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# Genes for anti-Müllerian hormone and androgen receptor are underexpressed in human cumulus cells surrounding morphologically highly graded oocytes

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## Abstract

**Objectives:** The aim of this study was to investigate the expression of genes crucial for the quality of the oocyte and whether expression levels of these genes in cumulus cells can be biological markers for the quality of the oocyte, zygote or embryo, or even for achievement of pregnancy after the assisted reproductive technology procedure. We examined the expression profile of the anti-Müllerian hormone (AMH) gene and its respective receptors: anti-Müllerian hormone receptor type 2 (AMHR2), follicle-stimulating hormone receptor (FSHR) and androgen receptor (AR) in cumulus cells (CCs) surrounding the oocyte, as well as AMH concentrations in follicular fluid of the associated follicle. The obtained gene expression levels were correlated with the morphological quality of the associated oocyte, zygote and embryo as well as with assisted reproductive technology outcome following the intracytoplasmic sperm injection procedure.

**Methods:** This study involved 129 cumulus cells and 35 follicular fluid samples, taken from 58 patients undergoing the intracytoplasmic sperm injection procedure. Oocytes, zygotes and embryos were assessed for morphological quality. The relative gene expression of AMH, AMHR2, FSHR and AR was calculated using the delta–delta Ct method. Anti-Müllerian hormone concentrations in follicular fluids were measured by enzyme-linked immunosorbent assay.

**Results:** The results yielded suggest a relationship between AMH, AR and oocyte morphology: AMH and AR gene expression levels in CCs surrounding morphologically optimal oocytes were significantly lower than in CCs surrounding oocytes with suboptimal morphology ($p = 0.011$ and $p = 0.008$, respectively). Statistically significant positive correlation was found between mRNA expression levels of AMH and FSHR ($p < 0.001$), AMH and AR ($p = 0.001$), AMHR2 and FSHR ($p < 0.001$), AMHR2 and AR ($p < 0.001$), as well as between FSHR and AR ($p < 0.001$).

**Conclusion:** Assessed results point to AMH and AR relation with oocyte maturity, but not with its fertilization potential, or with embryo quality.

## Keywords

Anti-Müllerian hormone, cumulus cells, follicular fluid, oocyte quality, oocyte morphology

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## Introduction

One of the key factors influencing the success rate of assisted reproductive technology (ART) is the quality of the oocytes. Through its development inside the follicle, oocyte is surrounded by somatic follicular cells – granulosa cells that proliferate during follicle growth and differentiation. With the development of the antral and pre-ovulation follicles, granulosa cells differentiate into mural cells surrounding the

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follicle wall and cumulus cells (CCs) surrounding the oocyte.[1,2] CCs are in close contact with the oocyte, together forming the cumulus–oocyte complex (COC) – a part of the microenvironment surrounding the developing oocyte.[3–6] Morphological assessment of the oocyte, commonly used during ART, is not always a good predictor of successful fertilization and the developmental capacity of the ensuing zygote.[1,7] Identification of specific genes or other biological components of the microenvironment surrounding each oocyte could potentially enable more accurate distinguishing between high- and low-quality oocytes, which could in turn enhance the success rate of the assisted reproduction procedures.[8]

Anti-Müllerian hormone (AMH) is a glycoprotein growth factor secreted by the granulosa cells.[9] The normal expression pattern of AMH implies that it is present at low levels in the primary follicles, followed by gradual growth up to maximum in the large pre-antral and small antral follicles, followed by decline as the growth of the follicle continues.[10] Based on this expression pattern, serum AMH was suggested to reflect the number of early growing follicles as its concentration declines with the decline in a pool of developing follicles.[11,12] Previous studies have investigated AMH gene expression levels in granulosa cells, along with its correlation with the oocyte quality, but the obtained results were inconsistent and contradictory.[13–16] Studies have also shown that AMH gene expression levels in CCs correlate with the concentration of AMH in the corresponding follicular fluid (FF).[14]

As with other members of the large transforming growth factor beta (TGF-β) family, AMH signals through two related transmembrane serine–threonine kinase receptors: type 1 (anti-Müllerian hormone receptor type 1 (AMHR1)) and type 2 (anti-Müllerian hormone receptor type 2 (AMHR2)).[17] AMHR2 is a ligand-specific receptor and is expressed in the Müllerian duct mesenchymal cells and gonads in both sexes. Its expression colocalizes with AMH gene expression levels in granulosa cells and the specific patterns of their gene expressions have important roles in follicle development and its functions.[17,18] AMHR2 was found to be a crucial factor for AMH signalling in AMHR2-deficient mice.[19] Moreover, correlation between gene expression levels of AMHR2 and AMH in granulosa cells has been previously demonstrated.[20]

The specific pattern of AMH expression in CCs, together with follicle-stimulating hormone (FSH), has a role in regulating the quantity of growing follicles as well as in the selection of the dominant follicle.[21] The action of FSH occurs through its binding to the follicle-stimulating hormone receptor (FSHR), which is localized on the surface of the granulosa cells.[22] Oestrogen secretion from granulosa cells stimulated by FSH causes an increase in the number of FSHR sites on the membrane. However, the exact mechanism that explains the interaction between FSH and FSHR leading to the activation of different signalling pathways in steroidogenesis is still unknown.

Studies across species reported that androgen receptor (AR) is expressed in the ovary, uterus and breast in females.[23] In the ovary, AR is expressed in follicular theca and granulosa cells as well as in the oocyte.[24] Studies on knockout mouse models have shown that androgens, via AR, have an important role in female fertility, influencing growth and development of the follicles as well as ovulation.[23–25]

A few studies have found connection between gene expression of AR, AMH and FSHR in CCs.[14,16] Expression of the AR gene in the primate ovary CCs is positively associated with mitosis and negatively with apoptosis in granulosa cells, and it is therefore highly expressed in healthy follicles.[26] FSHR gene expression levels are also positively correlated with AMH gene expression levels in granulosa cells, pointing to the existence of a sensitive balance between AMH and FSH inside the follicle.[27] Grøndahl et al.[14] showed that the expression of all these genes (AMH, AMHR2, FSHR and AR) is higher in follicles that were evaluated as healthy.

This study investigated the expression profiles of the AMH gene and its respective receptors (AMHR2, FSHR and AR) in the CCs of large antral follicles from healthy women undergoing the ART procedure. The aim of this study was to compare the studied expression profiles with the morphological characterization of oocytes from the same COC. The correlation between expression levels of these genes and the oocyte's morphological quality during assisted reproduction is examined, as well as potential correlation with the morphological characteristics of the zygotes and embryos and with the outcome of the ART procedure.

## Materials and methods

### *Subjects*

A total of 129 CCs and 35 FF samples were included in the study, taken from 58 patients undergoing assisted reproduction at the Department for Human Reproduction of the Clinic of Obstetrics and Gynaecology, Clinical Hospital Centre Rijeka, Croatia. The sample size was calculated by the OpenEpi calculator using the calculation for comparing two means. For smaller sample sizes, the confidence interval was adjusted. The study protocol was approved by the ethics committee of the Clinical Hospital Centre Rijeka, and written consent was obtained from all patients included. Patients were principally undergoing the intracytoplasmic sperm injection (ICSI) procedure as a result of infertility caused by the male factor. All women included in the study were healthy normally ovulatory, with body mass indexes between 18 and 28 $kg/m^2$ and ages between 28 and 45. The patients included in the study were undergoing different ovarian stimulation protocols during the ART procedure: 32 patients underwent the modified natural cycle, 23 patients received

gonadotropin-releasing hormone (GnRH) antagonist and 3 patients received GnRH agonist. Human chorion gonadotrophin (hCG; 5000 IU i.m.) was applied to all patients 34–36 h prior to the oocyte aspiration.

### CCs and FF collection

Each follicle from the patients included was aspirated separately under transvaginal ultrasound guidance. Its content was examined, and the COC was isolated. CCs were removed from the oocyte enzymatically with hyaluronidase, centrifuged at 200×*g* for 5 min and then immediately subjected to RNA extraction. Oocytes were cultivated separately. FFs were only included in the study if: they were from aspirated follicles in which COC belonging to the same follicle was identified, their volume was greater than 1 mL and they did not contain visible traces of blood. The obtained FFs were centrifuged at 500×*g* for 15 min, aliquoted and frozen at −20°C for later analysis. In this way, we were able to investigate the oocyte, CCs and FFs originating from a single follicle.

### Gene expression analysis

Total RNA was extracted using an RNeasy Micro Isolation kit (Qiagen). cDNA was synthesized using a High Capacity DNA Reverse Transcription Kit (Applied Biosystems). Purity and concentration of RNA and cDNA were determined spectrophotometrically and fluorometrically (Qubit fluorometer; Thermo Fisher Scientific and BioDrop DUO; BioDrop). AMH, AMHR2, FSHR and AR gene expression level analyses were conducted on a real-time polymerase chain reaction (RT-PCR) device (LightCycler 96 System; Roche). According to the literature, normalization was performed with two different controls genes (glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin genes).[28] TaqMan technology assays (TaqMan Gene Expression Assays; Applied Biosystems) were used to analyse gene expression levels of all tested genes: AMH (assay ID: Hs01006984_g1), AMHR2 (assay ID: hs00179718_m1), FSHR (assay ID: hs00174865_m1) and AR (assay ID: hs00171172_m1), as well as endogenous controls: GAPDH (assay ID: Hs03929097_g1) and β-actin (assay ID: Hs99999903_m1). Relative gene expression levels were calculated using the delta–delta Ct method (relative to the levels of the control genes) using the software of the RT-PCR device (LightCycler 96 Software, Version 1.1.0.1320; Roche).

### FF AMH concentrations

Concentrations of AMH in FFs were measured by enzyme-linked immunosorbent assay (AMH Gen II ELISA; Beckman-Coulter). AMH concentrations in a sample were calculated based on the calibrators' absorbance and calibration curve.

### Morphological assessment of oocytes, zygotes and embryos

Oocytes were assessed for morphological quality immediately before and during the ICSI procedure. The ICSI procedure was performed 3–6 h after oocyte retrieval, using metaphase II (MII) oocytes only. Zygotes and embryos were morphologically assessed 16–18 h and 64–66 h after fertilization, respectively. According to the previously described characteristics, oocytes, zygotes and embryos were separated into those with optimal and suboptimal morphology (Table 1).[3,7] The morphological assessment was performed by three embryologists from the Department for Human Reproduction of the Clinic of Obstetrics and Gynaecology, Clinical Hospital Centre Rijeka, Croatia.

### ART outcome

ART outcome was determined by measuring the level of serum βhCG 21 days after the embryo transfer. Values higher than 100 IJ/L were considered to demonstrate pregnancy and were denoted as positive ART outcome.

### Statistical analysis

Cross-analysis of gene expression levels between morphologically differently rated oocytes, zygotes and embryos was conducted using parametric or nonparametric statistical tests depending on the data characteristics (size, distribution, scaling etc.). REST 2009 Software (Relative Expression Software Tool V2.0.13; Qiagen) was used to examine differences in gene expression levels between morphologically differentially assessed oocytes, zygotes and embryos, as well as between oocytes with different ART procedure outcomes. To analyse statistical differences between FF AMH concentrations in morphologically differentially graded oocytes, zygotes and embryos, the Mann–Whitney nonparametric test was used. To analyse statistical correlation between FF AMH concentration and gene expression levels of AMH, AMHR2, FSHR and AR, as well as to analyse correlations between the investigated genes' expression levels, nonparametric Kendall's tau and Spearman's rank order tests were used. Student's t-test was applied to determine statistical differences between different data groups. All statistical procedures were run using Statistica Version 12 (StatSoft).

## Results

Expression of both AMH and AR mRNA in CCs surrounding morphologically optimal oocytes were statistically significantly lower than in morphologically suboptimal oocytes ($2^{-\Delta\Delta Ct}$(AMH) = 1.703; p = 0.011 and $2^{-\Delta\Delta Ct}$(AR) = 1.530; p = 0.008) (Table 2).

There was no significant difference in AMH or AR mRNA expression in CCs surrounding oocytes that developed to

**Table 1.** Morphological assessment of the oocytes, zygotes and embryos.

| | Morphology | Characteristics | N |
|---|---|---|---|
| Oocyte | Optimal | Mature MII oocyte (presence of 1PB in the PV) | 107 |
| | Suboptimal | MI oocyte (absence of GV in the cytoplasm and 1PB in the PV), PI oocyte (presence of GV in the cytoplasm) or atretic/degenerated oocyte (breakdown of ZP or dark vacuolated cytoplasm) | 22 |
| Zygote | Optimal | Adequate morphology, size and arrangement of the two PN, adequate arrangement of 4–6 NPBs and presence of the Halo effect | 73 |
| | Suboptimal | Inadequate morphology, size, arrangement and/or number of the PN, inadequate number, size and arrangement of NPBs or absence of the Halo effect | 44 |
| Embryo | Optimal | $\geqslant$7 blastomeres and $<$20% fragmentation | 29 |
| | Suboptimal | $<$7 blastomeres and $<$20% fragmentation or $\geqslant$7 blastomeres and $>$20% fragmentation | 73 |

MII: metaphase II; 1PB: first polar body; PV: perivitelline space; MI: metaphase I; GV: germinal vesicle; PI: prophase I; ZP: zona pellucida; PN: pronucleus; NPB: nucleolar precursor body.

zygotes and embryos, based on their morphology. No significant differences were found in AMHR2 or FSHR mRNA expression levels among different morphological groups of oocytes, zygotes and embryos. None of the investigated gene expression levels showed statistically significant differences with regard to ART outcome (Table 2).

The concentration of FF AMH did not differ significantly between different morphological groups of oocytes, zygotes and embryos, or between different ART outcomes ($p=0.082$, $p=0.230$, $p=0.486$ and $p=0.724$, respectively) (Table 3). There are no correlation between FF AMH concentration and mRNA expression levels of the AMH, AMHR2, FSHR or AR genes in associated CCs ($p=0.195$, $p=0.809$, $p=0.461$ and $p=0.240$, respectively).

Correlations between mRNA expression levels of the genes investigated are summarized in Table 4. Statistically significant correlation was found between AMH and FSHR mRNA expression levels ($p<0.001$) as well as between AMH and AR mRNA expression levels ($p=0.001$) and AMHR2 and FSHR ($p<0.001$), AMHR2 and AR ($p<0.001$) and FSHR and AR ($p<0.001$) mRNA expression levels.

## Discussion

The aim of this study was to analyse the expression patterns of biologically interconnected genes in COCs isolated from large antral follicles of healthy women during the ART procedure. We found that morphologically suboptimal oocytes had statistically significantly higher levels of AMH and AR gene expression in their associated CCs ($2^{-\Delta\Delta Ct}$(AMH)$=1.703$; $p=0.011$ and $2^{-\Delta\Delta Ct}$(AR)$=1.530$; $p=0.008$; Table 2). Up until now, AMH was normally considered to correlate with ovarian reserve and the primordial follicle recruitment, as well as with oocyte quality and ART procedure outcome.[29–32] Our results are consistent with the studies which found negative correlations between AMH gene expression in CCs and oocyte maturity, as well as elevated AMH gene expression in CCs of immature follicles.[14,16]

The specific expression pattern of AMH (low levels in primary follicles, gradual growth in pre-antral and early antral follicles followed by a decrease as the follicle continues to grow) implies its importance for regulating the quantity of growing follicles as well as for selection of the dominate follicle.[10,21,33,34] According to the previous studies, AMH might be one of the factors involved in determining the responsiveness of the follicle to FSH during cyclic recruitment and resumption of follicle growth and development.[17,18] It is therefore possible that AMH levels are used for regulating the growth of those follicles which contain oocytes of the adequate fertilization potential. In ART procedures, as a result of hormonal stimulation, larger numbers of follicles develop, while in the natural cycle their development would have already stopped. Furthermore, higher expression of AMH in CCs surrounding morphologically poorly rated oocytes might imply that those oocytes have difficulties with resuming meiosis. Indeed, AMH acting as a meiosis inhibitor has previously been observed in rat oocytes.[35] Grøndahl et al.[14] found that, in spite of a decrease in AMH expression in maturing follicles, there is still a certain amount of AMH expression left in the CCs of pre-ovulation follicles. This could indicate that AMH plays a yet unclarified role in oocyte maturation during the final stages of folliculogenesis.

AR and its expression in CCs were previously positively associated with follicular health in primates.[26,36] Grøndahl et al.[14] have also found higher AR expression levels in CCs of immature follicles, as well as positive correlation between AMH and AR expression levels, which is consistent with our results. The role of AR and androgens in general in follicular development is still insufficiently explained. Walters et al.[23,37] found that even a short-term increase in ovarian androgen levels can lead to permanent negative effects on follicular development in mouse models and that AR might have a role in unexplained anovulation. Negative correlation between AR expression in CCs and oocyte morphology can be explained by its direct effect on gene transcription and its indirect effect on cytoplasmic proteins involved in signal transmission. This

**Table 2.** AMH, AMHR2, FSHR and AR mRNA ΔCt differences between morphological groups of oocytes, zygotes and embryos as well as between different ART outcomes.

| Samples | Morphology | N | AMH | | AMHR2 | | FSHR | | AR | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | ΔCt (average) | p | ΔCt (average) | p | ΔCt (average) | P | ΔCt (average) | p |
| Oocyte | Optimal | 107 | 5.589 | 0.011* | 8.058 | 0.765 | 11.125 | 0.109 | 5.465 | 0.008* |
| | Suboptimal | 22 | 4.821 | | 7.982 | | 10.749 | | 4.851 | |
| Zygote | Optimal | 73 | 5.665 | 0.117 | 8.040 | 0.725 | 11.077 | 0.753 | 5.308 | 0.177 |
| | Suboptimal | 44 | 5.277 | | 8.115 | | 11.135 | | 5.566 | |
| Embryo | Optimal | 29 | 5.424 | 0.624 | 8.237 | 0.629 | 10.954 | 0.386 | 5.393 | 0.917 |
| | Suboptimal | 73 | 5.570 | | 8.117 | | 11.140 | | 5.416 | |
| ART outcome | Positive | 11 | 5.457 | 0.997 | 8.231 | 0.553 | 11.234 | 0.551 | 5.535 | 0.543 |
| | Negative | 118 | 5.458 | | 8.028 | | 11.045 | | 5.344 | |

AMH: anti-Müllerian hormone; AMHR2: anti-Müllerian hormone receptor type 2; FSHR: follicle-stimulating hormone receptor; AR: androgen receptor; mRNA: messenger RNA; ART: assisted reproductive technology.
*$p \leqslant 0.05$.

**Table 3.** FF AMH concentration differences between morphological groups of oocytes, zygotes and embryos as well as between different ART outcomes.

| Samples | Morphology | N | FF AMH | |
|---|---|---|---|---|
| | | | Concentration (ng/mL) | P |
| Oocyte | Optimal | 31 | 12.045 | 0.082 |
| | Suboptimal | 4 | 21.516 | |
| Zygote | Optimal | 22 | 11.195 | 0.230 |
| | Suboptimal | 10 | 15.208 | |
| Embryo | Optimal | 11 | 11.717 | 0.486 |
| | Suboptimal | 18 | 11.996 | |
| ART outcome | Positive | 5 | 12.066 | 0.724 |
| | Negative | 30 | 13.304 | |

FF: follicular fluid; AMH: anti-Müllerian hormone; ART: assisted reproductive technology.
Statistical significance $p \leqslant 0.05$.

can lead to disruption in communication between oocyte and CCs and consequently to poor oocyte quality.[38,39]

Our results suggest that gene expression levels of AMH and AR in CCs were not associated with morphological graduation of zygotes or embryos, which might point to a relationship of AMH and AR with oocyte maturity, but not with its fertilization potential.

There was no correlation between AMHR2 and FSHR gene expression levels in CC and morphological grade of oocytes, zygotes and embryos, or with ART procedure outcome. Moreover, no association was found with AMH FF concentration either. Previously conducted studies found similar results, although there are some inconsistencies among the reported results that can be explained by differences in presenting ART outcomes, as well as by the myriad of factors that can impact on fertilization, besides oocyte quality (e.g. sperm quality or ART procedure, patient selection).[16,40–42] The observed lack of correlation between FF AMH concentration and expression of the investigated genes in CCs from the same follicles might arise from the fact that the follicles studied were in the pre-ovulation phase (diameter 20 mm or more), by which time FF AMH levels have already decreased. Perhaps, more accurate results would be obtained using more precise measurement techniques, such as mass spectrometry.

Results regarding correlations between the relative expression levels of the genes reported here (Table 4) have been previously reported; however, that study was conducted on smaller, early antral follicles.[20] Nevertheless, we found no correlation between AMH and AMHR2 gene expression levels ($r = 0.14$; $p = 0.107$; Table 4). Similar results were reported by Catteau-Jonard et al.,[13] while Rice et al.[43] reported very low AMHR2 expression in pre-antral follicles, at the time when AMH expression reaches its maximum. To date, AMHR2, as a type II receptor, has been considered essential for AMH signalling.[17,18] It is thought that all members of TGF-β superfamily, as well as AMH, signal through two types of receptors: type I and II. Together they form the serine–threonine kinase receptor complex, composed of ligand-specific type II receptors and more general type I receptors.[18,44]

**Table 4.** Correlations between mRNA expressions of the AMH, AMHR2, FSHR and AR genes.

| | AMH | AMHR2 | FSHR | |
|---|---|---|---|---|
| AMH | | r = 0.14 | r = 0.40 | r = 0.30 |
| | | p = 0.107 | p < 0.001* | p = 0.001* |
| AMHR2 | r = 0.14 | | r = 0.42 | r = 0.63 |
| | p = 0.107 | | p < 0.001* | p < 0.001* |
| FSHR | r = 0.40 | r = 0.42 | | r = 0.49 |
| | p < 0.001* | p < 0.001* | | p < 0.001* |
| AR | r = 0.30 | r = 0.63 | r = 0.49 | |
| | p = 0.001* | p < 0.001* | p < 0.001* | |

mRNA: messenger RNA; AMH: anti-Müllerian hormone; AMHR2: anti-Müllerian hormone receptor type 2; FSHR: follicle-stimulating hormone receptor; AR: androgen receptor.
N = 129; r: correlation coefficient.
*$p \leq 0.05$.

However, the results described here might suggest a further investigation of the importance of the AMHR2 as well as of the type I receptors in the AMH signalling pathway. The other assessed correlations (Table 4) are consistent with previously reported results from similar studies.[13,14,20]

No significant differences in FF AMH concentration, or in AMH, AMHR2, FSHR and AR expression levels in CCs, were found between younger (aged < 35) and older (aged ⩾ 35) patients. However, despite the fact that the serum AMH concentration decreases with age, it is not the result of concentration, or the expression of AMH in each individual follicle, but the overall number of follicles that is greater in younger patients. Although the serum AMH level might be considered as an indicator of ovarian reserve, as it reflects the size of the developing follicles pool, it is not associated with the AMH level in individual follicles, or with the quality of the associated oocytes.[45–47]

The potential limitation of the study is the heterogeneity of the study population in terms of age and applied ovarian stimulation protocol. It has been previously shown that gene expressions can be affected by age and controlled ovarian stimulation.[48,49] Nevertheless, our results showed no differences in CC gene expressions regarding patients' age or ovarian stimulation protocol ($p > 0.05$; data not shown).

In this study, negative association was reported for the first time between AMH and AR gene expression levels in CCs isolated from healthy women during the ART procedure and the morphological quality of the oocyte from the same COC. This research reinforces the importance of the COC, namely as a means of communication between an oocyte and its microenvironment, for oocyte development is a prerequisite for successful fertilization and quality embryo formation. Further research, with larger groups of patients with stricter inclusion criteria, of the oocyte's microenvironment, such as CCs' gene expression and FF content profiling, are needed in order to clarify its importance for the oocyte maturation and fertilization potential.

## Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## Ethical approval

Ethical approval for this study was obtained from KBC Rijeka Ethics Committee (Approval No.: Kl: 003-05-14-1/26 Ur.br.: 2170-29-02/1-14-2).

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## Informed consent

Written informed consent was obtained from all subjects before the study.

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