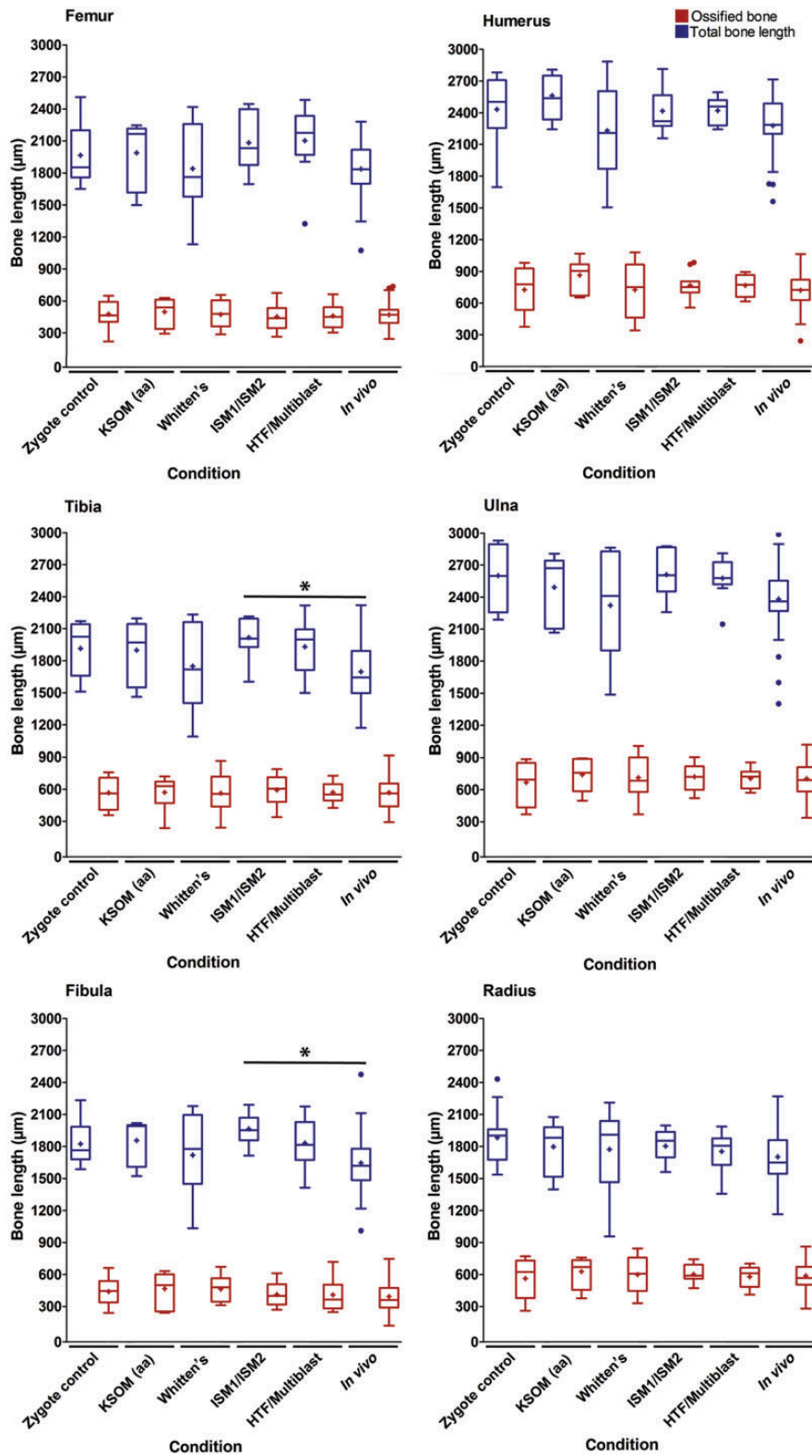
Figure 8 Bone length and ossified bone length in 15.5 dpc fetal femur, tibia, fibula, humerus, ulna and radius. The box depicts the median and quartiles and the whiskers extend for 1.5 × the inter-quartile distance, dots indicate outliers. Embryos were collected from B6C3F1 females mated with C57Bl/6 males or allowed to develop in *in vivo*. Collected embryos were replaced in pseudo-pregnant CD1 females (zygote control) or cultured for 3.5 days *in vitro* in one of the media before transfer at the blastocyst stage to pseudo-pregnant CD1 females. The length of tibia and fibula were significantly different between ISM1/ISM2 and *in vivo* fetuses (fibula: Kruskal–Wallis test, $P = 0.0025$, Dunn's test $P < 0.05$; tibia: Kruskal–Wallis test, $P = 0.0099$, Dunn's test $P < 0.05$). $P < 0.05$ is regarded as a trend (*); +, mean.

newly generated embryonic cells and tissues having normal developmental potentials now form the viable embryo.

Placental abnormalities have been reported to occur after ART (Delle Piane et al., 2010). Changes in the spongiotrophoblast/labyrinth ratio are associated with impaired placental function such as insufficient vascularization (Rossant and Cross, 2001). Placentae from embryos cultured in HTF/Multiblast, ISM1/ISM2, KSOM(aa) and the zygote control showed no gross abnormalities or different sizes in terms of morphology. Spongiotrophoblast and decidua proportions were indistinguishable from controls. Although the labyrinth area of HTF/Multiblast and KSOM(aa) placentae showed significant differences, the ratio was not different. Under our experimental conditions, the different ART media showed no diverse effects on placental morphology. This finding may be due to our strict selection as only the best blastocysts were transferred. These embryos contained normal TE leading to normal placenta development.

Fetal weight may be used as a parameter to assess the quality of different culture conditions as, for example, different oxygen concentrations have a direct influence on the weight of newborn mice (Feil et al., 2006). Changes in fetal weight after IVC have been detected in cattle. Large offspring syndrome is associated with serum supplementation and IVC-induced imprinting alterations (reviewed in Young et al., 1998; Sinclair et al., 2000). Also in the human, several reports have shown that IVC can induce departures in the weight of the newborn (Dumoulin et al., 2010; Nelissen et al., 2012). We were not able to confirm these findings as zygote control fetuses had the same weight as the fetuses after IVC. This is in accordance with recent findings reporting no differences in the weight of the offspring after IVC (Lin et al., 2013). In the mouse, preimplantation embryos are capable to compensate for poor culture conditions (Hogan et al., 1994; Calle et al., 2012). Furthermore, they are also able to compensate for substantial increases and decreases in cell numbers (Snow, 1981). The final size of mouse offspring is not affected by removing blastomeres from the preimplantation embryo. If a compensatory growth after impaired early development occurs, it happens at time points prior to 11.5 or 12.5 dpc (Burgoyne et al., 1983; Hogan et al., 1994). It obviously does not occur after 15.5 dpc, the time point when we analysed the fetuses. Although ISM1/ISM2 embryos showed lower mean cell numbers at the blastocyst stage (Schwarzer et al., 2012), they still implanted and formed fetuses with equal weight and CRL in comparison to other media and control groups. It might well be that compensatory growth has occurred at earlier time points being non-detectable at 15.5 dpc. Whether such compensatory growth also occurs in the human remains unknown.

CRL is a measure commonly determined in humans during pregnancy and upon birth. In this study, the CRL of the fetuses revealed differences between the in vivo control and the fetuses of the zygote culture group. The explanation for this observation is the higher number of in vivo fetuses limiting space and nutrition in an overcrowded uterus. However, we cannot exclude an additional effect resulting from the different background of the mother; in vivo fetuses were carried by a C57Bl6 mother, while all other fetuses were carried by a CD-1 foster mother. Zygote control and Whitten's fetuses were both showing a lower CRL than KSOM(aa) fetuses and were more similar to the in vivo fetuses. The reason for this is unclear and cannot be explained by correlation analysis (data not shown) or the number of fetuses per female. Since the zygote control fetuses were shorter than the KSOM(aa) fetuses, we cannot exclude a culture-associated effect. However, this effect was not observed in the other IVC groups. The Whitten's fetuses were shorter than the KSOM(aa) fetuses, a hint that the culture in Whitten's medium might impair growth, although this finding was associated with lower body weight.

Morphometric parameters and gross anatomical features, such as skeletal development, bone growth and ossification, are routinely used in toxicological studies (Schlabritz-Loutsevitch et al., 2004; Burdan et al., 2005). The assessment of these anatomical traits allows the detection of major anomalies in morphogenetic development in analogy to the toxicological studies. Equivalent methodology is easily applicable to mouse fetuses. Differences in cartilage and bone and differences in the length of the ossified bone may be a result of perturbed differences in growth rate. Especially, the length of femur (and humerus) is often used as reference value when the size of an animal or human fetus is described. In humans, the reduced length of the femur is a good anatomical indicator for several pathological conditions. For example, it is associated with skeletal dysplasia, chromosomal errors or might be a sign for growth retardation (Kurtz et al., 1990; Snijders et al., 2000; Bromley et al., 2002; Abdelhedi et al. 2012; Ventura et al., 2012). We found in ISM1/ISM2 media, fetuses with elongated tibia and fibula compared with their in vivo counterparts. Although ISM1/ISM2 fetuses were heavier and had a longer CRL compared with in vivo fetuses, it is surprising that elongated bone development was observed only in these two bones. Tibia and fibula are the slowest growing bones during fetal development of the limbs in mice. It seems that in ISM1/ISM2 fetuses, these bones developed faster than in the in vivo fetuses. This might also be a sign for compensatory growth as the precedent blastocysts had lower cell numbers.

At the moment, we cannot exclude epigenetic or behavioural effects of embryo culture as observed after culture in Whitten's medium (Doherty et al., 2000; Ecker et al., 2004; Giritharan et al., 2007, 2010). Further studies addressing such consequences of IVC are needed.

To the best of our knowledge, this study is the first to evaluate fetuses and implantation sites after IVC based on defined and clinically relevant histological and anatomical end-points. The normal morphological development and the lack of abnormalities in fetal development represent an encouraging outcome for the future use of ART procedures.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

S.A.H.: experiments and analysis, writing of the manuscript. C.S.: experimental mouse work, writing of the manuscript and discussion. M.B.: experimental design and discussion. J.E.: study design and discussion; writing of the manuscript. S.L.G.: experimental design and discussion.

S.S.: experimental design and discussion. V.N.: experimental design and discussion, writing of the manuscript.

Conflict of interest

None declared.

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