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PROGRESS IN REPRODUCTIVE BIOTECHNOLOGY IN SWINE

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ABSTRACT

This article summarizes recent progress in reproductive biotechnology in swine with special reference to in vitro production of embryos, generation of identical multiples, and transgenic pigs useful for xenotransplantation. In vitro production (in vitro maturation, in vitro fertilization, and in vitro culture) of viable porcine embryos is possible, although with much lower success rates than in cattle. The main problems are insufficient cytoplasmic maturation of porcine oocytes, a high proportion of polyspermic fertilization and a low proportion of blastocysts that, in addition, are characterized by a low number of cells, hampering their development in vivo upon transfer to recipients. Microsurgical bisection of morula and blastocyst stage embryos leads to a 2 to 3% monozygotic twinning rate of the transferred demi-embryos, which is similar to that in rabbits and mice but considerably lower than in ruminants. It was found that with decreasing quality an increasing proportion of demi-embryos did not possess an inner cell mass. Porcine individual blastomeres derived from 4- and 8-cell embryos can be cultured in defined medium to the blastocyst stage. Leukemia inhibitory factor has been shown to be effective at defined embryonic stages and supports the formation of the inner cell mass in cultured isolated blastomeres in a concentration-dependent manner. For maintaining pregnancies with micromanipulated porcine embryos, it is not necessary to transfer extraordinarily high numbers of embryos. Porcine nuclear transfer is still struggling from the inefficiency of producing normally functioning blastocysts. Blastomeres, blastocyst-derived cells, fibroblasts and granulosa cells have been employed as donor cells in porcine nuclear transfer and have yielded blastocysts. Recently, the generation of the first piglets from somatic cell nuclear transfer has been achieved. DNA-microinjection into pronuclei of porcine zygotes has reliably resulted in the generation of transgenic pigs, which have special importance for the production of valuable pharmaceutical proteins in milk and xenotransplantation. It has been demonstrated that by expression of human complement regulatory proteins in transgenic pigs the hyperacute rejection response occurring after xenotransplantation can be overcome in a clinically relevant manner. Although biotechnological procedures in swine have recently undergone tremendous progress, the development is still lagging behind that in cattle and sheep. With regard to genetic engineering, considerable progress will originate from the possibility of employing homologous recombination in somatic cell lines and their subsequent use in nuclear transfer. In combination with the increasing knowledge in gene sequences this will allow in the foreseeable future widespread use in the pig industry either for agricultural or biomedical purposes. © 2001 by Elsevier Science Inc.

Key words: swine, in vitro production of embryos, genetically identical multiples, transgenic, biomedicine

INTRODUCTION

Biotechnology in livestock comprises an arsenal of reproductive biotechnological procedures and molecular genetics. Reproductive biotechnology includes artificial insemination (AI), estrus synchronization, induction of parturition, embryo transfer (ET), cryopreservation of oocytes and embryos, sperm sexing, in vitro production of embryos (IVM/F/C), embryo bisection, nuclear transfer and microinjection of DNA constructs. Molecular genetics include genome analysis (e.g., sequencing, mapping and determination of polymorphisms of porcine genes), molecular diagnostics to identify genetic disorders, genetic identity and/or diversity; functional genomics (e.g., expression patterns, interaction of genes), and transgenic modification including either gain or loss of function. Molecular genetics are poorly developed in swine, mainly attributed to the few genes that have been sequenced and mapped from the porcine genome (61). On the reproductive biology side, AI has emerged as a growing business with approximately 50% of all sexually mature sows worldwide being served by AI. However, considerable differences among countries with regard to the frequency of AI application exist (73). Embryo transfer technology is routinely available and is mainly based on surgical embryo recovery and transfer procedures. However, registered commercial embryo transfer activity in swine is low; from 241 flushes approximately 8.071 embryos had been collected of which ~2.530 had been transferred (66). These numbers will likely increase in the future as significant progress has been made with regard to development of a reliable and efficient cryopreservation procedure for porcine embryos (12) and the advent of a robust nonsurgical embryo transfer technique (20). The chronological development of reproductive biotechnologies in swine is depicted in Table 1 and shows that offspring have been produced with all biotechnological procedures. To make full use of the anticipated increase in knowledge and techniques in molecular genetics, in vitro production of viable porcine embryos, the generation of identical multiples and the methods to generate transgenic pigs need substantial improvements. The following is an attempt to summarize recent progress in these fields (with emphasis on our own experiments) and to develop perspectives for future developments.

IN VITRO PRODUCTION (IVM/IVF/IVC) OF PORCINE EMBRYOS

Despite intensive research (15,32,40) in vitro production of viable porcine embryos is lagging behind the status in cattle where this technology is commercially applied (18,69). Prevalent problems of in vitro production of swine embryos are an insufficient cytoplasmic maturation of the oocyte, an unusually high degree of polyspermic fertilization, low numbers of viable blastocysts which, in addition, are frequently characterized by too few cells resulting in low development in vivo (15). Antral follicles from slaughterhouse ovaries obtained from prepubertal gilts regularly serve as a source of oocytes. The size of the follicle determines the developmental capacity of the oocyte, with oocytes from larger follicles having a high developmental potential (6), similar to observations made in cattle (45). For IVM, cumulus-oocyte-complexes are cultured for 42 to 48 h at 38.5 to 39°C in media such as TCM 199, NCSU37 or 23, supplemented with various hormones (LH, FSH, hCG, PMSG/hCG) and/or growth factors (e.g. EGF), follicular fluid, cumulus cells, follicular somatic cells and/or glutathione (42,50). However, the inherent tremendous variability of the biological material cannot yet (or can only partly) be overcome by these approaches. A two-step protocol aiming at

maximum synchronization of germinal vesicle stages allows high maturation rates. In this system oocytes are cultured in NCSU37 for the first 20 h in the presence of gonadotropins and dibutyryl cAMP. This is followed by addition of tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) for the next 24 h of IVM. TIMP-1 is secreted by porcine preovulatory granulosa cells and is thought to be essential for the oocyte to obtain the capacity for full preimplantation development. The improved quality of the matured oocytes leads to significantly elevated (34%) blastocyst yields (14).

Table 1. Animal biotechnology: The first live born offspring in swine.

Technology	Year of Publication	Authors
AI (fresh)	1936	Rodin & Lipatow (59)
AI (frozen/thawed)	1970	Polge et al. (49)
ET	1951	Kvasnickii (24)
Embryo freezing	1989 (contr.fr., hatch.bl.)	Hayashi et al. (19)
	1995 (z.p.i.)	Nagashima et al. (34)
Sexing	1991 (SI)	Johnson (22)
	1997 (IVF)	Rath et al. (53)
IVF	1985	Cheng (8)
	1989 (IVM)	Mattioli et al. (29)
Embryo bisection	1985 (4 cell)	Polge (48)
	1988 (mo/bl)	Nagashima et al. (35)
Blastomere proliferation	1991	Saito & Niemann (62)
Nuclear transfer	1989 (blastomeres)	Prather et al. (51)
	2000 (somatic cells)	Polejaeva et al. (47)
Transgenic	1985 (microinjection)	Hammer et al. (16)

SI = Surgical insemination; z.p.i. = zona pellucida intact; contr. fr. = controlled freezing; IVF = in vitro fertilization; IVM = in vitro maturation; hatch. bl. = hatched blastocysts; Mo/bl = morulae and blastocysts;

Whereas nuclear maturation can easily be assessed, cytoplasmic maturation can only be recorded by the ability of the oocytes to induce pronucleus formation after penetration by a single spermatozoon followed by a normal, early, preimplantation embryonic development. Timing of pronucleus formation has been found to be significantly different between in vivo and in vitro-matured porcine oocytes (28). Glutathione is beneficial for normal male pronucleus formation presumably by reduction of oxidative stress (76). Biosynthesis of glutathione depends on the presence of cysteine in the culture medium. Higher levels of glutathione in the maturation medium can be maintained by adding the thiol compounds cysteine, ß-mercaptoethanol or cysteamine (2,7,15). The intercellular connections between the oocyte and cumulus cells seem to be essential for exerting the physiological effects of glutathione (15). Thus, the previous low rates of male pronucleus formation were at least partly related to oxidative stress, which now can be prevented or greatly reduced. However, in vivo matured oocytes (e.g., those collected around ovulation) still possess a significantly higher capacity to develop into normal embryos than those from any of the IVM systems (57).

Freshly ejaculated as well as frozen/thawed boar semen has been successfully employed for in vitro fertilization. However, considerable variation has been observed with regard to rates of fertilization, seemingly attributed to differences in sperm capacitation. Boar seminal plasma contains decapacitation factors that can prevent in vitro capacitation of spermatozoa. To standardize semen production for IVF, epididymal semen after freezing and thawing can be successfully employed (55). However, this precludes use of semen from living boars with valuable genetics. Attempts to eliminate or at least to reduce the high proportion of polyspermic fertilization include co-culture of oocytes with epithelial cells, addition of oviductal fluid or preincubation of sperm with follicular fluid (15). An insufficient cytoplasmic maturation with a delayed or incomplete release of the cortical granules is thought to be involved in the high incidence of polyspermy. Recently, the addition of hyaluronic acid has been shown to increase the proportion of monospermic fertilization in IVF (65). This substance modulates sperm capacitation and is produced by cumulus oocyte complexes during the maturation process (60). In addition, an individual genetic factor cannot be fully excluded from contributing to polyspermic fertilization. In our experiments polyspermy rates hardly exceeded 7% irrespective of the IVM/IVF-system employed.

Normal preimplantation development with IVM/F derived embryos developing through the 4-cell block stage has been achieved and piglets have been obtained following IVF of oocytes matured either in vivo or in vitro (2,15,40,53,54; Table 1). Hypotaurine and taurine seem to be the crucial components of the widely used culture medium NCSU23. These substances mainly act as osmoregulators and pH stabilizers. In addition, serum has been shown to be an essential factor to obtain high rates of blastocyst development (46). When serum was replaced by BSA in BECM-3 medium, blastocyst development was enhanced (11), however, the ratio of ICM to trophoblast cells was altered (56). Although a great variety of IVC systems has been described, the number of blastocysts from IVM/F is low, varies considerably, and the quality of the embryos frequently is too poor to support regular fetal development upon transfer to recipients. Even in vivo fertilized porcine embryos cultured in vitro from the 2- and 4-cell stage to the blastocyst stage displayed distinct ultrastructural differences compared with their in vivo counterparts. These deviations were related to the decreased in vivo development of IVC porcine embryos (21). One plausible explanation for the lower developmental potential of porcine IVM/F/C embryos may be alterations in microfilament polymerization and organization. It has been shown that polymerization of G-actin into F-actin plays a crucial role in porcine oocyte mitosis and meiosis (72). The current results of IVM/F/C in swine can be summarized as follows: In vitro maturation, solely based on nuclear maturation: 70 to 95%; penetration rate: 50 to 90%; male pronuclear formation: 80 to 91%; polyspermy: 5 to 91%; cleavage rates: 20 to 75%; the proportion of blastocysts: 2 to 36% (15,40). On average, less than 3 to 5% of the transferred embryos develop to piglets.

This low efficiency of in vitro production of porcine embryos prevents numerous promising applications of this technology. Employing flow-cytometrically sorted semen could be one of the first areas by which offspring of a predetermined sex could be produced for targeted purposes (1,53,54). Further areas of application would be an acceleration of the genetic progress with a better exploitation of the female reproductive capacity and new options for the maintenance of genetic resources. In addition, a functional in vitro production system allows the study of basic molecular mechanisms in maturation, fertilization and early development that are in progress in the bovine and provide insight into alterations induced by in vitro techniques (39). In addition, a

reliable and efficient in vitro production system would facilitate and improve nuclear transfer and genetic engineering in a significant manner.

GENERATION OF IDENTICAL MULTIPLES

Progress has been made in improving technology for generating genetically identical pigs since the last review (38). We had shown that porcine isolated blastomeres derived from 4- and 8-cell embryos have inherent potential to grow into blastocysts and to undergo in vivo development upon transfer to recipients (62). However, the efficiency of the culture system was limited and could not be significantly improved by the addition of growth factors such as EGF, IGF-I and II (38). In a recent investigation we have shown that porcine intact embryos as well as isolated blastomeres can be cultured individually in defined medium to the blastocyst stage. By employing the differential staining procedure it was found that in blastomere-derived blastocysts the ratio of Inner Cell Mass (ICM) to trophectoderm cells was lower than in intact embryos. The presence of leukemia inhibitory factor (LIF) reduced blastocyst development in defined medium, but inhibited formation of blastocyst-like vesicular structures, indicating a supportive effect for the formation of ICM. The effects of LIF were dependent on the embryonic stage and the presence or absence of protein (14). We have also determined the presence or absence of the ICM in demi-embryos produced by simple bisection of morulae, early blastocysts and blastocysts (58,68). Interestingly, the percentage of demi-embryos without an ICM was 19 to 30% in demi-embryos generated from morulae and classified as excellent, fair and degenerate, and 4 to 30% for early blastocysts and blastocyst stages of corresponding quality. Thus, a considerable proportion of demi-embryos does not possess a functional ICM. The ratio of total cells to ICM was, however, similar between intact embryos and the various morphological categories of demi-embryos. Blastocysts were the optimal stage for obtaining maximum yields of viable porcine demi-embryos (68). When generating identical twin piglets for studies in biomedicine, we have tested the number of transferred embryos per recipient as a factor affecting the efficiency of the procedure. Our data clearly showed that the efficiency of obtaining pairs of identical twins was higher upon transfer of 20 pairs per recipient compared with 40 pairs per recipient (Table 2). When evaluating the efficiency of generating identical twins among species, it becomes evident that the pig resembles species such as the rabbit and mouse (having a low efficiency) but is considerably different from ruminants in which a high proportion of identical twins can be obtained (Table 3). Nevertheless, for specific biomedical and genetic purposes pairs of monozygotic piglets can be produced, although with low efficiency.

Table 2. Development of porcine embryos in relation to the number of demi-embryos transferred per recipient

Demi-embryos/recipient	20.0	30.0	40.0
Transfers	15.0	15.0	16.0
Pregnancies (%)	10.0 (67)	11.0 (73)	10.0 (63)
Piglets born	53.0	72.0	69.0
Average litter size	5.3	6.6	6.9
Survival rate of demi-embryos	17.6%	16.1%	10.7%
Survival of pairs	4.2%	2.8%	2.2%
Survival of pairs in farrowing recipients	6.4%	3.8%	3.5%
Monozygotic pairs	6.0	6.0	7.0

Table 3. Comparative aspects of in vivo development (%) of demi-embryos from various species (see reference 58 for details)

Species	Development to offspring	Incidence of twinning
Cattle	50 - 75	25 - 40
Sheep	40 - 65	40 - 45
Goat	30 - 60	15 - 45
Pig	20	2 - 3
Rabbit	20	2
Mouse	15 - 20	2 - 5

Nuclear transfer is the only procedure to generate potentially larger numbers of genetically identical animals of a single genotype. This technology has gained a great deal of public attention, and significant technical improvements in species as such cattle, sheep and even mouse have occurred since the first successful somatic nuclear transfer was demonstrated in sheep (75). In contrast, in the pig the development is lagging much behind that in cattle and sheep, although the first somatic nuclear transfer derived piglets were reported recently employing in vivo or in vitro derived oocytes and either the fusion or the injection technology (5,42,47). Blastomeres, blastocyst derived cells as well as fetal fibroblasts have been used for porcine nuclear transfer with the yield of blastocysts (13,17,31,33,51,66,67,70). As the success of somatic cell nuclear transfer critically depends on the cell cycle stage of the donor nucleus and the recipient cytoplast, the efficiency of serum deprivation as well as that of reversible cell cycle inhibitors to synchronize porcine fetal fibroblasts at the G_0/G_1-stage was assessed (25). Approximately 67 to 73% of porcine fetal fibroblasts were in G_0/G_1-stage in serum-supplemented medium. Serum deprivation for 24 to 74 h increased this proportion to 78 to 80%. A similar effect was observed using aphidicolin or butyrolactone I as reversible cell cycle inhibitors. However, extended serum deprivation of porcine fetal fibroblasts led to DNA-fragmentation with some characteristics of apoptosis (26), which currently is being studied in greater detail.

Both the fusion technology as well as the injection technology as described for nuclear transfer in the mouse (71) have been employed in swine without differences in the overall success rates. Several technical details have been identified as important for the success of nuclear transfer, including the surface and the size of the donor fibroblast. Small fibroblasts with a smooth surface derived from confluent serum-deprived cultures seem to be superior to other types of cells (66,67). The use of in vivo-derived oocytes as recipient cytoplasts usually yielded higher blastocyst rates than the use of in vitro produced oocytes, reflecting the insufficient conditions during IVM. Activation of porcine oocytes can be achieved by employing a variety of Ca^{2+} dependent and Ca^{2+} independent approaches. A two-step procedure employing thimerosal followed by DTT (dithiothreitol) was superior in inducing preimplantation development of nuclear transfer-derived porcine embryos than other protocols (52). The average efficiency of the different steps in somatic cell nuclear transfer currently can be summarized as follows:

enucleation: 60 to 85%; activation of the oocytes: 70 to 85%; fusion: 70 to 80%; initial cleavage: 30 to 50%; blastocysts: 1 to 15%, piglets: 1 to 2%.

TRANSGENIC PIGS FOR XENOTRANSPLANTATION

Microinjection of DNA constructs into pronuclei of porcine zygotes has been the preferred method to produce transgenic pigs, although efficiency is quite low. The transgenic technology has been applied in swine to enhance growth and development, to increase disease resistance, to produce foreign proteins in milk and for xenotransplantation (36,38,41). The latter area has undergone rapid progress in recent years (3,63). Allotransplantation (e.g., transplantation of organs [heart, kidney or liver] between individuals of the same species) has been very successful, leading to average survival of the patients of 10 to 15 years. This success led to an increase in the demand for suitable organs that cannot be met, since the willingness to donate organs remains stable or is declining in most western countries. Thus, increasing numbers of patients on the "waiting list" die because no appropriate organs can be transplanted within the needed short period of time. Pig organs are rather similar in their anatomy and physiology to human organs and, in addition, pigs offer the possibility to be raised under the highest possible hygienic standards. Due to their high fertility and prolificacy large numbers of offspring can be produced. Transplantation of organs between individuals of nonrelated species (i.e., xenotransplantation) results in a hyperacute rejection response (HAR) that destroys the transplanted organs within minutes (74). HAR is caused by an overreaction of the human complement system against cell membrane epitopes of porcine endothelial cells. As the gene sequences of three of the major complement regulating proteins are known, this information can be used to generate transgenic pigs that express human complement regulatory proteins. The goal is to overcome the complement-mediated HAR by overexpressing a human complement regulator in the pig, thereby making the organ suitable for human transplantation. After xenotransplantation of a porcine organ the patient would require an immunosuppressive treatment similar to that following allotransplantation (4). Indeed, it has been shown that expression of the human complement-regulating protein decay-accelerating-factor (hDAF) or human CD59 in transgenic pigs enables suppression of the complement attack in various in vitro models and even upon transplantation of porcine organs into primate recipients. Upon heterotopic transplantation of hDAF transgenic porcine hearts (i.e., transplantation in addition to the recipient's own heart), into cynomologous monkeys the average length of survival was 40 to 70 days (3,10). Even an orthotopic (i.e., life supportive) transgenic porcine heart transplantation led to an average survival of 10 days in the primate recipient (cynomologous monkey; 3). Similarly, following transplantation of porcine kidneys, normal kidney function was maintained for an average of 13 days in the primate recipient (77). These data clearly show that by applying this strategy, HAR can be overcome in a clinically relevant manner. Indeed, in several countries the rules for clinical trials in xenotransplantation are close to being finalized or are actively being developed.

In our experiments, the human complement regulator CD59 (hCD59) under the transcriptional control of the CMV promoter (Cytomegalovirus) has been expressed in transgenic pigs. Recently two lines with massive expression of the transgene in various organs were identified. The endothelial cells expressed the transgene and were protected from the deleterious effects of human complement in an in vitro test system (37). Kidneys from transgenic pigs of these lines maintained their physiological function upon perfusion with human blood

significantly longer compared with nontransgenic controls. To optimize individual steps of the procedure we had transferred different numbers of microinjected embryos per recipient and found no advantage for the higher number of embryos per recipient (Table 4). This coincides with our findings for demi-embryos (see Table 2). In addition, embryos could be transferred into one oviduct without reducing the results (Table 5), which avoids manipulation of the second ovary and oviduct and thereby prevents formation of adhesions.

Table 4. Effects of number of transferred microinjected porcine zygotes per recipient on pregnancy rates and litter size

Transferred zygotes/ recipient	20 – 30	31 – 40	>40	Total
Recipients	19.0	14.0	5.0	38.0
Pregnancies (%)	10.0 (53)	10 0 (71)	5.0 (100)	25.0 (66)
Piglets	53.0	55.0	27.0	135.0
Average litter size*	5.3	5.5	5.4	5.4
No. transgenic (%)	9.0 (17)	4.0 (7)	2.0 (7)	15.0 (11)

*Range: 1-11 piglets

Table 5. Effects of uni- and bilateral oviductal transfer of microinjected porcine zygotes

	One oviduct	Two oviducts
Zygotes transferred	703.0	495.0
Average no. zygotes/recip.	30.5	33.0
Transfers	23.0	15.0
Pregnancies (%)	16.0 (70.0)	10.0 (67.0)
Piglets (%)	94.0 (13.0)	47.0 (9.5)
Transgenic piglets (%)	9.0 (10.0)	6.0 (13.0)

It is anticipated that xenotransplantation using genetically modified porcine organs will move to clinical trials within the next 4 to 5 years, provided that the potential risks of pathogen transmission can be excluded. Recent data indicate that the risk of the transmission of endogenous retroviruses, originally thought to be the major obstacle to clinical xenotransplantation, obviously can no longer be regarded as a major cause of concern. No evidence for porcine retrovirus infection or activation was detected in patients who had porcine tissue transplants for long periods of time (44).

CURRENT STATUS AND FUTURE DEVELOPMENT

It is evident that in the pig, despite recent great progress, the status of reproductive biotechnology is not as advanced as it is in cattle and sheep. In particular, key technologies such

as cryopreservation, in vitro production of embryos and nuclear transfer are lagging behind the developments in cattle (Table 6). The future of pig reproductive biotechnology will heavily be affected by the development of a somatic cell nuclear transfer technology and increased knowledge in functional genomics. In comparison with human and mouse, not more than 500 genes have been sequenced and mapped in the pig (23). However, genomic maps in livestock will significantly benefit from the advancements made in human and mouse due to a high degree of homology between gene sequences of the various species.

Table 6. Current status of biotechnology in livestock

Biotechnique	Pig	Cattle	Sheep/Goat
A I	+++	+++	++
E T	++	+++	++
Embryo freezing	+	+++	+++
Sexing	+	++	+
IVM/F/C	+	++(+)	+
Embryo bisection	++	+++	++(+)
Nuclear transfer	+	++	+(+)
Transgenic animals (Microinjection)	++	++	++

+ = experimental stage, first offspring; ++ = field application possible; +++ = field application; ( ) = on the edge to next category

The application of somatic cell nuclear transfer in swine in combination with the increasing genomic information will allow for a significant improvement in the area of transgenesis and thus a highly diversified production. Transfection of donor cells and their subsequent use in nuclear transfer have been successful in sheep and cattle (9,64) and make this approach seemingly more effective for the generation of transgenic animals than the microinjection technology. In contrast, in the pig, the first transgenic blastocysts were obtained recently upon transfection with a reporter gene construct (27). However, integration of the transgene into the host genome occurs at random upon transfection (as is the case for microinjection) leading to position effects with the possibility for an insufficient or aberrant expression pattern of the transgene. With the renaissance of homologous recombination in somatic cells and their use in nuclear transfer, targeted genetic modifications will become possible and will open a completely new horizon for the production of transgenic pigs both for biomedical and/or agricultural purposes. This will enable, for example, a knockout of the α-galactosyltransferase gene, a gene that is primarily responsible for the formation of the main xenoreactive epitopes. Such a knockout ultimately would allow the generation of pigs specifically tailored for xenotransplantation. At the beginning of the new millennium targeted homologous recombination was shown to be feasible in sheep (30). In addition, a whole arsenal of sophisticated molecular tools developed in mice awaits application in livestock (36). The following years will see the emergence of new technologies such as bioinformatics and cDNA-arrays, which will be essential tools in functional genomics in livestock that will revolutionize

our understanding and the possibilities to alter the genetic make-up of pigs in a significant manner.

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